INSULIN-LIKE GROWTH FACTORS AND GROWTH OF THE FETAL SHEEP

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CHAPTER 3

IGF-I and -II gene expression in fetal tissues: Relationships to circulating IGFs and fetal growth in sheep.

ABSTRACT

Growth of the mammalian fetus is dependent on the fetal genome and its interaction with various modulating factors, primarily the placental supply of essential fetal substrates. The insulin-like growth factors (IGF-I, IGF-II) are implicated as potential mediators of the effects of external influences, such as placental substrate supply, on fetal growth. Restriction of placental size in sheep retards fetal growth by limiting the delivery of nutrients and oxygen to the fetus. Placental growth can be experimentally restricted by surgical removal of most endometrial caruncles, the placental attachment sites, from the uterus of the non-pregnant ewe. To investigate the role of the IGFs as mediators of the influence of placental substrate supply on growth *in utero*, the relationship between fetal growth, circulating IGF-I and -II proteins and the expression of the genes for IGF-I and -II in fetal tissues was examined in fetal sheep with normal and restricted placental size.

Concentrations of IGF-I and -II proteins were measured in arterial blood samples collected from fetal sheep with normal and restricted placental size at 120 and 127 days gestation (term≈150 days), via indwelling catheters. Concentrations of IGF-I in fetal blood at 120 and 127 days gestation correlated positively with fetal weight, placental weight and the weights of individual fetal tissues, measured at 130 days gestation. Fetal plasma IGF-I was positively related to fetal arterial blood pO_2 and blood glucose at both gestational ages. In contrast, concentrations of IGF-II were related to fetal weight, placental weight and to fetal blood pO_2 and glucose, at 127 days gestation, but not 120 days gestation. Therefore, the supply of substrates to the fetus is suggested as a regulator of the circulating concentration of IGF-I in late gestation, and very late in gestation of IGF-II, in the sheep fetus. These results are consistent with the suggestion that circulating IGFs may mediate, in part, the effect of altered substrate supply on growth of the fetal sheep.

However, changes in the concentrations of the IGF proteins in fetal blood may be the result of altered production at one or more tissue sites, as many fetal tissues and the placenta contain IGF-I and -II mRNAs. To determine whether altered fetal growth was associated with altered expression of the genes for IGF-I or IGF-II in fetal tissues, IGF-I and -II mRNAs were

measured in tissues from a further group of normal and growth-retarded fetal sheep at 121 days gestation. IGF-I mRNA was detected in the ovine placenta and all six fetal tissues examined (liver, lung, quadriceps muscle, heart, kidney, cerebellum). IGF-II mRNA was not detected in fetal sheep cerebellum, but was present in placenta and in the other fetal tissues examined. The highest relative abundance of IGF-I mRNA was observed in skeletal muscle and liver, while IGF-II mRNA was most abundant in kidney and lung. When estimates were made of the total amount of IGF mRNAs in fetal tissues, using tissue mass, liver and muscle were identified as quantitatively major sites of IGF-I production, while skeletal muscle, placenta and lung were indicated as major sites of IGF-II synthesis.

The relative abundance of IGF-I mRNA was reduced in skeletal muscle, kidney and lung of growth-retarded fetal sheep. IGF-I mRNA abundance in fetal liver, skeletal muscle and kidney correlated positively with fetal plasma IGF-I, implicating these tissues as potential sources of circulating IGF-I *in utero*. Therefore, altered production of IGF-I at a number of tissue sites in the growth-retarded fetus may contribute to retarded fetal growth, either through reduced local actions and/or as a consequence of reduced circulating levels. In fetal liver, IGF-I mRNA abundance correlated positively with fetal arterial blood pO_2. However, plasma insulin, but not plasma glucose was related to hepatic IGF-I mRNA abundance. Therefore, restricted nutrient supply may regulate IGF-I production in fetal liver, at least in part via changes in plasma insulin. These results are consistent with regulation of expression of the IGF-I gene in fetal sheep tissues by substrate supply.

In contrast to IGF-I, the abundance of IGF-II mRNA was not altered in any of the fetal tissues examined at 120 days gestation in the growth-retarded fetus. No associations were observed between IGF-II mRNA abundance in fetal tissues and IGF-II protein concentrations in fetal blood, and it is possible that a number of fetal tissues may be sources of IGF-II protein in fetal blood. Furthermore, no associations were observed between the concentrations of substrates in fetal blood and expression of IGF-II in fetal tissues, suggesting that at this gestational stage, substrate supply is not an important regulator of IGF-II production.

Muscle and liver were indicated as potential sources of circulating IGF-I in the fetus. In the adult, negative feedback regulation of hepatic IGF-I production is suggested. Therefore, to determine whether IGF-I regulates IGF-I or -II gene expression in the fetus, the effect of intravenous infusion of IGF-I into the fetal sheep on the abundance of IGF-I and -II mRNAs in fetal liver and skeletal muscle was determined. Recombinant human IGF-I (26 ug/kg/hr) or saline was infused continuously into the fetal tarsal vein from 120 to 130 days gestation. Plasma IGF-I was increased by approximately three-fold by infusion of IGF-I into the fetus, but plasma IGF-II concentrations did not change. Infusion of IGF-I reduced the abundance of IGF-I and -II mRNAs in fetal liver by approximately 50%, but, had no effect on IGF-I and -II mRNAs in skeletal muscle. This suggests that IGF-I inhibits the expression of both IGF-I and -II genes in fetal liver, but not in fetal muscle. However the concentration of insulin in fetal blood was also reduced by IGF-I infusion, suggesting that the inhibition of hepatic IGF-I expression may be mediated indirectly through insulin.

In summary, these current studies indicate that retarded fetal growth in sheep, associated with restricted supply of substrates to the fetus, is accompanied by reduced concentrations of IGF-I in fetal blood and decreased production of IGF-I in several major fetal tissues. This suggests that both altered endocrine and autocrine/paracrine actions of the IGFs may contribute to retarded growth of the fetus when placental supply of substrates is limited. Although many tissues may contribute to circulating IGF-I proteins in the fetus, liver, and possibly muscle, were suggested as likely sources of IGF-I protein in fetal sheep blood and in the former IGF-I expression may be subject to negative feedback regulation. In contrast, concentrations of IGF-II in fetal blood are reduced in later gestation only, suggesting that any role of IGF-II in mediating the influence of placental substrate supply on fetal growth occurs very late in gestation.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give my consent to this copy of my thesis, when deposited in the University Library, being available for photocopying and loan.

Signed

Date

13th February 1995

Karen Kind

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in fetal blood were measured and Ms Linda Mundy performed all of the metabolite assays. I gratefully acknowledge the contribution, collaboration and assistance of all of these people.

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ABBREVIATIONS

cAMP	cyclic 3', 5'-adenosine monophosphate
Ci	Curie
cpm	counts per minute
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
F:P ratio	fetal to placental weight ratio
g	gram
h	hour
Hb	haemoglobin
hIGF	human IGF
ID	internal diameter
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor-II
IGFBP 1-6	insulin like growth factor binding proteins 1-6
kb	kilobases
kDa	kilodaltons
l	litre
mA	milliampere
min	minute
ml	millilitre
mRNA	messenger ribonucleic acid
n	number of animals
^{o}C	degrees Celsius
OD	outside diameter
oIGF	ovine IGF
pCO_2	partial pressure of carbon dioxide
PCR	polymerase chain reaction
pO_2	partial pressure of oxygen

RIA	radioimmunoassay
RNA	ribonucleic acid
RNAse	ribonuclease
RRA	radioreceptor assay
TBE	Tris-borate EDTA buffer
TCA	trichloroacetic acid
v/v	volume/volume
w/v	weight/volume
wt	weight
µg	micro-gram
µl	microlitre

PUBLICATIONS ARISING FROM THE RESEARCH IN THIS THESIS

Papers

Owens J.A., Kind K.L., Carbone F., Robinson J.S. and Owens P.C. (1994) Circulating insulin-like growth factors-I and -II and substrates in fetal sheep following restriction of placental growth. *Journal of Endocrinology*. **140**, 5-13.

Kind K.L., Owens J.A., Robinson J.S., Quinn K.J., Grant P.A., Walton P.E., Gilmour R.S. and Owens P.C. (1995) Effect of restriction of placental growth on expression of insulin-like growth factors in fetal sheep: relationship to fetal growth, circulating insulin-like growth factors and binding proteins. *Journal of Endocrinology*.(in press)

Kind K.L., Owens J.A., Lok F., Robinson J.S., Quinn K.J., Mundy L., Gilmour R.S. and Owens P.C. Intravenous infusion of insulin-like growth factor-I (IGF-I) in fetal sheep reduces hepatic IGF-I and -II mRNAs. (submitted, 1995, *Molecular and Cellular Endocrinology*).

Abstracts

Kind K.L., Owens J.A., Robinson J.S., Gilmour R.S. and Owens P.C. (1992) Potential sources of insulin-like growth factor (IGF)-I in fetal sheep. *Proceedings of the Endocrine Society of Australia*. **35**, Abstract 9.

Kind K.L., Owens J.A., Robinson J.S., Gilmour R.S. and Owens P.C. (1993) Insulin-like growth factor (IGF-II) gene expression in normal and growth-retarded fetal sheep. *Proceedings of the Endocrine Society of Australia*.. **36**, Abstract 51.

Kind K.L., Owens J.A., Robinson J.S., Gilmour R.S. and Owens P.C. (1993) Restriction of placental size reduces insulin-like growth factor-I mRNA but not insulin-like growth factor-II mRNA in the late gestation fetal sheep. *Proceedings of the.Australian Society for Medical Research*. **32**, Abstract O34.

Kind K.L., Owens J.A., Robinson J.S., Gilmour R.S. and Owens P.C.(1994) Insulin-like growth factor-I and -II gene expression in normal and growth-retarded fetal sheep. *Growth Regulation* **4**, Supplement 1, Abstract I110.

Kind K.L., Owens J.A., Lok F., Robinson J.S., Gilmour R.S. and Owens P.C. (1994) Intravenous infusion of insulin-like growth factor-I (IGF-I) to fetal sheep reduces IGF-I and IGF-II mRNA abundance in fetal liver, but not muscle. *Proceedings of the Endocrine Society of Australia*.. **37**, Abstract 19.

CHAPTER 1

INTRODUCTION

INTRODUCTION

Fetal growth is regulated by the interaction of the genetic drive to growth, as determined by the fetal genome, with modulating external factors, particularly the placental supply of essential fetal substrates. Furthermore, the influence of substrate supply on fetal growth is partly mediated by the fetal and placental endocrine systems. Postnatally, the regulation of growth by nutrition, growth hormone and other hormones is mediated, at least in part, by the peptide growth factors, the insulin like growth factors (IGFs). A major role for this family of growth factors in mediating the impact of various influences on fetal growth and development is also suggested. This review will provide a brief overview of the major factors known to regulate fetal growth (1.1), describe the insulin-like growth factors with a focus on their expression, regulation and function in postnatal life (1.2) and review the current understanding of the potential roles and regulation of the IGFs *in utero* (1.3).

1.1 REGULATION OF FETAL GROWTH

The regulation of fetal growth by substrate supply and endocrine factors has been extensively reviewed (Gluckman & Liggins, 1984; Milner & Hill, 1984; Gluckman, 1986) and is summarised here.

1.1.1 *Substrate supply*

The major substrates utilised by the fetus, for oxidative metabolism and tissue accretion are glucose, lactate, amino acids and oxygen (reviewed by Fowden, 1994). The size, function and metabolism of the placenta are important determinants of the rate of supply of these substrates to the fetus (Owens & Robinson, 1988). In sheep, the placenta attains its maximal weight prior to 90 days of gestation (term ≈ 150 days), while the fetus continues to grow rapidly throughout the third trimester (Owens & Robinson, 1988). In late gestation, an association between fetal weight and placental size is evident in sheep, particularly when placental growth is restricted (Alexander, 1964; Harding *et al.*, 1985; Owens *et al.*, 1989a). Placental metabolism also influences the amount of available maternal substrate which reaches the fetus. A proportion of the substrates extracted from maternal blood are metabolised by the placenta, which consumes

oxygen and glucose, and releases lactate into the uterine and umbilical circulations (Fowden, 1994). Altered placental size or a change in functional characteristics of the placenta are common causes of intra-uterine growth retardation in the human (Owens *et al.*, 1989).

1.1.2 *Endocrine regulation of fetal growth*

Most hormones, including insulin and growth hormone, do not cross the placenta and the major hormones influencing fetal growth are of fetal origin. Maternal cortisol is converted into biologically inactive cortisone by the 11β-hydroxy-dehydrogenase enzyme within the placenta, limiting the passage of cortisol from the mother to the fetus (Gluckman & Liggins, 1984).

Pituitary hormones

Concentrations of growth hormone are high in fetal blood, and growth hormone receptors are present in fetal tissues in several species (Bassett *et al.*, 1970; Klempt *et al.*, 1993; Werther *et al.*, 1993). However, growth *in utero* appears to be independent of the actions of growth hormone, since newborn human infants with anencephaly and congenital absence of the pituitary are not growth-retarded (see Gluckman & Liggins, 1984). Hypophysectomy of fetal sheep in mid-gestation results in retarded fetal growth at term (Deayton *et al.*, 1993). Weights of the heart and lung, but not those of brain, kidney or liver are reduced in hypophysectomised fetal sheep suggesting that pituitary hormones influence fetal growth in a tissue specific manner (Deayton *et al.*, 1993). Hypophysectomy of fetal sheep, early in late gestation restricts the growth and maturation of the appendicular skeleton, but did not alter tissue weights (Mesiano *et al.*, 1987). The effects of hypophysectomy on fetal growth may be related to a concomitant fall in thyroid hormones. The role of thyroid hormones in the regulation of fetal growth is species specific. Thyroidectomy retards the growth of fetal sheep and alters development of the skeleton and skin (Hopkins & Thorburn, 1972). In the human fetus, thyroid hormone deficiency does not alter fetal growth, but delays skeletal maturation (Gluckman & Liggins, 1984).

Insulin

The role of insulin in the regulation of fetal growth has been extensively reviewed (Gluckman, 1986; Milner & Hill, 1984; Fowden, 1989). Insulin deficiency, associated with congenital

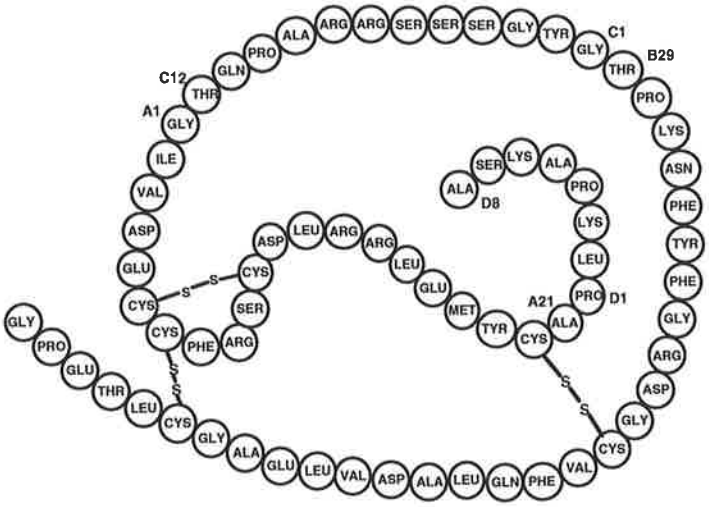
absence of the pancreas in humans, or experimental ablation of the pancreas in animals, produces fetal growth retardation, indicating that insulin is necessary for normal fetal growth (Fowden & Comline, 1984; see Gluckman, 1986). However, fetal hyperinsulinaemia is associated with a moderate increase in fetal weight, due predominantly to an increase in fat deposition (see Gluckman, 1986), suggesting that insulin may have a permissive role in the regulation of fetal growth. A primary influence of insulin on fetal growth is exerted through its effects on fetal metabolism, including stimulation of fat deposition and of uptake of nutrients into fetal tissues (Fowden, 1989; 1994).

Cortisol

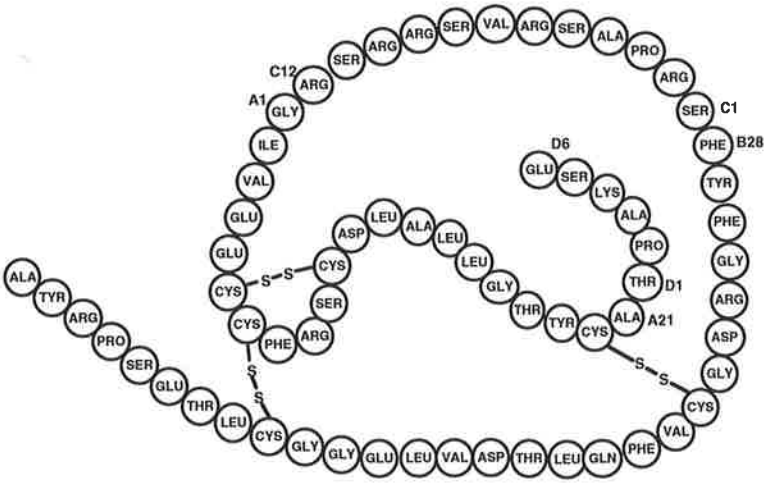
Cortisol concentrations in fetal blood increase rapidly immediately before birth with the maturation of the fetal adrenal. In the sheep a gradual increase in blood cortisol concentrations commences around 125 to 130 days gestation (term≈150 days), and the final prepartum cortisol surge occurs over the last three to five days before birth (Bassett & Thorburn, 1969; Silver, 1990). The increase in circulating cortisol concentration is suggested to influence a number of developmental changes in the fetus, including pulmonary maturation and surfactant production, increased glycogen deposition, maturation of the small intestine and induction of gluconeogenic enzymes and other enzymes in liver (Silver, 1990; Nathanielsz, 1976).

1.2 INSULIN-LIKE GROWTH FACTORS

Insulin-like growth factor-I and -II are single chain peptides cross-linked by three disulphide bonds, with molecular weights of 7649 and 7471 respectively (Rinderknecht & Humbel, 1978 a,b). IGF-I is a 70 amino acid basic protein, while IGF-II consists of 67 amino acids and is slightly acidic. The IGF proteins are identical for 62% of their amino acid sequence and share 47-49% homology with the A and B chains of insulin. Unlike proinsulin, the connecting peptide of the IGFs is not cleaved during formation of the mature protein, and the IGFs contain an additional carboxy-terminal D domain (Figure 1.1).



IGF-I



IGF-II

FIGURE 1.1 Human insulin-like growth factor -I and -II

Amino acid sequence and primary structure of human IGF-I and -II. B C A and D domains are indicated. IGF-I consists of 70 amino acids and IGF-II of 67 amino acids.

Adopted from Sara & Carlsson-Skwirut (1988)

Three lines of investigation led to the isolation of the IGF peptides from serum. Salmon and Daughaday, in 1957, observed that the incorporation of [^{35}S] sulphate into cartilage *in vitro* was stimulated by addition of normal rat serum, but serum from hypophysectomised rats had no such effect. Treatment of hypophysectomised rats with growth hormone restored this activity. The presence of sulphation factors (Salmon & Daughaday, 1957) or somatomedins (Daughaday *et al.*, 1972), serum factors which mediated the actions of growth hormone on cartilage was postulated. Subsequently, peptides which stimulated the replication of fibroblasts *in vitro* were extracted from serum (Pierson & Temin, 1972) and conditioned medium of a rat hepatocyte cell line (Dulak & Temin, 1973), and termed multiplication-stimulating activity. In addition, others had detected factors in serum which exerted insulin-like metabolic actions on adipose tissue and muscle, and observed that these actions could not be suppressed by anti-insulin serum (non-suppressible insulin-like activity) (Froesch *et al.*, 1963). These peptides were purified from human serum and sequenced in 1978 (Rinderknecht & Humbel, 1978 a,b) and designated IGF-I and IGF-II (Rinderknecht & Humbel, 1978 a,b). IGF-I was found to be identical to somatomedins C and A (Klapper *et al.*, 1983; Enberg *et al.*, 1984) while multiplication stimulating activity was determined to be rat IGF-II (Marquardt *et al.*, 1981).

Isolation of the first human IGF-I and IGF-II cDNAs (Jansen *et al.*, 1983; Bell *et al.*, 1984) indicated that the IGFs are formed as larger precursor proteins, with an additional amino terminal leader peptide and a carboxyl terminal extension or E domain (Figure 1.2, 1.4).

1.2.1 *Insulin-like growth factor-I*

1.2.1.1 *IGF-I peptides*

The sequence of IGF-I has been determined for seven mammalian species and is highly conserved (reviewed in Rotwein, 1991). Human (Rinderknecht & Humbel, 1978a), bovine (Honegger & Humbel, 1986), porcine (Francis *et al.*, 1989a; Tavakkol *et al.*, 1988) and guinea pig IGF-I (Bell *et al.*, 1990) have identical amino acid sequences. A single substitution occurs in ovine IGF-I (Francis *et al.*, 1989b), while rat IGF-I (Shimatsu and Rotwein, 1987a) differs by three amino acids from human IGF-I and mouse by four (Bell *et al.*, 1986). In all of these

species, IGF-I is a 70 amino acid protein consisting of a 29 residue amino terminal B domain, a C domain of 12 amino acids, an A domain of 21 amino acids and a carboxyl terminal D region of 8 amino acids (Figure 1.1).

Several sequences for pre-pro-IGF-I proteins are predicted. Alternative splicing of exons 1 and 2 of the IGF-I gene (1.2.2.1), and the existence of multiple in-frame translation initiation codons suggest the presence of IGF-I precursor proteins with different amino terminal leader peptides. A high degree of homology is observed in a predicted leader peptide of 48 amino acids in human, rat and porcine pre-pro-IGF-I, and of 49 amino acids in bovine and ovine pre-pro-IGF-I (due to the addition of a glutamine at position -28) (Rotwein, 1991; Simmen, 1991). An alternate leader peptide of 32 or 33 amino acids is predicted in human, rat, sheep and pigs (Tobin *et al.*, 1990; Roberts *et al.*, 1987b; Wong *et al.*, 1989; Weller *et al.*, 1993). In addition a 22 amino acid leader peptide in the human, rat and mouse or 25 amino acid leader protein in the cow, pig, sheep and chicken is potentially encoded by all IGF-I mRNAs. All of the precursor proteins predicted in the rat are translated in cell free studies of *in vitro* transcribed IGF-I mRNAs. However, fewer IGF-I precursor proteins containing the 22 amino acid protein leader sequence are translated in these studies (Rotwein *et al.*, 1987a; Simmons *et al.*, 1993).

Two different carboxyl terminal extension peptides (Ea, Eb domains) are produced as a result of alternative splicing of exons 5 and 6 of human, rat and mouse IGF-I genes (Rotwein *et al.*, 1986; Roberts *et al.*, 1987a; Bell *et al.*, 1986; 1.2.1.2). In other species, a single extension peptide, corresponding to the Ea domain, has been characterised. The Ea domain consists of 35 amino acids and a high degree of homology is observed between species, with 30 of these amino acids invariant. In the human the alternative Eb domain contains 77 amino acids with the first 16 residues identical to those of the Ea domain. In rodents, the Eb domain contains 41 amino acids and has less than 50% homology with the human equivalent.

Two potential N-glycosylation sites, which are functional *in vitro*, are found in the IGF-I Ea domain of the rat and mouse pre-protein (Bach *et al*, 1990). In the rat, the IGF-I Ea domain is glycosylated *in vitro* in pre-pro-peptides with 32 and 22 amino acid leader peptides, but not in those with 48 residue leaders (Simmons *et al.*, 1993). Human, sheep, pig and chicken IGF-I

Ea domains contain the first glycosylation site only (Bach *et al.*, 1990). No studies have determined whether this site is functional in these species. The IGF-I Eb precursor is not glycosylated (Bach *et al.*, 1990).

The mechanism by which pre-pro-IGF-I peptides are processed to produce IGF-I is not clear. *In vitro* the leader peptides are removed by cotranslational cleavage, following addition of microsomal membranes to cell-free translation systems (Rotwein *et al.*, 1987; Kajimoto & Rotwein, 1989; Simmons *et al.*, 1993). IGF-I precursor proteins containing the E-domain are secreted by human fibroblasts (Clemmons & Shaw, 1986; Conover *et al.*, 1989) and the pro-IGF-I Ea protein and free E-peptide are detected in human plasma (Powell *et al.*, 1987), suggesting that cleavage of the E domain occurs after secretion.

1.2.1.2 *IGF-I gene and mRNAs*

IGF-I is encoded by a single gene mapped to human chromosome 12 (Tricoli *et al.*, 1984; Brissenden *et al.*, 1984). The IGF-I gene has been described for several mammalian species including rat, human, mouse, pig, sheep and cow (Bell *et al.*, 1986; Rotwein *et al.*, 1986; Rotwein, 1986; Shimatsu & Rotwein, 1987a,b; Roberts *et al.*, 1987a,b; Wong *et al.*, 1989; Dickson *et al.*, 1991; Hall *et al.*, 1992; Weller *et al.*, 1993). The gene is large, spanning over 80-90 kb of chromosomal DNA. Human and rat IGF-I genes are the most completely characterised and consist of six exons (Rotwein *et al.*, 1986; Tobin *et al.*, 1990; Hall *et al.*, 1992; Roberts *et al.*, 1987b). The exons and the mRNAs they encode are named according to a consensus reached in 1991 (Holthuizen *et al.*, 1991). Processing of the IGF-I gene is complex, with multiple transcription initiation sites, alternate splicing patterns and differential polyadenylation site usage producing IGF-I mRNAs, which vary in their 5' and 3' ends (Figure 1.3). Multiple forms of IGF-I mRNAs, ranging in size from approximately 7.5 kb to 0.8 kb, are transcribed in all mammalian species studied (Murphy *et al.*, 1987a,b; Han *et al.*, 1988; Lund *et al.*, 1986; Bell *et al.*, 1986).

The mature IGF-I protein is encoded by exons 3 and 4 of the IGF-I gene. Exon 3 encodes the first 25 amino acids of the B domain and exon 4 codes for the remainder of the mature protein

(B, C, A, D domains). Exon 3 also contains coding region for 27 amino acids of the leader peptide in the rat, human and pig and 28 amino acids in the sheep and cow. The first 16 residues of the carboxy-terminal extension peptide in each species are encoded by exon 4.

Mutually exclusive exons 1 and 2 have been described in the human (Tobin *et al.*, 1990), rat (Roberts *et al.*, 1987b), porcine (Muller & Brem, 1990; Weller *et al.*, 1993) and ovine IGF-I genes (Wong *et al.*, 1989, Dickson *et al.*, 1991). Exons 1 and 2 are alternately spliced to exon 3. Each of these leader exons codes for unique 5' untranslated regions (UTR) and the amino-terminal amino acids of a signal peptide (exon 1, encodes 21 amino acids, exon 2, encodes 5 amino acids) (1.2.1.1). Therefore, class 1 mRNAs, containing the exon 1 sequence, encode the 48 or 49 residue leader peptide. Class 2 mRNAs, containing the exon 2 sequence, contain a translation initiation codon predicting the 32 or 33 amino acid leader. In addition, all IGF-I mRNA transcripts contain the methionine codon(s) in exon 3, which predicts leader peptides of 22 and/or 25 amino acids. In the rat, deletion of a 186 base pair region of exon 1, encodes a further potential 5' untranslated sequence for IGF-I mRNAs (class 1 deletion), while a third leader exon, defined as exon 1W, has been described in the ovine gene (Wong *et al.*, 1989). Both of these exons contain only the initiation codon for the 22/25 amino acid signal protein.

Exons 5 and 6 contain coding region for the remainder of the carboxyl terminal E domain (Rotwein *et al.*, 1986; Shimatsu & Rotwein, 1987a; Hall *et al.*, 1992; Roberts *et al.*, 1987a). As described in Section 1.2.1.1, two forms of the E domain (Ea, Eb) exist in the human, rat and mouse. In the human gene, exons 5 and 6 are mutually exclusive and are alternately spliced to exon 4 (Rotwein *et al.*, 1986). Exon 6 codes for the final 19 amino acids of IGF-I Ea while exon 5 codes for 61 amino acids of the IGF-I Eb. In the rat and mouse IGF-I Ea mRNAs are also encoded by exon 6. However, the mRNA encoding IGF-I Eb peptides results from the insertion of a 52 bp exon 5 at a splice donor site within exon 6 in these species (Roberts *et al.*, 1987a; Bell *et al.*, 1986). Thus the sequence of rodent Eb domains diverge from that of human IGF-I Eb pre-pro-peptide. No evidence has been found for exon 5 sequence in the ovine IGF-I gene (Dickson *et al.*, 1991; Ohlsen *et al.*, 1993).

In the human, exons 5 and 6 encode the 3' untranslated region (Rotwein *et al.*, 1986). In the rat, this sequence is contained within exon 6 only (Hall *et al.*, 1992). Two alternative polyadenylation sites are found in exon 6 of the human gene, while in the rat and sheep, exon 6 contains 3 sites (Shimatsu & Rotwein, 1987a; Steenbergh *et al.*, 1991; Dickson *et al.*, 1991; Hoyt *et al.*, 1992). Differences in the lengths of the 3' untranslated regions account for much of the size heterogeneity of IGF-I mRNA transcripts (Steenbergh, *et al.*, 1991; Lund *et al.*, 1989; Hall *et al.*, 1992). Human and rat exon 6 encode approximately 6.4-6.6 kb of 3' untranslated region (Steenbergh *et al.*, 1991; Hoyt *et al.*, 1992) and this long 3' untranslated region provides the majority of the sequence of the large 7.5 kb IGF-I mRNA. The half life of the 7.5 kb IGF-I mRNA species is shorter than that of smaller IGF-I mRNAs *in vitro* and *in vivo* (Hepler *et al.*, 1990; Hoyt *et al.*, 1992). Altered stability of this mRNA may be due to the presence of multiple AT-rich regions and several inverted repeats within the 3' UTR, as these features have been implicated in the regulation of mRNA stability (Brawerman, 1987).

Multiple transcription initiation sites are present in both leader exons of the IGF-I gene, in all species studied (Adamo *et al.*, 1991a; Jansen *et al.*, 1991; Kajimoto & Rotwein, 1991; Kim *et al.*, 1991; Hall *et al.*, 1992; Weller *et al.*, 1993; Pell *et al.*, 1993; Ohlsen *et al.*, 1993). Transcription initiation in exon 1 occurs at several sites spread over a disperse region 170-390 nucleotides from the 3' end of the exon. A further site at 1155 nucleotides has been reported in human exon 1 (Jansen *et al.*, 1991), but was not detected by others (Kim *et al.*, 1991). Exon 2 contains a more defined region of transcription initiation, 60-80 nucleotides from the 3' end of the exon (Jansen *et al.*, 1991; Adamo *et al.*, 1991a; Hall *et al.*, 1992; Pell *et al.*, 1993; Weller *et al.*, 1993). An upstream start site at 750-850 nucleotides is present in rat, sheep and human exon 2 (Adamo *et al.*, 1991a; Jansen *et al.*, 1991; Ohlsen *et al.*, 1993), however the majority of transcription is initiated at the downstream site.

The regions upstream of both exons 1 and 2 lack distinct regulatory regions. Typical promoter regions, such as a TATA box, are absent and the region is not GC rich. However, putative promoter regions, which span several hundred basepairs, have been described for both exons 1 (promoter 1) and 2 (promoter 2) in the rat and human IGF-I gene (Kim *et al.*, 1991; Hall *et al.*, 1992; Lowe & Teasdale, 1992; Jansen *et al.*, 1992; Adamo *et al.*, 1993). Transcriptional

activity of rat promoter 1, but not promoter 2, is detected in transfection studies using a human neuroephithelioma cell line (SK-N-MC) (Hall *et al.*, 1992). However, both promoters of the rat gene are active when transfected into CHO cells (Adamo *et al.*, 1993). Both promoters of the human gene enhance transcription of a linked reporter gene in SK-N-MC cells and in ovarian carcinoma cells (Jansen *et al.*, 1992). Promoter 1 is more active than promoter 2 in SK-N-MC cells while the reverse is true in the ovarian cell line, indicating that the activity of the promoters is cell type specific. Preliminary inspection of the DNA sequence proximal to the human IGF-I promoter 1 has identified consensus binding sites for several transcription factors (Kim *et al.*, 1991). However, the specific *cis*-acting elements and *trans* acting factors which regulate the expression of the IGF-I gene remain to be determined.

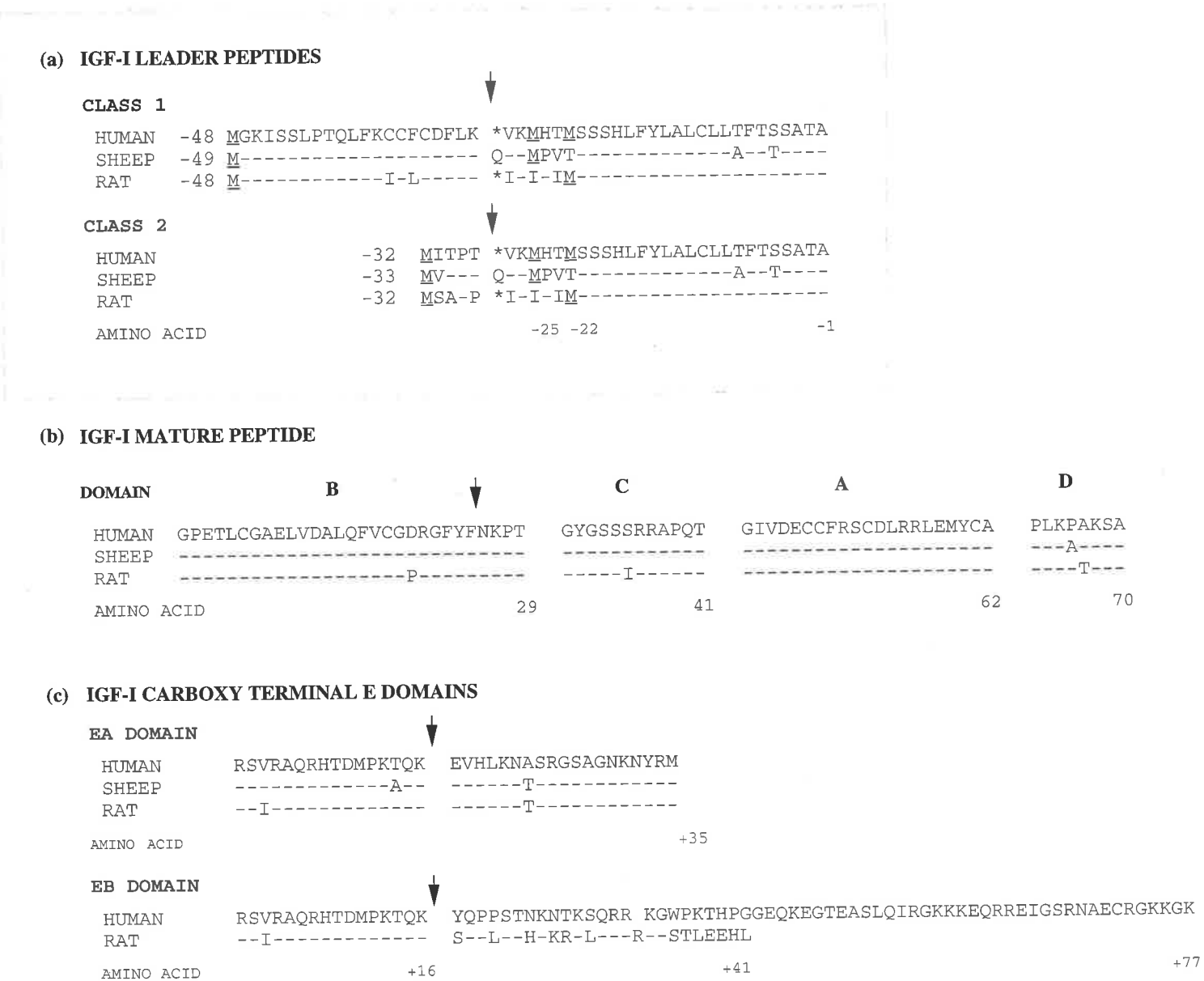
The human IGF-I gene, and its mRNAs are depicted in Figure 1.2. The significance of the multiple IGF-I mRNAs and the different precursor proteins which they encode is uncertain. As noted, IGF-I mRNAs containing the entire 3' untranslated region have a reduced stability. Foyt *et al.* (1991) report that class 1 deletion IGF-I mRNAs in the rat are associated with polysomes to a greater extent than class 1 and 2 variants, while IGF-I mRNAs containing the longest 3' untranslated region are not detected in polysomal RNA. Thus IGF-I mRNAs with different 5' and 3' regions may be translated differently. Expression of the alternative IGF-I mRNA forms is differentially regulated and occurs in a developmental and tissue specific manner suggesting that these regions have a physiological significance. In addition, it has been suggested that the alternative leader peptides of the IGF-I pre-pro-peptides may have a role in targeting of the IGF-I protein, with IGF-I pre-pro-peptides translated from class 2 IGF-I mRNAs targeted to the circulation, while class 1 IGF-I mRNAs produce locally acting IGF-I (Lund, 1994; Gilmour; 1994). In support of this view, hepatic class 2 IGF-I mRNA expression parallels changes in circulating IGF-I when nutritional or growth hormone status is altered in sheep and pigs (Pell *et al.*, 1993; Weller *et al.*, 1994).

FIGURE 1.2 Amino acid sequence of insulin-like growth factor-I.

The amino acid sequence of the mature IGF-II protein from sheep, rat and human is shown in (b). In each species IGF-II contains 70 amino acids, and the B, C, A and D domains are indicated.

Several sequences are suggested for pre-pro-IGF-I. Putative methionine translation initiation sites (M) are indicated. Leader peptides of 48 amino acids (class 1) or 32 amino acids (class 2) are predicted in human and rat. In sheep class 1 leader peptides contain 49 amino acids due to the addition of a glutamine (*). Leader peptides of 25 amino acids in human and sheep IGF-I or 22 amino acids in rat are also suggested (Section 1.2.1.2).

Exon arrangement of the human IGF-I gene is indicated in Figure 1.3. The initial 21 amino acids of class 1 IGF-I mRNAs are encoded by exon 1. The initial 5 amino acids of class 2 IGF-I mRNAs are encoded by exon 2. The remaining 27 amino acids in the leader peptides and the initial 25 amino acids of the mature peptide (B domain) are encoded by exon 3. The remainder of the mature peptide and the initial 16 amino acids of the carboxy terminal E domain are encoded by exon 4. In each species exon 6 encodes the final 19 amino acids of the Ea domain. No sequence for exon 5 has been identified in sheep. In the human exon 5 encodes the final 61 amino acids of the Eb domain. A 41 amino acid Eb domain is encoded in rat by the insertion of exon 5 sequence within a splice donor site in exon 6.


(a) IGF-I LEADER PEPTIDES
CLASS 1
HUMAN -48 MGKISSLPTQLFKCCFCDFLK *VKMHTMSSSHLFYLALCLLTFTSSATA
SHEEP -49 M---------- Q--MPVT----------A--T----
RAT -48 M----------I-L----- *I-I-IM----------
CLASS 2
HUMAN -32 MITPT *VKMHTMSSSHLFYLALCLLTFTSSATA
SHEEP -33 MV--- Q--MPVT----------A--T----
RAT -32 MSA-P *I-I-IM----------
AMINO ACID -25 -22 -1
(b) IGF-I MATURE PEPTIDE
DOMAIN B C A D
HUMAN GPETLCGAELVDALQFVCGDRGFYFNKPT GYGSSSRRAPQT GIVDECCFRSCDLRRLEMYCA PLKPAKSA
SHEEP ---A----
RAT ----------P--------- -----I------ ----T---
AMINO ACID 29 41 62 70
(c) IGF-I CARBOXY TERMINAL E DOMAINS
EA DOMAIN
HUMAN RSVRAQRHTDMPKTQK EVHLKNASRGSAGNKNYRM
SHEEP ----------A-- ------T----------
RAT --I---------- ------T----------
AMINO ACID +35
EB DOMAIN
HUMAN RSVRAQRHTDMPKTQK YQPPSTNKNTKSQRR KGWPKTHPGGEQKEGTEASLQIRGKKKEQRREIGSRNAECRGKKGK
RAT --I---------- S--L--H-KR-L---R--STLEEHL
AMINO ACID +16 +41 +77

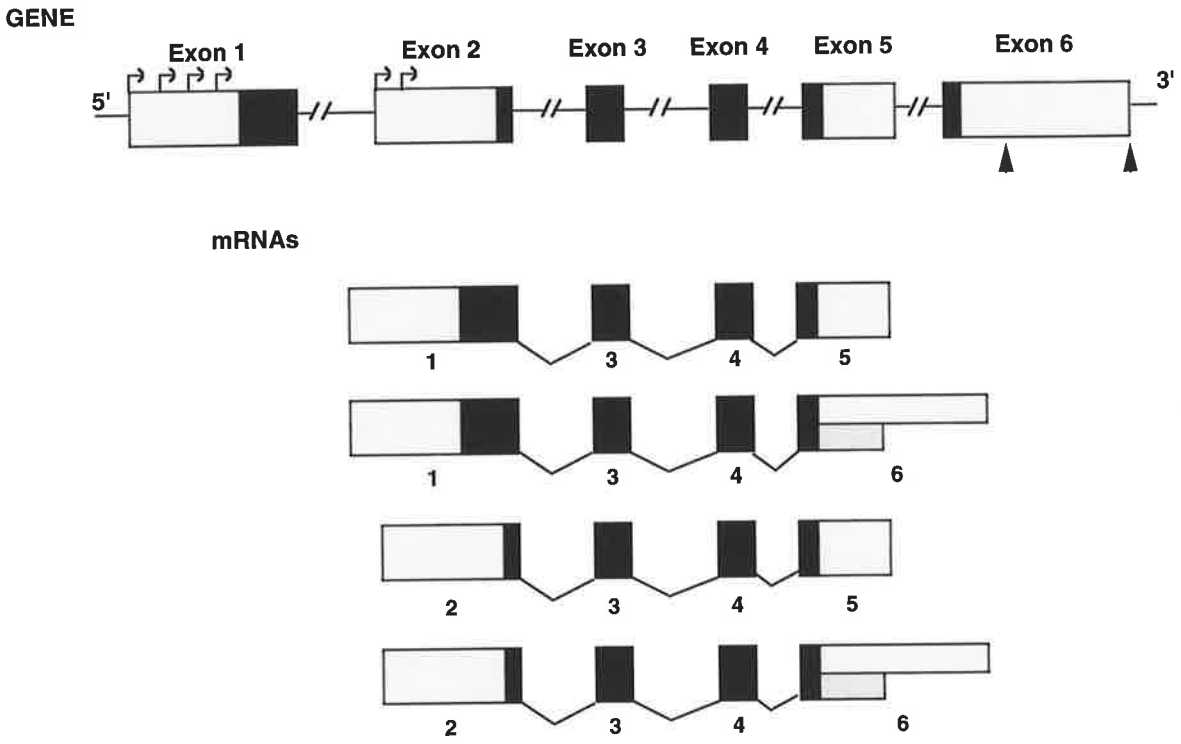


FIGURE 1.3 Human IGF-I gene and mRNAs.

Exons are indicated by boxes. Solid regions represent coding sequence for the IGF-I pre-pro-protein. Hatched regions represent untranslated regions of the IGF-I mRNAs.

Alternative polyadenylation sites in exon 6 are indicated by arrows ▲. Multiple transcription initiation sites ↱ are present in exons 1 and 2.

Alternative splicing of exons 1 and 2 produces IGF-I mRNAs containing different 5' untranslated regions which potentially encode IGF-I pre-pro-proteins with alternate leader peptides. Alternative splicing of exons 5 and 6 in the human gene produces IGF-I mRNAs containing different 3' untranslated regions and encoding alternate carboxy-terminal E domains (see Figure 1.2).

This figure is not drawn to scale. The figure was adapted from data reviewed in Section 1.2.1.2, Jansen *et al.*, 1983; Rotwein, 1986; Rotwein, 1991).

1.2.2 *Insulin-like growth factor-II*

1.2.2.1 *IGF-II peptide.*

The sequence of the IGF-II peptide has been directly determined (Rinderknecht & Humbel, 1978b; Marquadt *et al.*, 1981; Francis *et al.*, 1988, 1989a,b) or deduced from cDNAs (Bell *et al.*, 1984; Dull *et al.*, 1984; Stempien *et al.*, 1986; Soares *et al.*, 1986; Levinovitz *et al.*, 1992a; O'Mahoney & Adams, 1989) in seven mammalian species. As for IGF-I, the sequence is highly conserved, with 60 of the 67 amino acids invariant. Porcine IGF-II differs in one amino from human IGF-II (Francis *et al.*, 1989a), while three substitutions occur in bovine IGF-II (Francis *et al.*, 1988), four in rat and ovine IGF-II (Soares *et al.*, 1986; Francis *et al.*, 1989b) and six in mouse IGF-II (Stempien *et al.*, 1986). IGF-II is a 67 amino acid peptide consisting of a 28 residue amino terminal B domain, a C domain of 12 amino acids, an A domain of 21 amino acids and a carboxyl terminal D domain of 6 amino acids (Rinderknecht & Humbel, 1978b) (Figure 1.4).

In contrast to IGF-I, a single 180 amino acid precursor protein for IGF-II is produced in the rat, mouse and human (Bell *et al.*, 1984; Dull *et al.*, 1984; Stempien *et al.*, 1986). Pre-pro-IGF-II consists of a 24 amino acid signal peptide, the 67 residue IGF-II and an 89 amino acid carboxyl terminal extension peptide. The E domain in the ovine and bovine pre-pro-IGF-II contains 88 amino acids (Brown *et al.*, 1990; O'Mahoney & Adams, 1989). Sixty-seven of the 89 amino acids in the E domain are invariant across these species.

IGF-II mRNA from a number of fetal rat tissues (Romanus *et al.*, 1988) and from cultures of the BRL-3A rat liver cell line (Acquaviva *et al.*, 1982; Rechler *et al.*, 1985a) directs the synthesis of 22 000 molecular weight pre-pro-IGF-II in cell free translation studies. Pre-pro-rat IGF-II is cotranslationally processed to 20 000 molecular weight pro-IGF-II in the presence of microsomal membranes (Yang *et al.*, 1985), consistent with the removal of the signal peptide. Pro-IGF-II does not appear to be secreted, but is processed into intermediate forms of ~19, ~15, ~10, and ~8 kDa proteins and the mature 7.5 kDa IGF-II (Yang *et al.*, 1985). The intermediate forms are detected in media and intracellularly following biosynthetic labelling of intact BRL-3A cells, indicating that processing occurs both before and after secretion (Yang *et*

al., 1985). A 40 amino acid peptide derived from the IGF-II E domain is detected in serum of neonatal rat pups (Hylka *et al.*, 1987).

Higher molecular weight forms of IGF-II are found in adult and fetal human plasma (Zumstein *et al.*, 1985; Gowan *et al.*, 1987; Hill, 1990; Daughaday & Trivedi, 1992) and incompletely processed forms of IGF-II are produced and secreted by human tumours (Haselbacher *et al.*, 1987; reviewed by Daughaday; 1991).

1.2.2.2. *IGF-II gene and mRNAs*

The single gene for IGF-II is located on the short arm of human chromosome 11 (Brissenden *et al.*, 1984; Tricoli *et al.*, 1984), 1.4 kb downstream of and contiguous with the insulin gene (de Pagter-Holthuizen, 1987). The human gene is 30 kb in size and consists of 9 exons (de Pagter-Holthuizen *et al.*, 1988; Holthuizen *et al.*, 1990). Rat and mouse IGF-II genes have 6 exons and span approximately 12 kb (Frunzio *et al.*, 1986; Soares *et al.*, 1986; Ueno *et al.*, 1987, 1989; Rotwein & Hall, 1990) (Figure 1.5). In each species the coding region for pre-pro-IGF-II is contained within the final three exons (human, exons 7-9; rat, 4-6). Exon 7 of the human gene encodes the signal peptide and the B domain of the IGF-II peptide. Exon 8 contains sequence for the remaining 39 amino acids of IGF-II and for the first 11 residues of the E domain. The final 78 amino acids of the E domain are encoded by exon 9. All coding IGF-II mRNAs produce the same IGF-II precursor protein.

Transcription of the IGF-II gene is complex and, as for IGF-I, multiple size forms of IGF-II mRNA are produced. IGF-II mRNAs range in size from 6.0 to 1.8 kb. The various mRNA forms differ only in the 5' and 3' untranslated regions. Different length 5' untranslated regions are produced by alternative splicing of the 5' non-coding leader exons (exons 1-6 in the human gene, 1-3 in the rat and mouse gene). The human IGF-II gene contains four promoters located 5' to exons 1, 4, 5 and 6. IGF-II mRNAs containing 5' untranslated regions specified by exons 1-3, 4, 5 or 6, alternately spliced to the coding exons 7-9 are transcribed (Figure 1.4). Similar processes occur in the rat gene, with three promoters adjacent to each of the 5' exons resulting in mRNAs containing exons 1, 2 or 3 alternatively spliced to the coding exons, 4-6.

The rat gene lacks the most 5' of the promoters of the human IGF-II gene and the first three associated exons.

The final coding exon (human exon 9, rat exon 6), also transcribes the 3' untranslated region. At least two polyadenylation sites are present within this region in the human IGF-II gene (de Pagter-Holthuizen *et al.*, 1988) and a number of sites have been described in the rat gene (Chiariotti *et al.*, 1988; Ueno *et al.*, 1989). In addition, a polyadenylated IGF-II mRNA species of 1.8 kb, that does not encode the IGF-II protein, is expressed in fetal and adult human tissues, and in rat and mouse tissues (de Pagter-Holthuizen *et al.*, 1988; Holthuizen *et al.*, 1993). This mRNA consists of 3' untranslated sequence encoded by exon 9 in the human (exon 6 in rodents) and is formed through endonucleolytic cleavage of IGF-II mRNAs (Miensma *et al.*, 1991; 1992).

The human IGF-II gene and mRNAs are depicted in Figure 1.4. In man, major IGF-II mRNAs of 6.0, 5.0, 5.3, 4.8, 2.2 and 1.8 kb are transcribed. In adult liver, promoter 1 is activated, while the other three promoters are repressed, and only the 5.3 kb IGF-II mRNA is expressed. Promoters 2, 3 and 4 are expressed in human fetal tissues and adult non-hepatic tissues. Three major IGF-II mRNAs of 3.8 kb (encoded by exons 1, 4, 5, 6), 4.6 kb (exons 2, 4, 5, 6) and 3.6 kb (exons 3, 4, 5, 6) are detected in fetal tissues and/ or rat cell lines (Frunzio *et al.*, 1986; Soares *et al.*, 1986; Gray *et al.*, 1987; Chiariotti *et al.*, 1988). Minor size forms also result from the use of alternative polyadenylation sites (Ueno *et al.*, 1989; Holthuizen *et al.*, 1993).

The nucleotide sequences of the human IGF-II promoters have been examined for characteristic promoter elements. Promoter 1 does not contain TATA or CCAAT boxes, but has a GC rich region of around 80 nucleotides. The 184 nucleotides upstream of exon 1 are necessary for maximum promoter 1 activity (Van Dijk *et al.*, 1991) and a binding site for the liver specific transcription factor CCAAT/Enhancer binding protein (C/EBP) is found within this region (Van Dijk *et al.*, 1992a). Regions upstream of these 184 nucleotides repress promoter 1 activity in transfected cells (Van Dijk *et al.*, 1991). A 67 nucleotide inverted repeat located within this region may be involved in the negative regulation of promoter 1 expression (de Pagter -Holthuizen *et al.*, 1987; Van Dijk *et al.*, 1991).

Promoter 3, in contrast, contains a TATA box, a CCAAT box and binding sites for a number of transcription factors (Van Dijk *et al.*, 1991, 1992b; Raizis *et al.*, 1993; Holthuizen *et al.*, 1993). Five of these regions resemble the consensus sequence for Sp1, a general transcription factor (Raizis *et al.*, 1993). In addition, at least three putative binding sites for the early growth response factors ERG-1 and ERG-2 are found within promoter 3 (Van Dijk *et al.*, 1992b; Raizis *et al.*, 1993). The zinc finger protein encoded by the Wilms tumour suppressor gene, WT1, also binds to this consensus sequence (Madden *et al.*, 1991). While the ERG proteins act as transcriptional activators (Lemaire *et al.*, 1990), WT1 has been shown to suppress the activity of IGF-II promoter 3 in transient transfection assays of Hep3B cells (Drummond *et al.*, 1992). Extensive methylation of promoter 3 in cells with low activity of this promoter, suggests that DNA methylation may also be involved in regulation of IGF-II gene expression (Raizis *et al.*, 1993). Human promoter 4 contains a TATA-like sequence 25 bases upstream from the cap site (Van Dijk *et al.*, 1991; Hyun *et al.*, 1993). Two negative regulatory regions, a positive regulatory region and binding sites for Sp1 have been identified in the 5' flanking sequence of promoter 4 (Hyun *et al.*, 1993). Promoter 2 is not active following transient transfection into Hep3B or HeLa cells and the promoter elements have not been characterised (Van Dijk *et al.*, 1991). Promoter 2 produces a 5.0 kb IGF-II mRNA which is a minor form in fetal liver, but is expressed in certain tumour cell lines (Holthuizen *et al.*, 1990).

Promoter regions upstream of exons 1, 2 and 3 of the rat gene have been described (Frunzio *et al.*, 1986; Ueno *et al.*, 1987; Evans *et al.*, 1988; Matsuguchi *et al.*, 1990). These promoters are homologous to human promoters 2, 3 and 4 and some of the promoter elements described for the human promoters are present in the rat gene.

A further 5' non-coding exon, located between exons 4 and 5 of the human IGF-II gene, has been described in human histiocytoma tissue (Ikejiri *et al.*, 1991). A 5.0 kb IGF-II mRNA species containing sequence from exon 4, the additional exon, and exons 7-9 is expressed in this tissue. Therefore two exon splicing arrangements are proposed for the 5.0 kb human IGF-II mRNA (Holthuizen *et al.*, 1990; Ikejiri *et al.*, 1991). Ikejiri *et al.* (1991) propose a nomenclature for a ten exon human IGF-II gene in which their novel exon is incorporated as exon 5 and the coding exons become exons 8-10.

A similar nomenclature has been used by Ohlsen *et al.* (1994) to describe the ovine IGF-II gene. The ovine gene resembles the human IGF-II gene, and consists of nine known exons which share between 70-90% homology with the equivalent human exons (O'Mahoney *et al.*, 1989, 1991, Brown *et al.*, 1990; Demmer *et al.*, 1993, Ohlsen *et al.*, 1994). The sheep gene contains an exon homologous to the novel exon 5 described by Ikejiri *et al.*, 1991, but lacks an exon homologous to human exon 2 (Ohlsen *et al.*, 1994). The expression pattern of the ovine IGF-II gene is similar to the human gene, with an mRNA of 5.3 kb or 5.1 kb expressed only in adult liver (O'Mahoney *et al.*, 1991b; Demmer *et al.*, 1993; Delhanty & Han, 1993). This transcript originates from the adult liver specific promoter 1 (Ohlsen *et al.*, 1994). Expression of ovine IGF-II exon 4 was not observed in adult or fetal sheep liver. In contrast to the novel exon 5 of the human gene (Ikejiri *et al.*, 1991), which is expressed in tumour tissue only, the equivalent exon of the sheep gene is present in both fetal and adult liver. Similarly, mRNAs equivalent to those produced from the human IGF-II promoters 3 and 4 are detected in pre- and postnatal sheep hepatic RNA by polymerase chain reaction analysis (Ohlsen *et al.*, 1994). However, Demmer *et al.* (1993) report that these promoters become transcriptionally inactive at 28 weeks postnatal age in sheep and only the 5.1 kb IGF-I mRNA (derived from promoter 1) is seen in adult liver by Northern blot analysis (Demmer *et al.*, 1993; Delhanty & Han, 1993). The distance between the insulin and IGF-II genes in sheep (762 nucleotides) is less than that of humans, and a number of the potential promoter 1 elements identified in the human gene, including the binding site for the CCAAT/Enhancer binding protein, are absent from the sheep gene. No detailed analysis of the promoters of the ovine IGF-II gene has been performed.

The significance of the different size forms of IGF-II mRNAs is not certain. However, in a number of human cell lines and fetal liver a proportion of the 6.0 kb and 2.2 kb IGF-II mRNAs are found in the untranslated free messenger ribonucleoprotein particle fraction, while the 5.0 kb and 4.8 kb mRNAs are located in the polysomes (Nielsen *et al.*, 1990; De Moor *et al.*, 1994). This suggests that the different IGF-II mRNA forms have a differential translation efficiency. The observation that the amount of IGF-II mRNA in human carcinoma tissue is not related to the abundance of IGF-II protein supports the suggestion that IGF-II production can be regulated by post-transcriptional mechanisms (Haselbacher *et al.*, 1987).

FIGURE 1.4 Amino acid sequence of insulin-like growth factor-II.

The amino acid sequence of the mature IGF-II protein from sheep, rat and human is shown in (b). In each species IGF-II contains 67 amino acids, and the B, C, A and D domains are indicated.

IGF-II is translated as a 180 amino acid pre-pro-protein in the human and rat, consisting of a 24 amino acid leader peptide (a), the mature IGF-II protein (b) and an 89 amino acid carboxy-terminal E domain (c). Ovine pre-pro-IGF-II contains an 88 amino acid E domain. * indicates insertion of a threonine in the sequence of the E domain in sheep. The pre-pro-IGF-II protein in sheep contains a total of 179 amino acids.

Exon 7 of the human IGF-II gene codes for the leader peptide and the initial 28 amino acids of the mature IGF-II protein, exon 8 encodes the remainder of the mature peptide (C A D) domains and the initial 11 amino acids of the E domain. The remainder of the E domain is coded by exon 9 (Section 1.2.2.2). Exon boundaries are designated by arrows above the amino acid sequence. These exons are designated exons 8 (human 7), 9 (human 8) and 10 (human 9) of the ovine gene, according to the nomenclature of Ohlsen *et al.*, 1994. Sequence data taken from Rotwein, 1991; O'Mahoney & Adams, 1989.

(a) IGF-II LEADER PEPTIDE

```
HUMAN     MGIPMGKSMLVLLTFLAFASCCIA
SHEEP     ---TA-----A--A--------Y-
RAT       ----V--------IS--P-L----

AMINO ACID                    +24
```

(b) IGF-II MATURE PEPTIDE

```
DOMAIN          B                          C              A                    D

HUMAN     AYRPSETLCGGELVDTLQFVCGDRGFYF   SRPASRVSRRSR   GIVEECCFRSCDLALLETYCA   TPAKSE
SHEEP     ----------------------------   ---S--IN----   --------------------   A-----
RAT       ----------------------S-----   ---S--AN----   --------------------   ------

AMINO ACID                        +28            +40                  +61       +67
```

(c) IGF-II CARBOXY TERMINAL E DOMAIN

```
HUMAN     RDVSTPPTVLPDNFPRYPVGKFFQYDTWKQSTQRLRRGLPALLRARRGHVLAKELEAFREAKRHRPLIALPTQDPA*HGGAPPEMASNRK  +89
SHEEP     ----ASTT----D-TA--------S----------------F------RT-------L----S-------------T----SS-AS-D    +88
RAT       -----SQA----D----------KF---R--AG---------------RM------------------V--PK---*----SS--S--HQ  +89

                                                                                                AMINO ACID
```

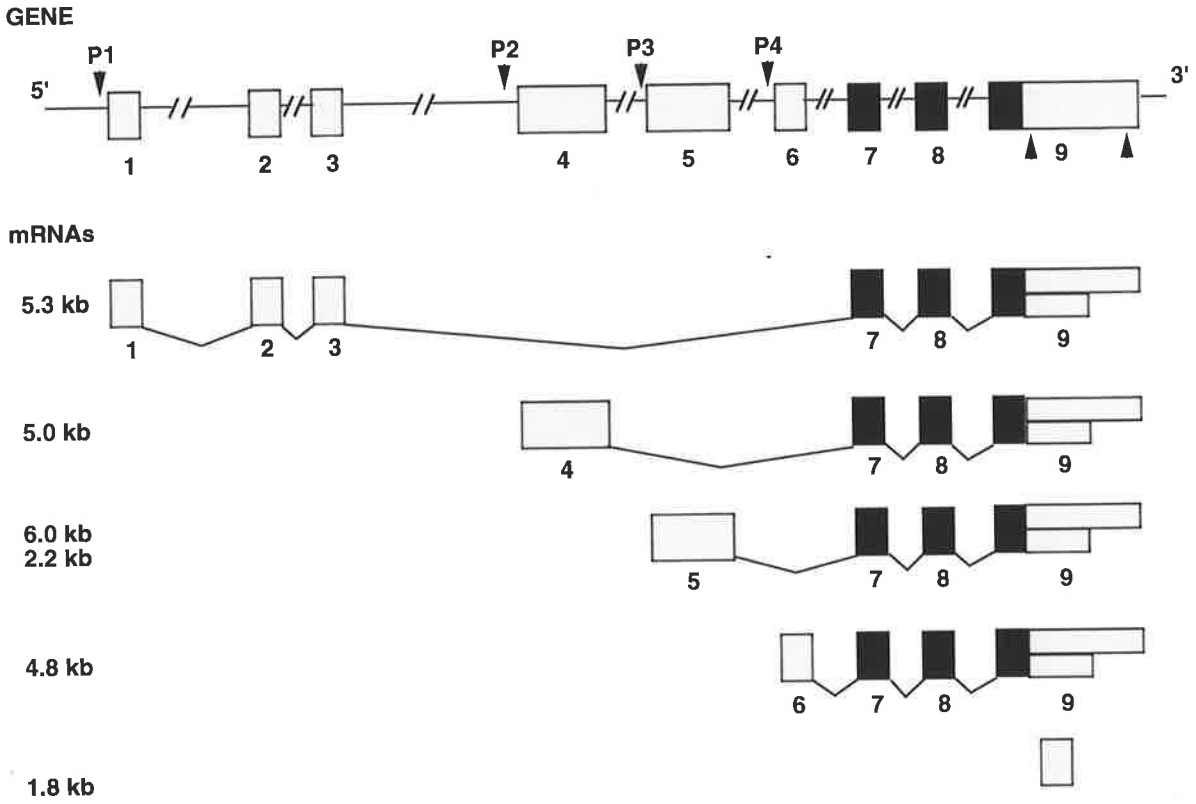


FIGURE 1.4 Human IGF-II gene and mRNAs.

Exons are indicated by boxes. Solid regions represent coding sequence for the IGF-II pre-pro-protein, which is contained within exons 7, 8 and 9 (see Figure 1.3).

Hatched regions represent untranslated regions of the IGF-II mRNAs. Alternative splicing of the IGF-II gene, due to the presence of four promoters (P1 to P4) produces IGF-II mRNAs, with alternative 5' untranslated regions, as indicated. Two polyadenylation sites are indicated ▲ in exon 9. A 1.8 kb IGF-II mRNA is also produced by endonucleolytic cleavage within exon 9.

This figure is not drawn to scale. The figure was adapted from De Pagter-Holthuizen et al., 1987, 1988; Holthuizen, 1990; Rotwein, 1991).

1.2.3 *Regulation of production of the insulin-like growth factors*

The following section reviews current knowledge of the factors regulating IGF-I and -II gene expression and circulating protein levels in postnatal life. The factors regulating prenatal production of the IGFs are reviewed subsequently in Section 1.3.

1.2.3.1 Factors regulating production of IGF-I.

Tissue specific expression of the IGF-I gene

IGF-I mRNA is detected in many adult rat tissues, but the highest levels of expression are found in adult liver (Murphy *et al.*, 1987a; Mathews *et al.*, 1986). The non-hepatic tissue(s) containing the highest abundance of IGF-I mRNA in adult rodents are reported in separate studies to be the uterus and ovary (Murphy *et al.*, 1987a), pancreas (Mathews *et al.*, 1986) and adipose tissue (Moller *et al.*, 1991; Gosteli-Peter *et al.*, 1994). Hepatic IGF-I mRNA abundance is at least 50 times greater than that of skeletal muscle in the rat (Murphy *et al.*, 1987a; Moller *et al.*, 1991) and at least 20 fold greater in young sheep and cattle (Pell *et al.*, 1993; Hannon *et al.*, 1991). In contrast, in humans and pigs, the relative abundance of IGF-I mRNA in muscle and liver is similar, and IGF-I mRNA is most abundant in adipose tissue (Moller *et al.*, 1991; Coleman *et al.*, 1994). Therefore the tissue pattern of expression of IGF-I varies between species. Within a tissue, IGF-I expression varies with cell type and region. For example, IGF-I mRNA is detected in granulosa cells of the rat ovary (Hernandez *et al.*, 1989) and in epithelial cells of the thick ascending limbs of Henle's loops in rat kidney (Chin *et al.*, 1992).

The liver is proposed as the major source of IGF-I protein in the blood of rats. Studies of incorporation of ^{35}S-cysteine into the IGF-I molecule by the isolated perfused rat liver indicate that IGF-I is continuously synthesised and secreted by the liver, and that the rate of hepatic IGF-I synthesis can account for the steady state level of IGF-I in rat plasma (Schwander *et al.*, 1983). In addition, plasma concentrations of IGF-I correlate with hepatic IGF-I mRNA abundance in a variety of physiological states in rats (Emler & Schalch, 1987; Goldstein *et al.*, 1988). However, the liver is not the major site of IGF-I expression in pigs and humans (Moller

et al., 1991; Coleman *et al.*, 1994) and it is possible that other tissues act as sources of circulating IGF-I in these species.

Developmental regulation of IGF-I

Expression of IGF-I is developmentally regulated. Developmental changes in plasma levels of IGF-I and expression of the IGF-I gene by fetal tissues are discussed in detail in Section 1.3.2. In general, plasma IGF-I and hepatic IGF-I gene expression increase throughout fetal life. In the human fetus, IGF-I concentrations at birth are lower than adult levels (Lassarre *et al.*, 1991), while in the ovine fetus at term concentrations of IGF-I are similar to adult levels (Gluckman & Butler, 1983; Carr *et al.*, 1995). Expression of IGF-I in liver is low during fetal life and increases postnatally (Lund *et al.*, 1986; Dickson *et al.*, 1991; Yang *et al.*, 1991). IGF-I mRNA is 10-50 times more abundant in adult rat liver than in fetal rat liver (Lund *et al.*, 1986). However, IGF-I mRNA is present in many fetal tissues in addition to liver (Lund *et al.*, 1986; Han *et al.*, 1988; Adamo *et al.*, 1989; Dickson *et al.*, 1991). In the human fetus many extrahepatic tissues contain IGF-I mRNAs in greater abundance than the liver (Han *et al.*, 1988). In a number of tissues, in the rat and sheep fetus, including muscle and lung, IGF-I expression is highest during fetal life (Adamo *et al.*, 1989; Yang *et al.*, 1991).

Regulation of IGF-I by growth hormone

Postnatally, growth hormone is a major regulator of circulating IGF-I (Daughaday & Rotwein, 1989) and IGF-I gene expression (Murphy *et al.*, 1987b; Roberts *et al.*, 1986; Lowe *et al.*, 1987; Lowe *et al.*, 1988; Hall *et al.*, 1992). Growth hormone treatment increases IGF-I mRNA abundance in cultured hepatocytes (Johnson *et al.*, 1989), other primary cell lines (Doglio *et al.*, 1987) and in the liver of normal animals (Grant *et al.*, 1991; Pell *et al.*, 1993; Mathews *et al.*, 1986). IGF-I mRNA abundance is reduced in liver and non-hepatic tissues of growth hormone deficient hypophysectomised rats and *lit/lit* mice, and is restored by growth hormone treatment (Roberts *et al.*, 1986; Mathews *et al.*, 1986; Murphy *et al.*, 1987b; Lowe *et al.*, 1988). Administration of growth hormone to normal sheep and pigs increases hepatic IGF-I mRNA and serum IGF-I protein, but does not alter muscle IGF-I gene expression (Grant *et al.*, 1991; Pell *et al.*, 1993; Coleman *et al.*, 1994). In growing pigs, growth hormone administration increases IGF-I mRNA in adipose tissue (Coleman *et al.*, 1994). In

hypophysectomised rats, growth hormone increases IGF-I mRNA abundance through a rapid increase in gene transcription (Bichell *et al.*, 1992).

Regulation of IGF-I by nutrition

Nutritional status is an important influence on circulating IGF-I and on IGF-I gene expression in tissues (reviewed by Clemmons & Underwood, 1991; Thissen *et al.*, 1994; Straus, 1994). Plasma IGF-I is reduced in children with protein-energy malnutrition (Soliman *et al.*, 1986), in fasted human volunteers (Clemmons *et al.*, 1981) and in experimental animals which have been fasted (Phillips & Young, 1976; Maes *et al.*, 1983a) or maintained on protein restricted diets (Maes *et al.*, 1984; Maiter *et al.*, 1988; VandeHaar *et al.*, 1991). Concentrations of IGF-I are restored by refeeding (Phillips & Young, 1976; Clemmons *et al.*, 1981; Maes *et al.*, 1983a; Soliman *et al.*, 1986) and in human volunteers, plasma IGF-I correlates positively with nitrogen balance during fasting and refeeding (Clemmons *et al.*, 1981).

Fasting reduces the abundance of IGF-I mRNA in liver (Emler & Schalch, 1987; Bornfeldt *et al.*, 1989; Straus & Takemoto, 1990a) and extrahepatic tissues in rats (Lowe *et al.*, 1989; Bornfeldt *et al.*, 1989). A positive association between plasma IGF-I and IGF-I gene expression in liver is observed in fasted rats (Emler & Schalch, 1987), suggesting that reduced hepatic production is responsible for the decrease in plasma IGF-I. Restriction of dietary protein in rats also reduces IGF-I mRNA in liver (Straus & Takemoto, 1990b; VandeHaar *et al.*, 1991), and muscle (VandeHaar *et al.*, 1991), but does not alter the abundance of IGF-I mRNA in heart, kidney, brain and aorta (Bornfeldt *et al.*, 1989). Decreasing concentrations of amino acids in culture media of rat hepatocytes are associated with reduced IGF-I mRNA abundance, indicating that amino acid availability can regulate IGF-I expression (Pao *et al.*, 1993).

Nutrient restricted rats are resistant to the actions of growth hormone (Phillips & Young, 1976, Maiter *et al.*, 1988; Maes *et al.*, 1988), thus decreased IGF-I production in undernourished animals may be due, at least in part, to growth hormone resistance. Growth hormone resistance in fasted rats is associated with a reduction in growth hormone receptor number (Baxter *et al.*, 1981; Maes *et al.*, 1983a; Straus & Takemoto, 1990a). In contrast, intracellular, post-receptor

resistance to growth hormone actions is implicated during protein restriction (Maiter *et al.*, 1988; Maes *et al.*, 1988; Thissen *et al.*, 1990a,b). Growth hormone levels are also elevated in malnourished children, concomitant with reduced plasma IGF-I and poor growth (Soliman *et al.*, 1986).

In fasted rats, all IGF-I mRNA size forms decrease in a co-ordinate manner (Straus & Takemoto, 1990a), and IGF-I gene expression is regulated at the transcriptional level (Hayden *et al.*, 1994). In contrast, dietary protein restriction preferentially decreases the 7.5 kb IGF-I mRNA (Straus & Takemoto, 1990b; Thissen *et al.*, 1991). As previously discussed (1.2.1.2), altered stability of the 7.5 kb IGF-I mRNA, under conditions such as protein restriction, is associated with the long 3' UTR of this mRNA. Injection of growth hormone to protein restricted rats for 7 days restores liver IGF-I mRNA, but not plasma IGF-I, suggesting that other post-transcriptional mechanisms can regulate IGF-I production during protein restriction (Thissen *et al.*, 1991). For example, protein restriction increases clearance of IGF-I from the blood of rats (Thissen *et al.*, 1992).

Regulation of IGF-I by insulin

Plasma levels of IGF-I and hepatic abundance of IGF-I mRNA are reduced in streptozotocin diabetic rats and pigs and are restored towards normal by administration of insulin (Goldstein *et al.*, 1988; Leaman *et al.*, 1990; Yang *et al.*, 1990). Insulin stimulates IGF-I gene expression in cultured hepatocytes (Johnson *et al.*, 1989; Phillips *et al.*, 1991a) and potentiates the effect of growth hormone on IGF-I expression in these cells (Tollet *et al.*, 1990). IGF-I gene transcription is reduced in the liver of diabetic rats and increases with insulin treatment (Pao *et al.*, 1992), and is stimulated by addition of insulin to cultured hepatocytes (Pao *et al.*, 1993), indicating that this regulation occurs at the transcriptional level. However, plasma levels of IGF-I are not increased when growth hormone is administered to diabetic rats (Maes *et al.*, 1986; Scott & Baxter, 1986), suggesting a growth hormone resistant state. Both reduced hepatic growth hormone binding and a post-receptor defect are suggested as possible causes of growth hormone resistance in diabetic rats (Baxter *et al.*, 1980; Maes *et al.*, 1983b; Maes *et al.*, 1986). Reduced clearance of IGF-I from the blood of diabetic rats is also suggested (Scott &

Baxter, 1986). In contrast to the fall in hepatic IGF-I expression observed in insulin-deficient diabetic rats, renal IGF-I mRNA increases (Catanese *et al.*, 1993).

Regulation of IGF-I by other hormones

A number of other hormones are implicated in the regulation of IGF-I gene expression. Thyroid hormones potentiate the effect of growth hormone on IGF-I gene expression *in vivo* (Wolf *et al.*, 1989) and *in vitro* (Tollet *et al.*, 1990). The increase in hepatic IGF-I mRNA, which occurs during the perinatal period in rats, is suppressed in hypothyroid neonates (Gallo *et al.*, 1991), and IGF-I mRNA abundance is reduced in the liver of hypothyroid adult rats (Thomas *et al.*, 1993). Dexamethasone reduces IGF-I mRNA abundance in several tissues of normal rats and suppresses the increase in IGF-I mRNA, which occurs in response to growth hormone in hypophysectomised rats (Luo & Murphy, 1989).

Other hormones regulate IGF-I production at the level of their target tissue. Estrogen is the major trophic stimulus for IGF-I production in the uterus (reviewed by Murphy, 1991). Estrogen increases uterine IGF-I mRNA and protein in immature and ovariectomised rats and pigs (Murphy *et al.*, 1987c; Norstedt *et al.*, 1989; Simmen *et al.*, 1990). In contrast, chronic estrogen treatment of ovariectomised/hypophysectomised rats attenuates the normal growth hormone induction of hepatic IGF-I mRNA (Murphy *et al.*, 1988). Uterine IGF-I expression is positively regulated by progesterone (Norstedt *et al.*, 1989; Simmen *et al.*, 1990) and estrogen and progesterone are determinants of IGF-I production by ovarian granulosa cells (Hernandez *et al.*, 1989). Parathyroid hormone and prostaglandin E_2 stimulate IGF-I expression in oesteoblast enriched cultures from fetal rat bones (McCarthy *et al.*, 1989; 1991). Infusion of adrenocorticotropic hormone (ACTH) reduces IGF-I mRNA abundance in the adrenal gland of intact rats (Townsend *et al.*, 1990).

Negative feedback inhibition of IGF-I production is also suggested. IGF-I infusion reduces hepatic IGF-I mRNA in energy restricted rats (Schalch *et al.*, 1989), growth hormone deficient dwarf rats (Butler *et al.*, 1994) and hypophysectomised rats (Gosteli-Peter *et al.*, 1994), but not in normal rats (Butler *et al.*, 1994). Administration of IGF-I reduced IGF-I gene expression in kidney of dwarf rats, but did not affect IGF-I mRNA in cardiac or skeletal

muscle (Butler *et al.*, 1994). However, infusion of IGF-I into hypophysectomised rats slightly increased IGF-I mRNA in skeletal muscle (Gosteli-Peter *et al.*, 1994). High concentrations of IGF-II in rats carrying IGF-II producing tumours are associated with a decrease in serum IGF-I concentrations, but no change in hepatic IGF-I mRNA (Wilson *et al.*, 1989). Addition of serum to cultured rat fibroblasts reduces IGF-I mRNA (Lowe *et al.*, 1990), due at least in part to the actions of heat-labile proteins.

Regulation of IGF-I during tissue regeneration and hypertrophy

Factors other than hormones and nutrition influence IGF-I gene expression. IGF-I mRNA is increased in rat skeletal muscle during work-induced hypertrophy and during regeneration following ischemic injury (DeVol *et al.*, 1990; Edwall *et al.*, 1989), and is elevated in smooth muscle of hypertrophied rat bladder and portal vein (Chen *et al.*, 1994). Cardiac IGF-I mRNA levels are increased in the hypertrophied left ventricle in association with pressure overload (Hanson *et al.*, 1993) and renal hypertension (Wahlander *et al.*, 1992), and in the hypertrophied right ventricle which occurs with chronic hypoxia (Russell-Jones *et al.*, 1993). IGF-I mRNA is increased in regenerating rat pancreas (Smith *et al.*, 1991) and in experimentally demyelinated regions of the mouse central nervous system and decreases in association with subsequent remyelination (Komoly *et al.*, 1992). Compensatory growth of the kidney, following unilateral nephrectomy in rats, is associated with an increase in kidney IGF-I mRNA and protein (Fagin & Melmed, 1987). However, others report an increase in kidney IGF-I protein, but not mRNA (Lajara *et al.*, 1989).

Molecular mechanisms of regulation of IGF-I synthesis

As discussed (1.2.1.2) two promoter regions of the IGF-I gene have been described but are as yet poorly characterised. However, expression of the alternative promoters and the expression of IGF-I mRNAs containing alternative 5' untranslated regions is regulated in a tissue specific and developmental manner. In adult rats, class 1 IGF-I mRNAs (regulated by promoter 1) are expressed in liver and non-hepatic tissues, while class 2 IGF-I mRNAs (regulated by promoter 2) are predominantly expressed in liver (Lowe *et al.*, 1988). Only class 1 IGF-I mRNAs are expressed in fetal rat tissues (Adamo *et al.*, 1989; Adamo *et al.*, 1991b). Class 2 transcripts are detected in postnatal tissues in the rat, particularly the liver, from around 15 days of age.

However, whether class 1 transcripts are exclusively expressed after birth in other species is not certain. Class 2 IGF-I mRNAs are detected in multiple tissues of the mid-gestation sheep fetus by polymerase chain reaction analysis (Ohlsen *et al.*, 1993) and class 2 transcripts are observed in non-hepatic sheep tissues between birth and 4 weeks of age, after which time, class 2 expression is predominantly confined to the liver (Saunders *et al.*, 1991).

Early studies suggested that expression of class 1 and class 2 IGF-I mRNAs was differentially regulated by growth hormone. Growth hormone increases class 1 IGF-I transcripts in non-hepatic tissues of hypophysectomised rats approximately two-fold, but stimulates hepatic class 2 IGF-I expression seven to eight fold (Lowe *et al.*, 1987). Hepatic class 2 transcripts are also preferentially increased following growth hormone treatment in lambs (Saunders *et al.*, 1991; Pell *et al.*, 1993). Similarly, IGF-I mRNAs containing the Eb form of the E domain increase to a greater extent than Ea IGF-I mRNAs in the liver of growth hormone treated hypophysectomised rats (Lowe *et al.*, 1988; Adamo *et al.*, 1991c). However, a co-ordinate increase in class 1 and class 2 IGF-I mRNAs and in Ea and Eb IGF-I mRNAs has been observed immediately following growth hormone injection of hypophysectomised rats (Bichell *et al.*, 1992; Hall *et al.*, 1992) and a co-ordinate increase in hepatic class 1 and 2 IGF-I mRNAs is observed following two weeks growth hormone treatment of normal female rats (Hall *et al.*, 1992).

As noted previously the specific molecular mechanisms through which the promoter regions of the IGF-I gene are regulated have not been determined. The stimulatory effects of a number of hormones on IGF-I gene expression appear to be mediated through activation of cAMP (McCarthy *et al.*, 1990; Bichell *et al.*, 1993). The inhibitory effect of serum on IGF-I expression in fibroblasts is mediated through activation of the protein kinase-C pathway (Lowe *et al.*, 1992; Lowe *et al.*, 1993). Treatment of the human macrophage-like cell line U937 with phorbol esters, which activate protein kinase-C also decreases steady state abundance of IGF-I mRNA (Nagaoka *et al.*, 1990). Increased intracellular calcium levels also reduce IGF-I mRNA in cultured rat fibroblasts, independent of the activation of protein kinase-C (Hovis *et al.*, 1993).

Preliminary examination of DNA sequence of the putative promoter regions of the rat and human IGF-I gene has not identified binding sites for the estrogen receptor, glucocorticoid receptor or a putative growth hormone response element (Yoon *et al.*, 1990; Kim *et al.*, 1991; Bichell *et al.*, 1993). Estrogen regulation of the chicken IGF-I promoter occurs through enhancement of Fos-Jun binding to an AP1 motif (Umayahara *et al.*, 1994), however, this motif was not observed in the human promoter (Kim *et al.*, 1991). Preliminary analysis has revealed a sequence resembling the cAMP-response element (CRE) (Habener, 1990) within rat promoter 1 (Bichell *et al.*, 1993). In addition, a growth hormone responsive DNase I hypersensitive site, in chromatin of the second IGF-I intron, and DNase I sites which become hypersensitive coincident with developmental activation of the IGF-I gene, have been described (Bichell *et al.*, 1992; Kikuchi *et al.*, 1992). Binding of nuclear proteins within these regions may be involved in growth hormone and developmental regulation of IGF-I expression.

1.2.3.2 *Factors regulating production of IGF-II*

Development and tissue specific regulation of IGF-II expression

The major factor regulating expression of the IGF-II gene is development. Developmental regulation of IGF-II gene expression is highly species specific. In rat, concentrations of IGF-II mRNA in tissues and IGF-II protein in blood are high during fetal life, and decrease after birth. IGF-II protein is barely detectable in blood of adult rats (Moses *et al.*, 1980) and in the majority of adult rat tissues IGF-II mRNAs cannot be detected by Northern analysis (Frunzio *et al.*, 1986; Soares *et al.*,1986; Lund *et al.*, 1986; Chiariotti *et al.*, 1988; Gray *et al.*, 1987; reviewed by Rechler, 1991). Expression of IGF-II in adult rat brain is lower than *in utero*, but remains detectable in contrast to other tissues (Murphy *et al.*, 1987a; Gray *et al.*, 1987; Hynes *et al.*, 1988). IGF-II mRNAs in rat brain are localised to the choroid plexus and leptomeninges (Hynes *et al.*, 1988; Stylianopoulou *et al.*, 1988a).

IGF-II mRNAs are also abundant in many tissues of human, sheep, guinea pig and pig fetuses and IGF-II expression is down-regulated in most tissues at birth (Gray *et al.*, 1987; Han *et al.*, 1988; O'Mahoney *et al.*, 1991; Delhanty & Han, 1993; Levinovitz *et al.*, 1992a; Lee *et al.*, 1993). However in contrast to rodents, IGF-II protein is present and is the most abundant IGF

in postnatal blood in these species and IGF-II mRNA can be detected in a number of adult tissues (reviewed by Rechler, 1991). In adult humans, IGF-II mRNAs are present in liver, skin, peripheral nerves, muscle and adrenal, while levels in kidney, colon, uterus and stomach are low to undetectable (Gray *et al.*, 1987; Irminger *et al.*, 1987). In sheep, IGF-II expression is detected in liver, adrenal, kidney and choroid plexus (O'Mahoney *et al.*, 1991; Delhanty & Han, 1993). Choroid plexus is the only ovine tissue in which comparable levels of IGF-II mRNA are observed in the fetus and adult (Delhanty & Han, 1993). Expression of IGF-II in postnatal human and ovine liver is due to the presence of an adult liver specific promoter (1.2.2.2; Ohlsen *et al.*, 1994). In adult guinea pigs, IGF-II expression is present in muscle, liver, kidney and brain cortex, and in contrast to other species, IGF-II mRNA is most abundant in muscle (Levinovitz *et al.*, 1992). Thus the tissue specific pattern and developmental regulation of IGF-II expression is species specific.

Regulation of IGF-II by growth hormone

Growth hormone does not appear to be as important a determinant of IGF-II production as it is for IGF-I. Plasma IGF-II is normal in human patients with endogenous growth hormone excess or following growth hormone injection (Zapf *et al.*, 1981; Davenport *et al.*, 1988). However, IGF-II is reduced in blood from growth hormone deficient subjects (Zapf *et al.*, 1981). IGF-II mRNA is reduced in the brains of hypophysectomised rats and increases following injection of growth hormone into the lateral ventricle (Hynes *et al.*, 1987). In addition, IGF-II mRNA is increased in skeletal and cardiac muscle, but not liver, of postnatal rats inoculated with growth hormone secreting cells (Turner *et al.*, 1988).

Regulation of IGF-II by nutrition

IGF-II plasma levels are not altered by short term fasting in human subjects (Davenport *et al.*, 1988). However, chronic protein-energy malnutrition in children is associated with reduced concentrations of IGF-II (Soliman *et al.*, 1986). Malnourished neonatal rats also have reduced plasma levels of IGF-II (Phillips *et al.*, 1989; Donovan *et al.*, 1991). In young growing rats, brain IGF-II mRNA is not altered by 72 hours fasting (Straus & Takemoto, 1990a), but is reduced following longer term restriction of protein (Straus & Takemoto, 1990b) or energy (Straus & Takemoto, 1991a). Thus chronic, but not acute, nutrient deprivation can regulate

IGF-II production. Maintenance of BRL-3A rat liver cells in culture medium deficient in a single essential amino acid reduces the abundance of the 3.6 kb IGF-II mRNA (Straus & Takemoto, 1988). Regulation of IGF-II expression by amino acid restriction appears to be post-transcriptional, as the transcription rate of the IGF-II gene was not altered.

Regulation of IGF-II by insulin

IGF-II mRNA abundance is not altered in liver, muscle and heart of diabetic pigs (Leaman *et al.*, 1990). However, acute infusion of insulin into rats, under euglycaemic conditions increases IGF-II protein abundance in the dorsomedial hypothalamus, suprachiasmatic nucleus and in plasma and reduces the IGF-II content of the lateral hypothalamus, supraoptic nucleus and paraventricular nucleus. Injection of insulin for four days into rats reduces the abundance of IGF-II mRNA in the ventral hypothalamus and increases IGF-II mRNA in the lateral and dorsal hypothalamus (Lauterio *et al.*, 1990). This suggests that peripheral insulin concentrations may influence IGF-II production in the rat brain, but in region specific manner.

Regulation of IGF-II by steroid hormones, other hormones and factors

Injection of one day old rats with dexamethasone or nine day old rats with cortisone reduces hepatic IGF-II mRNA, suggesting that the postnatal decrease in hepatic IGF-II expression in rats may be associated with the postnatal increase in plasma glucocorticoids (Beck *et al.*, 1988; Levinovitz & Norstedt, 1989). Treatment of rats with ACTH is associated with a decrease in adrenal IGF-II mRNA, and it is uncertain whether this is related to a a concomitant increase in plasma corticosterone (Townsend *et al.*, 1990). The decline in hepatic IGF-II mRNA observed during the neonatal period in rats is delayed in hypothyroid neonates (Gallo *et al.*, 1991).

Negative feedback regulation of IGF-II production is suggested, as IGF-I or IGF-II treatment of differentiating myoblasts *in vitro* suppresses IGF-II mRNA (Magri *et al.*, 1994). A number of other growth factors, including transforming growth factor-β1 (TGFβ1) and basic fibroblast growth factor (bFGF) inhibit IGF-II expression in cultured osteoblasts (Gabbitas *et al.*, 1994). Follicle-stimulating hormone (FSH) increases IGF-II mRNA abundance in human granulosa cells (Voutilainen & Miller, 1987). Ovariectomy and subsequent estrogen replacement do not alter IGF-II mRNA in rat uterus (Norstedt *et al.*, 1989), however, 17-β-estradiol increases

IGF-II expression in an estrogen responsive breast cancer cell line (Yee *et al.*, 1988). Progesterone and promegestone, a synthetic progestogen, stimulate IGF-II production in human oesteoblast cells (Tremollieres *et al.*, 1992). IGF-II mRNA is induced in regenerating muscle of postnatal rats (Levinovitz *et al.*, 1992b) and increases during work-induced hypertrophy of rat skeletal muscle (DeVol *et al.*, 1990). In addition, the IGF-II gene is expressed at high levels in a variety of human tumours (reviewed by Rechler, 1991). In a neuroblastoma cell line retinoic acid has been shown to stimulate IGF-II gene expression (Matsumoto *et al.*, 1992).

1.2.4 *Insulin-like growth factor binding proteins*

The IGFs are present in the circulation and other biological fluids, bound to specific IGF binding proteins (reviewed by Clemmons, 1991; Baxter, 1991; McCusker & Clemmons, 1992; Cohick & Clemmons, 1994). To date, six IGF binding proteins, designated IGFBP-1 through -6, have been described (Shimasaki *et al.*, 1991a). The IGFBPs are proteins of 200 to 300 amino acids, with molecular weights ranging from 24 to 33 kDa. Considerable sequence homology exists in the amino and carboxy terminal ends of the IGFBPs, including the alignment of 18 cysteine residues (Shimasaki *et al.*, 1991a). Human and rat IGFBPs have been most extensively characterised, however IGFBPs have been detected in blood and biological fluids of other species, including the sheep (Lord *et al.*, 1991; Carr *et al.*, 1994, 1995).

Human IGFBP-1 is a 259 amino acid protein, with a molecular weight of 25 kDa, which migrates at approximately 29 kDa on SDS polyacrylamide gel electrophoresis (see McCusker & Clemmons, 1992). IGFBP-1 is present in adult blood at around 2 nmol/l (see Baxter, 1991) and has similar affinities for IGF-I and -II. In the adult, IGFBP-1 is predominantly produced in liver, decidua and uterus. The principal regulators of serum levels of IGFBP-1 are glucose and insulin. Plasma levels of IGFBP-1 show a diurnal rhythm, inversely related to blood glucose levels (Yeoh & Baxter, 1988). Insulin-induced hypoglycaemia and fasting increase plasma IGFBP-1 (Yeoh & Baxter, 1988). Conversely, insulin suppresses IGFBP-1 levels under euglycaemic conditions in humans (Suikkari *et al.*, 1988) and inhibits IGFBP-1 production in rats (Ooi *et al.*, 1990; Unterman *et al.*, 1991; Powell *et al.*, 1991).

Glucocorticoids stimulate IGFBP-1 production (Luo *et al.*, 1990), and insulin and glucocorticoids appear to interact as regulators of IGFBP-1 expression (Unterman *et al.*, 1993).

Human IGFBP-2 consists of 289 amino acids and has a molecular weight of 31 kDa (see McCusker & Clemmons, 1992). The concentration of IGFBP-2 in adult blood is approximately 5 nmol/l (see Baxter, 1991). IGFBP-2 has a three fold higher affinity for IGF-II than for IGF-I, and has a higher affinity for both IGF proteins than IGFBP-1. In rats, IGFBP-2 is developmentally regulated, with high plasma levels and gene expression falling at birth (Orlowski *et al.*, 1990). IGFBP-2 is expressed in liver, kidney and choroid plexus of adult sheep (Delhanty & Han, 1993) and IGFBP-2 mRNA remains abundant in adult rat brain (Tseng *et al.*, 1989). Fasting and hypophysectomy increase IGFBP-2 protein and hepatic mRNA in rats, while dexamethasone inhibits IGFBP-2 production (Orlowski *et al.*, 1990).

IGFBP-3 is a 29 kDa glycoprotein, which when separated under reducing conditions, migrates as a 39-43 kDa doublet (see Baxter, 1991; McCusker & Clemmons, 1992). In blood, IGFBP-3 associates with either IGF-I or -II protein and an acid-labile glycoprotein of 85 kDa to form a 150 kDa ternary complex (Baxter & Martin, 1989). IGFBP-3 has a high affinity for both IGF proteins. Its concentration in plasma is 20-50 times higher than that of IGFBP-1 and 2 and the majority of the IGF proteins in adult blood are carried in the 150 kDa complex (Baxter, 1991). The IGFBP-3 gene is expressed in a number of adult rat tissues including liver, kidney, stomach, placenta, ovary and uterus (Albiston & Herington, 1992). Plasma IGFBP-3 is growth hormone dependent (Baxter & Martin, 1986; Blum *et al.*, 1990), reaches its highest levels at puberty in humans (Baxter & Martin, 1986) and is reduced by chronic nutrient restriction in sheep (Gallaher *et al.*, 1992). In children, positive associations are observed between the molar concentration of IGFBP-3 levels and the molar concentration of IGF-I plus IGF-II (Baxter, 1988; 1991). In rats, reduced serum IGFBP-3 following protein restriction are restored by IGF-I infusion, implicating IGF-I in the regulation of IGFBP-3 production in rats (Clemmons *et al.*, 1989).

Human IGFBP-4 contains 237 amino acids, has a predicted molecular weight of 26 kDa and migrates at 24 and 29 kDa on acrylamide gels in SDS (Shimasaki *et al.*, 1990; LaTour *et al.*, 1990). IGFBP-4 was initially isolated from human and rat serum and conditioned media of adult bone cells (Mohan *et al.*, 1989; Shimonaka *et al.*, 1989; Kiefer *et al.*, 1991). In the rat and sheep, the IGFBP-4 gene is expressed in a number of tissues, including liver, kidney, lung and heart (Shimasaki *et al.*, 1990; Carr *et al.*, 1994). IGFBP-4 has a high affinity for both IGF-I and IGF-II. IGFBP-5 contains 252 amino acids, has a molecular weight of 28 kDa and migrates as a doublet of 31 to 32 kDa, although the BP does not appear to be glycosylated (Shimasaki *et al.*, 1991b; see Cohick & Clemmons, 1993). IGFBP-5 has the highest affinity for IGF-I and -II of all the IGFBPs. In adult rats, IGFBP-5 mRNA is expressed in a range of tissues, with high levels of expression observed in kidney, but its concentrations in blood are low (Shimasaki *et al.*, 1991a). IGFBP-6 is a 216 amino acid protein with a molecular weight of 22 kDa (Shimasaki *et al.*, 1991c; see Cohick & Clemmons, 1993). IGFBP-6 was originally isolated from cerebrospinal fluid and conditioned medium of fibroblast cell lines (Roghani *et al.*, 1989; Forbes *et al.*, 1990), and is present in human serum, amniotic and follicular fluids (Baxter & Saunders, 1992). IGFBP-6 has a 10 to 100 fold higher affinity for IGF-II than for IGF-I (Roghani *et al.*, 1989; Forbes *et al.*, 1990).

As discussed above, the majority of the IGF proteins in adult blood are present in the bound form, associated with IGFBP-3 in the 150 kDa ternary complex. Unsaturated IGF binding sites are found within the smaller molecular weight IGFBPs. A number of functions are indicated for the IGFBPs. The IGFBPs prolong the half-life of the IGFs in the circulation. The 10 min half-life of free IGF-I in blood is extended to 30 minutes when IGF-I is bound to smaller molecular weight IGFBPs, and to greater than 10-15 hours, when IGF-I is carried by the 150 kDa ternary complex (Guler *et al.*, 1989; Davis *et al.*, 1989). Analogs of IGF-I which have a reduced affinity for IGF binding proteins are cleared more rapidly from blood (Ballard *et al.*, 1991a).

The IGFBPs act as both inhibitors and potentiators of IGF actions. IGFBPs 1-4 inhibit IGF stimulated actions in a range of cell types (Zapf *et al.*, 1979; Ritvos *et al.*, 1988; Ross *et al.*, 1989; Mohan *et al.*, 1989; see Clemmons 1991). The IGFBPs inhibit the actions of the IGFs

by reducing their interaction with their cell surface receptors. In man, the total circulating pool of IGF proteins has a hypoglycaemic potential 50 to 100 times greater than that of circulating insulin (Baxter, 1988). Association of IGFs in blood with IGFBPs limits the amount of free IGF protein in the circulation and protects against acute metabolic actions of free IGFs, including their hypoglycaemic potential. However, other studies report a potentiation of IGF actions following exposure of cells to IGFBPs *in vitro*. IGFBP-1 potentiates IGF-I stimulated DNA synthesis in fibroblasts and porcine smooth muscle cells (Elgin *et al.*, 1987). Preincubation of fibroblasts with IGFBP-3 potentiates the action of IGF-I, while coincident addition of IGF-I and IGFBP-3 inhibits IGF-I actions (DeMellow & Baxter, 1988). Preincubation with IGFBP-3 increases the amount of cell-associated IGFBP-3 (Conover, 1992). The affinity of cell surface associated IGFBP-3 for IGFs is lower than that of IGFBP-3 in solution, suggesting that its potentiation of IGF-I action may be due to increased interaction of IGF-I with its receptor, when IGF-I is bound by cell surface associated IGFBP-3 (Conover, 1992). Other modifications of IGFBPs also affect their function. Phosphorylation of IGFBP-1 increases the affinity of this protein for IGF-I (Jones *et al.*, 1991). In addition, specific proteolytic activity for IGFBPs -2, -3, -4 or -5 has been observed in plasma and/or conditioned media of a range of cell types (Clemmons *et al.*, 1993)

IGFBPs may also act to target the IGFs to their sites of action. IGFBP-1 and -2 contain an Arg-Gly-Asp (RGD) sequence, which may mediate the attachment of these proteins to cell surface integrin receptors. IGFBPs -1, -2, -3 and -4 have been shown to cross the capillary boundaries of the rat heart (Bar *et al.*, 1990; Boes *et al.*, 1992), suggesting a potential mechanism by which IGF proteins may leave the circulation. Expression of the IGFBPs in many tissues is region and cell type specific (see McCusker & Clemmons, 1992), supporting the suggestion that the IGFBPs have important functions in regulating both bioavailability and targeting of the IGFs.

1.2.5 *Insulin-like growth factor receptors*

The IGFs exert their actions by binding to cell surface receptors (reviewed in Rechler & Nissley, 1985b; Nissley *et al.*, 1991; Kornfeld, 1992; Moxham & Jacobs, 1992). Two specific

receptors (type 1 and 2 IGF receptors) have been described for the IGFs, and both IGFs also bind the insulin receptor. The type 1 IGF receptor is a glycosylated heterotetramer, with structural homology to the insulin receptor, consisting of two 135 kDa α subunits and two 90 kDa β subunits linked by disulphide bridges (Ullrich *et al.*, 1986). The α subunits are entirely extracellular and contain the ligand binding site. The β subunits contain the transmembrane domain, an ATP binding site and an intracellular tyrosine kinase domain. The type 2 IGF receptor shares no homology with the type 1 IGF or insulin receptors, but is identical to the cation independent mannose-6-phosphate receptor (Morgan *et al.*, 1987; McDonald *et al.*, 1988). The receptor consists of a single polypeptide chain of approximately 250 kDa, composed of a large extracellular domain, a transmembrane domain and a short cytoplasmic region. The extracellular domain contains 15 repeating segments of approximately 147 amino acids each. Separate binding sites for mannose-6-phosphate and IGF-II are located within the extracellular region.

The type 1 IGF receptor binds both IGF-I and -II proteins with high affinity. The affinity of the type 1 IGF receptor is reported to be highest for IGF-I, although in some studies similar binding affinities have been observed for both IGF proteins (Roth *et al.*, 1988). Insulin binds to the type 1 IGF receptor with 100 to 500 fold lower affinity than the IGF proteins. Both IGFs can also bind to the insulin receptor, but with a low affinity when compared to insulin. The type 2 IGF receptor preferentially binds IGF-II, and insulin does not bind to this receptor (Roth *et al.*, 1988). Actions of both IGF proteins are mediated through their association with the type 1 IGF receptor (Moxham & Jacobs, 1992). The signal transduction mechanism of the type 1 IGF receptor resembles that of the insulin receptor. IGF binding results in autophosporylation of the β subunit of the receptor and phosphorylation of endogenous cellular substrates, via the intrinsic tyrosine kinase activity (Czech, 1989). A number of endogenous substrates for the insulin and IGF-I receptor kinases have been described (Sun *et al.*, 1991; Myers & White, 1993, Condorelli *et al.*, 1989). Hybrid receptors, consisting of one insulin α and β subunit and one type 1 IGF receptor α and β subunit have been detected in certain tissues and cultured cells (Moxham & Jacobs, 1992). Hybrid receptors have a greater affinity for IGF-I than insulin.

The principal function of the type 2/mannose 6 phosphate receptor is to bind mannose-6-phosphate bearing lysosomal enzymes and to target them to lysosomes (Kornfeld, 1992). In addition, the receptor regulates degradation of IGF-II by receptor mediated internalization (Oka *et al.*, 1985). The majority of the actions of IGF-II are mediated through the type 1 IGF receptor, however several studies report effects of IGF-II which are mediated by the type 2 IGF receptor (see Nissley *et al.*, 1991; Kornfeld, 1992). These include stimulation of glycogen synthesis in a human hepatoma cell line (Hari *et al.*, 1987) and stimulation of Ca^{2+} influx in Balb/c 3T3 cells (Nishimoto *et al.*, 1987). In chickens and frogs, the type 2 IGF receptor lacks the binding site for IGF-II (Clairmont & Czech, 1989), suggesting that these properties of the receptor are confined to mammals. The suggested mechanism of signal transduction via the type 2 IGF receptor involves activation of G-proteins, as the type 2 receptor has been shown to interact with $G_{i\alpha 2}$, a GTP binding protein (Kornfeld, 1992).

1.2.6 *Actions of the insulin-like growth factors*

A wide range of biological actions of the IGF peptides have been described *in vitro* and *in vivo* (reviewed by Lowe, 1991). The IGFs are mitogenic and stimulate DNA synthesis in/or proliferation of a wide variety of cultured cells, including chondrocytes, fibroblasts, oesteoblasts, myoblasts and muscle cells (Lowe, 1991). In BALB-c/ 3T3 cells, IGF-I acts as a progression factor in the cell cycle, working in synergy with competence factors (platelet derived growth factor, fibroblast growth factor) to promote the progression of cells through the G_1 phase into S phase (Stiles *et al.*, 1979).

The IGFs exert a range of insulin-like metabolic actions *in vitro*. The IGFs stimulate glucose and amino acid uptake, promote glycogen synthesis and enhance RNA and protein synthesis in a variety of cell types (see Lowe, 1991). In cultured muscle and/or adipose tissue, the IGFs have been shown to enhance glucose uptake and metabolism, promote lipid and glycogen synthesis and inhibit lipolysis (Froesch *et al.*, 1985).

In addition, the IGFs promote cellular differentiation of certain cell types. Both IGFs are implicated in the terminal differentiation of muscle cells to form myotubes (Florini *et al.*,

1991). IGF-I promotes differentiated function of ovarian granulosa cells (Adashi *et al.*, 1985), and stimulates differentiation of osteoblasts (Schmid *et al.*, 1984), adipocytes (Smith *et al.*, 1988), erythroid cells (Claustres *et al.*, 1987) and oligodendrocytes (McMorris *et al.*, 1986). More specific actions of the IGFs occur in particular cell types. For example, the IGFs stimulate proteoglycan synthesis in chondrocytes and collagen synthesis and matrix formation in bone cells (see Lowe, 1991) and augment FSH stimulated steroid hormone synthesis in ovarian granulosa cells (Adashi *et al.*, 1985).

The mitogenic effects of IGFs are observed *in vivo*. Continuous infusion of IGF-I for 6 days stimulates body weight and tibial epiphyseal width in hypophysectomised rats (Schoenle *et al.*, 1982) and exogenous IGF-I enhances body weight and/or length in growth hormone deficient Snell mice and dwarf rats (Van Buul-Offers *et al.*, 1986; Skottner *et al.*, 1989). IGF-I also stimulates the growth of normal rats (Hizuka *et al.*, 1986; Tomas *et al.*, 1993). IGF-II infusion into hypophysectomised rats increases tibial epiphyseal width, but does not alter body weight, suggesting that IGF-II is not as potent a growth promotant as IGF-I in rodents (Schoenle *et al.*, 1985). This view is supported by studies comparing the effects of IGF-I and -II on the growth of normal rats (Conlon *et al.*, 1994). Administration of IGF-I to rats in a variety of physiological conditions, which are characterised by reduced plasma IGF-I, retarded growth and/or a catabolic state, enhances body weight gain (Schweiller *et al.*, 1986; Ballard *et al.*, 1991b). Specific effects of IGFs on the pattern of organ growth are also observed. In particular, IGF-I treatment enhances the growth of the spleen, thymus and kidneys (Guler *et al.*, 1988; Skottner *et al.*, 1989; Ballard *et al.*, 1991b).

Infusion of IGF-I into experimental animals and humans lowers blood glucose levels (Zapf *et al.*, 1986; Guler *et al.*, 1987; Jacob *et al.*, 1989; Douglas *et al.*, 1991) and suppresses plasma insulin (Jacob *et al.*, 1989; Clemmons *et al.*, 1992). In fasted rats or sheep, acute infusion of IGF-I increases glucose uptake and stimulates glycogen synthesis (Jacob *et al.*, 1989; Douglas *et al.*, 1991). Insulin infusion also stimulates glucose clearance, but in contrast to IGF-I infusion, suppresses hepatic glucose production (Jacob *et al.*, 1989; Douglas *et al.*, 1991). In fasted lambs and rats, acute infusion of IGF-I inhibits protein breakdown (Jacob *et al.*, 1989; Douglas *et al.*, 1991). In lambs, this effect is observed with doses of IGF-I that do not alter

blood glucose levels, and is accompanied by an increase in protein synthesis in skeletal and cardiac muscle and liver (Douglas *et al.*, 1991). Inhibition of protein breakdown is also observed in human subjects (Clemmons *et al.*, 1992; Turkalj *et al.*, 1992) but no effect on protein anabolism is evident at low IGF-I doses (Mauras *et al.*, 1992). IGF-I also alters kidney function, and an increase in glomerular filtration rate is observed following IGF-I infusion (Guler *et al.*, 1989).

These anabolic and metabolic activities of exogenous IGFs, together with positive associations between circulating IGFs and growth in a variety of physiological states are consistent with endocrine actions of the IGFs. The original somatomedin hypothesis proposed that growth hormone regulated somatic growth by stimulating hepatic production of somatomedins or IGFs which then mediated the actions of growth hormone, at the target tissue, in an endocrine fashion (Salmon & Daughaday, 1957). However, the subsequent observation that IGFs are produced by many tissues (D'Ercole *et al.*, 1984), including the growth plate (Isaksson *et al.*, 1987), indicated that locally produced IGFs can also act in an autocrine/paracrine manner within tissues. The demonstration that production of the IGFs is regulated by numerous other factors (1.2.3), including nutrition and trophic hormones in their target tissue, suggests that the IGFs are mediators of the actions of a number of growth regulators and can act in an autocrine or paracrine fashion. Therefore, the IGFs appear to have both endocrine and autocrine/paracrine actions (Holly & Wass, 1989; Daughaday & Rotwein, 1989; Gluckman *et al.*, 1991).

1.3 INSULIN-LIKE GROWTH FACTORS IN THE FETUS

A role for the IGFs in fetal growth and development is implicated by a range of evidence (reviewed by D'Ercole, 1991; Han & Hill; 1992). This section reviews the current understanding of the production, regulation and actions of the IGFs during fetal life.

1.3.1 *IGF-I and -II in fetal blood*

IGF-I and -II proteins are detected from 12-15 weeks of gestation in human fetal plasma (Ashton *et al.*, 1985; D'Ercole *et al.*, 1986) and from 51 days of pregnancy (term≈150 days) in

the blood of fetal sheep (Gluckman & Butler, 1983). In all species studied concentrations of IGF-II in fetal blood are higher than those of IGF-I (Gluckman & Butler, 1983; Mesiano *et al.*, 1989; Daughaday *et al.*, 1982, 1986; Lassarre *et al.*, 1991; Lee *et al.*, 1991; Carr *et al.*, 1995). However, the developmental profile of IGF-I and -II proteins in fetal blood differs between species.

Plasma concentrations of IGF-I in the fetus increase throughout gestation in the human (Ashton *et al.*, 1985; Bennett *et al.*, 1983; Lassarre *et al.*, 1991), sheep (Handwerger *et al.*, 1983; Van Vliet *et al.*, 1983; Gluckman & Butler, 1983), rat (Daughaday *et al.*, 1982), pig (Lee *et al.*, 1991) and guinea pig (Daughaday *et al.*, 1986). Concentrations of IGF-I in human and rat fetuses are lower than adult levels at term (Sara *et al.*, 1980; Bennett *et al.*, 1983; Ashton *et al.*, 1985; Glasscock *et al.*, 1990; Lassarre *et al.*, 1991). In the term human fetus plasma IGF-I is approximately 50 to 60% of adult levels (Bennett *et al.*, 1983; Lassarre *et al.*, 1991). Plasma IGF-I increases throughout the neonatal period in these species, and in the human maximal concentrations of IGF-I in blood are attained at puberty (Hall *et al.*, 1980; Daughaday & Rotwein, 1989). The concentrations of IGF-I in the blood of fetal sheep, at term, are similar to adult levels (Gluckman & Butler, 1983; Mesiano *et al.*, 1989; Carr *et al.*, 1995). In contrast to the rat and human, IGF-I increases markedly 3 to 4 days after birth in sheep and the highest plasma levels of IGF-I are recorded during the neonatal period (Gluckman & Butler, 1983; Van Vliet *et al.*, 1983; Mesiano *et al.*, 1989).

Fetal plasma IGF-II increases with advancing gestation (Bennett *et al.*, 1983; Lassarre *et al.*, 1991; Daughaday *et al.*, 1982, 1986; Carr *et al.*, 1995). Concentrations of IGF-II in blood of porcine and human fetuses at term are approximately 50% lower than adult levels, and increase after birth (Ashton *et al.*, 1985; Bennett *et al.*, 1983; Lassarre *et al.*, 1991; Lee *et al.*, 1991). Maximal plasma IGF-II levels in the human are attained in early childhood (Sara & Hall, 1990). In contrast, the amount of IGF-II in blood of late gestation fetal sheep, guinea pigs and rats is significantly elevated in comparison to adult blood (Moses *et al.*, 1980; Daughaday *et al.*, 1982, 1986; Gluckman & Butler, 1983; Mesiano *et al.*, 1989; Carr *et al.*, 1995). Plasma IGF-II in fetal sheep and guinea pigs begins to fall shortly before birth and decreases rapidly in the early perinatal period (Gluckman & Butler, 1983; Daughaday *et al.*, 1986; Mesiano *et al.*,

1989; Carr *et al.*, 1995). In the rat, plasma IGF-II begins to fall around six days of postnatal age (Daughaday *et al.*, 1982; Glasscock *et al.*, 1990) and is almost unmeasurable in the blood of adult rats (Moses *et al.*, 1980).

Higher molecular weight forms of immunoreactive IGF-II, of between 8 and 15 kDa, are found in human fetal plasma (Hill, 1990). Similarly, acid-gel chromatography of plasma from fetal and newborn lambs reveals a higher molecular weight form of IGF-II (Carr *et al.*, 1995).

1.3.2 *Production of IGF-I and -II by fetal tissues and placenta*

IGF-I and -II do not cross the placenta, suggesting that IGF proteins in the fetal circulation are produced by fetal tissues and/or the placenta. Synthesis of IGF-I and -II by fetal tissues was initially demonstrated *in vitro* using a variety of fetal cell types (Adams *et al.*, 1983a,b; Hill *et al.*, 1985a; Richman *et al.*, 1985) and organ explants (Hill *et al.*, 1987; Bryson *et al.*, 1989). Explants of fetal mouse tissues, including intestine, heart, brain, kidney, liver and lung, were found to release IGF-I into culture media (D'Ercole *et al.*, 1980), and IGF-I and -II peptides were extracted from a range of mid gestation human fetal tissues (D'Ercole *et al.*, 1986; Hill *et al.*, 1990). Subsequently, with the isolation of cDNAs for IGF-I and -II, multiple fetal tissues from several species have been shown to contain IGF-I and -II mRNAs (Han *et al.*, 1988; Lund *et al.*, 1986; Adamo *et al.*, 1989; Dickson *et al.*, 1991; O'Mahoney *et al.*, 1991; Delhanty *et al.*, 1993; Lee *et al.*, 1993).

IGF-I mRNA is detected in mouse embryos from the oocyte stage (Doherty *et al.*, 1994) and IGF-II mRNA from the two-cell stage onwards (Rappolee *et al.*, 1992). IGF-I and -II genes are expressed throughout the embryonic period in rats and mice (Rotwein *et al.*, 1987b; Beck *et al.*, 1987; Stylianopoulou *et al.*, 1988b; Bondy *et al.*, 1990; Lee *et al.*, 1990). In human fetal tissues IGF-II mRNA is present from early pregnancy, being detected from at least 18 days gestation (Brice *et al.*, 1989).

As previously noted (1.2.3), the expression of the IGF genes is developmentally regulated. In the mid gestation human fetus, IGF-I mRNAs are detected in many fetal tissues (Han *et al.*, 1988). In contrast to postnatal life, fetal liver is amongst the tissues with the lowest expression

of the IGF-I gene. The highest expression of IGF-I in the human fetus is detected in the stomach, spleen, thymus and adrenal, followed by muscle and heart. Lower levels of expression are evident in intestine, hypothalamus, lung, pancreas, kidney, liver, skin and regions of the brain. IGF-I mRNAs are present in a range of tissues in the fetal rat (Lund *et al.*, 1986; Adamo *et al.*, 1989). IGF-I mRNA increases eight-fold between days 11 and 13 of gestation (term=21 days) in the rat embryo, however, total IGF-I mRNA levels in the mid-gestation rat fetus are approximately 12% of that in adult liver (Rotwein *et al.*, 1987b). The abundance of IGF-I mRNA in liver, heart, and kidney increases after birth in the rat, while the amount of IGF-I mRNA in muscle, stomach, testes and lung are highest during fetal and early postnatal life (Adamo *et al.*, 1989). IGF-I mRNA has been detected in the liver and lung of fetal sheep from 60 days of gestation to term and increases in liver, but decreases in lung, as a function of gestational age (Yang *et al.*, 1991). IGF-I mRNA has also been detected from 84 days gestation in skeletal muscle of fetal sheep and decreases with gestational age (Dickson *et al.*, 1991).

IGF-II gene expression is highest during fetal life (de Pagter-Holthuizen *et al.*, 1987, 1988; Gray *et al.*, 1987; Scott *et al.*, 1985; Han *et al.*, 1988; Lund *et al.*, 1986; reviewed by Rechler, 1991) and IGF-II mRNAs are present in greater abundance than IGF-I mRNAs, in most fetal tissues, in all species studied (Lund *et al.*, 1986; Han *et al.*, 1988; Yang *et al.*, 1991). The highest levels of IGF-II expression in the mid-gestation human fetus are found in liver, adrenal and muscle (Han *et al.*, 1988; Brice *et al.*, 1989; de Pagter-Holthuizen, 1988; Scott *et al.*, 1985). Kidney, skin and pancreas express intermediate amounts of IGF-II mRNA, and low expression is evident in heart, stomach, lung, brain stem and spleen (Han *et al.*, 1988). Several studies failed to detect IGF-II mRNA in human fetal brain in early gestation (Scott *et al.*, 1985; Brice *et al.*, 1989). Han *et al.* (1988) report that IGF-II mRNA is present in brain stem, but not cortex or hypothalamus in mid-gestation human fetuses, while others have detected low levels of IGF-II mRNA in total brain RNA (Gray *et al.*, 1987) and in brain sections (Hirvonen *et al.*, 1989) from human fetuses.

In the rat, high levels of expression of IGF-II mRNA are observed in fetal liver, muscle, intestine and skin, and lower levels are found in heart, kidney, lung and regions of the brain

(Frunzio *et al.*, 1986; Chiariotti *et al.*, 1988). IGF-II mRNA is abundant in ovine fetal tissues (O'Mahoney *et al.*, 1991; Delhanty & Han, 1993). IGF-II mRNA levels are highest at mid-gestation in the majority of fetal sheep tissues. In late gestation, IGF-II mRNA is most abundant in fetal kidney, lung, adrenal, liver and muscle, with lower levels observed in spleen, heart, intestine and placenta (O'Mahoney *et al.*, 1991; Delhanty & Han, 1993). IGF-II mRNA is not detected in the cortex, hypothalamus or cerebellum regions of fetal sheep brain, but is present in choroid plexus (Delhanty & Han, 1993).

Multiple IGF-II transcripts are present in tissues of the fetal sheep, human and rat (1.2.2.2). The 6.0 kb and 2.2 kb size forms of IGF-II mRNA, produced from promoter 3 of the human IGF-II gene, are predominant in human fetal tissues (De Pagter-Holthuizen *et al.*, 1988). A 6.0 kb IGF-II mRNA is the major IGF-II mRNA form in fetal sheep tissues and placenta and additional IGF-II mRNAs of 5.9, 3.8, 2.9, 2.3, 1.9 and 1.2 kb are also detected (O'Mahoney *et al.*, 1991; Delhanty & Han, 1993). In contrast to the human, rat promoter 3 (equivalent to human promoter 4), which produced the 3.6 kb IGF-II mRNA, is the most active in fetal rat tissues.

In situ hybridisation studies have localised IGF-I and -II mRNAs to connective tissue or cells of mesenchymal origin in 14 tissues of mid-gestation human fetuses (Han *et al.*, 1987a). IGF mRNAs were present in perisinusoidal cells of the liver, perichondrium of cartilage, sclera of the eye and connective tissue layers, sheaths, septa and capsules of each organ. Using immunocytochemistry, IGF peptides are generally detected in differentiated epithelial and mesodermal cells in mid gestation, including hepatocytes, skeletal and cardiac muscle fibres, intestine and kidney tubules and adrenal cortical cells (Han *et al.*, 1987b). Thus the cellular localisation of IGF peptides is distinct from that of IGF-I and -II mRNAs. The antibodies used in this study did not distinguish between IGF-I and -II immunoreactivity (Han *et al.*, 1987b), however, higher levels of IGF-II are found in extracts of human fetal tissues (Hill, 1990).

Others suggest that the pattern of IGF expression in the human fetus differs with the stage of development. In the early gestation human fetus (6-8 weeks), IGF-II mRNA is detected in immature hepatocytes, haematopoietic cells, the fetal and definitive adrenal cortex and the

metanephric blastema of the developing kidney (Brice *et al.*, 1989). In these regions of the adrenal cortex and kidney the abundance of IGF-II mRNA decreases with advancing gestation, and by mid-gestation the pattern of expression in these tissues resembles that previously reported for mid-gestation human fetuses (Han *et al.*, 1988). Similarly, in the bovine fetus, IGF-II mRNA is detected in developing muscle cells in early to mid-gestation and becomes localised primarily to connective tissue in the third trimester (Listrat *et al.*, 1994).

Cells of mesodermal origin are also the predominant site of IGF-I and -II expression in the rat fetus. In mid-gestation rat embryos, IGF-I mRNA is detected in undifferentiated mesenchymal tissue in the vicinity of sprouting nerves and spinal ganglia, in mesenchyme of bronchi walls, bowel and diaphragm, in areas of active tissue remodelling such as the cardiac outflow tract, and in aggregations of undifferentiated mesenchyme surrounding developing muscle and cartilage (Bondy *et al.*, 1990). In late gestation fetal rats IGF-I mRNA is also present in the olfactory bulb (Ayer-Le Lievre *et al.*, 1991). Abundant IGF-II mRNAs are detected in tissues of mesodermal origin, including developing muscle and cartilage, dermis, vascular tissue, and epicardium and myocardium of the heart in rat embryos between days 10 to 21 of gestation (Beck *et al.*, 1987; Stylianopoulou *et al.*, 1988b; Bondy *et al.*, 1990). IGF-II mRNAs are also found in endodermally derived tissues in the rat fetus, including liver, bronchial and gut epithelium and ectodermally derived tissues including choroid plexus and Rathke's pouch, the primordial anterior and intermediate pituitary (Beck *et al.*, 1987; Stylianopoulou *et al.*, 1988b; Bondy *et al.*, 1990; Ayer-Le Lievre *et al.*, 1991). IGF-II mRNA is not expressed in the remaining central and peripheral nervous system in the rat fetus (Beck *et al.*, 1987). As observed in the human fetus, IGF-II mRNA abundance decreases with the appearance of a differentiated cell type in certain tissues. For example, IGF-II mRNA in chondrocytes is reduced prior to ossification (Beck *et al.*, 1987).

IGF-II mRNA is abundant in the rat, mouse and human placenta (Zhou & Bondy, 1992; Shen *et al.*, 1986; Wang *et al.*, 1988; Redline *et al.*, 1993). Major IGF-II mRNAs of 6.0, 4.9, 3.2 and 2.2 are expressed in human placenta (Daimon *et al.*, 1992) indicating the use of promoters 3 and 4 of the IGF-II gene in placenta. IGF-I mRNA has been detected in rat and human placenta (Wang *et al.*, 1988; Pescovitz *et al.*, 1991), however others suggest that IGF-I mRNA

is present only in the myometrium, and not the placenta (Zhou & Bondy, 1992). IGF-II mRNA is abundant in the trophoblast derived elements of the rat placenta, from the time of implantation. IGF-II mRNA is detected in undifferentiated trophoblast in early gestation and in both the inner labyrinthine and outer basal layers during mid-gestation, the period of rapid placental growth (Zhou & Bondy, 1992). Within the labyrinthine zone IGF-II mRNA is present in mesenchyme, trabecular trophoblast, cytotrophoblast and fetal blood vessels. Abundance of IGF-II mRNA decreases in the basal layer in late gestation (Zhou & Bondy, 1992). Similarly, in the mouse placenta expression of IGF-II is highest in the spongiotrophoblast (basal layer) at mid-gestation, when its development is maximal, while IGF-II mRNA is most abundant in vasculogenic mesenchyme in late gestation (Redline *et al.*, 1993). In human placenta IGF-I and -II mRNA abundance is highest during the first and second trimester (Shen *et al.*, 1986; Wang *et al.*, 1988). IGF-II mRNA is abundant in the cytotrophoblasts of the superficial basal plate in the human placenta, particularly in early gestation (Ohlsson *et al.*, 1989; Zhou & Bondy, 1992). In the ovine placenta the abundance of IGF-I and -II mRNA is not altered between 60 days gestation and term (150 days) (Bassett & Challis, 1992). In sheep, IGF-I and -II immunoreactive peptides are present in the fetal trophectoderm and feto-maternal syncytium between 60 and 100 days of gestation, but are found only in fetal trophoblast tissue between 100 days gestation and term (Bassett & Challis, 1992). IGF-II mRNA is present in fetal stromal cells and fibroblasts underlying the trophoblastic cells and in the endothelium of fetal blood vessels in the ovine placenta (Bassett & Challis, 1992).

1.3.3 *Regulation of production of the insulin-like growth factors in the fetus*

The majority of studies aimed at determining the factors regulating production of the IGFs by fetal tissues have measured blood levels of the growth factors. In rodents, limited amounts of fetal tissue and low levels of IGF-I gene expression *in utero* make analysis of IGF mRNA abundance difficult and suitable DNA clones for use in studies of IGF production in larger mammals have only become available in recent years (Dickson *et al.*, 1991; O'Mahoney *et al.*, 1991). Thus, much of the current understanding of prenatal IGF-I and -II regulation is based

on IGF protein concentrations in fetal blood. However, multiple fetal tissues produce IGFs and the source of circulating growth factors *in utero* is not known. Furthermore, many of the early studies measuring blood levels of the IGFs used acid-ethanol extraction methods to separate the IGFs from their binding proteins. Incomplete removal of binding proteins from fetal sheep plasma has been documented with this method (Mesiano *et al.*, 1988; Breier *et al.*, 1991) and this problem can be amplified because developmental, endocrine or nutritional pertubations which alter the amount of binding proteins in fetal blood.

Regulation of fetal IGF-I and -II by growth hormone

In contrast to postnatal life, growth hormone does not appear to be a major determinant of IGF-I or -II production in the fetus. IGF-I and -II concentrations are not different in anencephalic human fetuses (Ashton *et al.*, 1985). Human growth hormone does not stimulate IGF-I production by fetal rat fibroblasts (Adams *et al.*, 1983b), human fetal fibroblasts or myoblasts (Hill *et al.*, 1985; Swenne *et al.*, 1987). However, growth hormone does increase IGF-I release into culture medium by human fetal hepatocytes (Strain *et al.*, 1987). Hypophysectomy of fetal sheep reduces plasma IGF-I, but not IGF-II (Mesiano *et al.*, 1989). However, IGF-I levels are partially restored by thyroxine infusion, suggesting that reduced secretion of IGF-I may be related to the fall in thyroid hormone following hypophysectomy (Mesiano *et al.*, 1989). Similarly, hypophysectomy of fetal pigs reduces plasma IGF-I and IGF-I protein content of several fetal tissues and IGF-I protein concentrations are partially restored by thyroxine replacement (Latimer *et al.*, 1993).

Regulation of fetal IGF-I and -II by substrate supply

Experimental restriction of nutrient supply in fetal sheep (Jones *et al.*, 1988; Bassett *et al.*, 1990; Oliver *et al.*, 1993; Iwamoto *et al.*, 1992), rats (Vileisis & D'Ercole, 1986; Davenport *et al.*, 1990; Straus *et al.*, 1991b; Unterman *et al.*, 1993) and guinea pigs (Jones *et al.*, 1987; Jones *et al.*, 1990) reduces the concentration of IGF-I in fetal blood. In sheep, circulating IGF-I is reduced by 72 hours maternal fasting (Bassett *et al.*, 1990) and concentrations of IGF-I can be restored by infusion of glucose, but not amino acids to the fetus (Oliver *et al.*, 1993). Fetal plasma IGF-I is also reduced following four hours of maternal hypoxia in sheep (Iwamoto *et*

al., 1992). Thus, both glucose and oxygen can independently regulate circulating IGF-I in the sheep fetus.

Fetal plasma IGF-I is reduced by 30% by 72 hours maternal fasting in rats, but the abundance of IGF-I mRNA in fetal rat liver and lung does not change (Davenport *et al.*, 1990). This suggests that production of IGF-I is regulated at a post-transcriptional level in the rat fetus (Davenport *et al.*, 1990). However, Straus *et al.* (1991) report a 55% decrease in hepatic IGF-I mRNA in fetuses from dams fasted for 96 hours. Similarly, the concentration of IGF-I protein in the liver, but not lung, of fetal rats is reduced by up to 50%, when nutrient supply is limited by uterine artery ligation (Vileisis & D'Ercole, 1986). This suggests that liver may be a major site of production of circulating IGF-I in the fetal rat. In the sheep fetus, reduction of maternal uterine blood flow for 24 hours results in fetal hypoxia, but does not alter plasma glucose or insulin levels (McLellan *et al.*, 1992). Reduction of maternal uterine blood flow reduces hepatic IGF-I mRNA but plasma levels of IGF-I and IGF-I mRNA abundance in fetal lung, muscle, thymus and kidney are not altered (McLellan *et al.*, 1992). Therefore oxygen supply may regulate IGF-I expression in fetal liver.

In contrast to the reduction in plasma levels of IGF-I when nutrient supply to the fetus is limited, plasma levels of IGF-II are reported to remain unchanged (Davenport *et al.*, 1990; Unterman *et al.*, 1993), decrease (Jones *et al.*, 1990; Straus *et al.*, 1991b) or increase (Jones *et al.*, 1987; 1990). IGF-II concentrations in fetal rat blood and IGF-II mRNA in fetal liver are not altered by 72 hours maternal starvation (Davenport *et al.*, 1990). However, starvation of the pregnant rat for 96 hours reduces fetal plasma IGF-II, but not hepatic IGF-II mRNA (Straus *et al.*, 1991b). Maternal undernutrition also decreases plasma IGF-II concentrations in the guinea pig fetus (Jones *et al.*, 1990). Plasma IGF-I decreases by 71% with 96 hours of starvation in fetal rats, indicating that IGF-I is more sensitive to nutrient restriction than IGF-II.

In contrast to the rat, hepatic IGF-II mRNA is increased at 135 days gestation in fetal sheep by maternal underfeeding from early pregnancy and by insulin-induced maternal hypoglycaemia from mid-pregnancy onwards (Townsend *et al.*, 1992a). This effect is tissue specific since kidney IGF-II mRNA is reduced by maternal feed restriction (Townsend *et al.*, 1992a). In

contrast, short term fasting of the pregnant ewe near term at 140 days gestation reduces hepatic and renal IGF-II expression (Li *et al.*, 1993). Therefore, the effect of limited nutrient supply on IGF-II gene expression in fetal sheep may be dependent on gestational age. However, in sheep maternal fasting in late gestation is associated with a rise in fetal blood cortisol, and cortisol has been shown to negatively regulate hepatic IGF-II mRNA (Li *et al.*, 1993). Therefore, the effect of limited nutrient supply may depend on the fetal endocrine response to restriction.

Restriction of uterine blood flow in fetal sheep and guinea pigs increases fetal plasma IGF-II (Jones *et al.*, 1988; Jones *et al.*, 1990). Hepatic IGF-II mRNA increases in fetal rats following uterine artery ligation (Price *et al.*, 1992a). Therefore an increase in hepatic production may account for the rise in plasma IGF-II, when nutrient and oxygen supply to the fetus is limited. Exposure of pregnant rats to a hypoxic environment from day 14 of gestation also increases fetal plasma IGF-II (Tapanainen *et al.*, 1994). However, in fetal sheep, no change in plasma IGF-II or IGF-II mRNA abundance in several fetal tissues was observed following 24 hours restricted uterine blood flow (McLellan *et al.*, 1992). Thus in rodents, plasma IGF-II and hepatic IGF-II mRNA increase when oxygen alone or in combination with nutrients is restricted (Jones *et al.*, 1990; Price *et al.*, 1992a), but do not change or decrease when nutrients alone are limited (Davenport *et al.*, 1990; Jones *et al.*, 1990; Straus *et al.*, 1991b).

Regulation of fetal IGF-I and -II by insulin and cortisol

Pancreatectomy of fetal sheep reduces IGF-I concentrations and increases IGF-II levels in fetal plasma (Gluckman *et al.*, 1987). Both insulin and IGF-I concentrations are reduced when substrate supply to the fetus is limited (Jones *et al.*, 1990; Oliver *et al.*, 1993). Therefore, insulin is suggested as a potential regulator of IGF-I production in the fetus. However, IGF-I concentrations are elevated only in association with excessive hyperinsulinaemia and IGF-I production in fetal cells is stimulated by addition of high concentrations of insulin (see Han & Hill, 1992), suggesting that in some fetal tissues the actions of insulin on IGF-I production may be permissive. Insulin treatment of fetal sheep hepatocytes increases IGF-II mRNA abundance (Townsend *et al.*, 1991), indicating that insulin may regulate expression of IGF-II in fetal liver. However, plasma IGF-II correlates positively with glucose and negatively with insulin in pancreatectomised fetal sheep (Gluckman *et al.*, 1987). Insulin concentrations are

reduced when substrate supply to the fetus is limited and no relationship is observed between fetal plasma insulin and IGF-II in fetal rats with restricted nutrient supply (Unterman *et al.*, 1993).

Glucocorticoid treatment of neonatal rats has been shown to reduce hepatic IGF-II expression (Beck *et al.*, 1988) (1.2.3.2). Increasing the concentration of cortisol in fetal sheep, by intra-fetal cortisol infusion for five days in late gestation, suppresses hepatic IGF-II mRNA (Li *et al.*, 1993). Blood cortisol levels are negatively correlated with hepatic IGF-II mRNA (Li *et al.*, 1993). Cortisol infusion also reduces skeletal muscle IGF-II mRNA (Li *et al.*, 1993). Similarly, infusion of ACTH or cortisol to fetal sheep at 120-125 days gestation reduces the abundance of IGF-II mRNA and peptide in the adrenal gland (Lu *et al.*, 1994). Thus, the cortisol surge observed in late gestation fetal sheep is implicated as a potential mediator of the fall in IGF-II gene expression around the time of birth.

Regulation of fetal IGF-I and -II by other hormones and factors

Ovine placental lactogen stimulates IGF-II synthesis in fetal rat fibroblasts (Adams *et al.*, 1983b) and promotes IGF-I release into culture medium by fibroblasts, myoblasts and hepatocytes (Hill *et al.*, 1985b; Strain *et al.*, 1987). A number of studies examining IGF-I and -II regulation (1.2.3.2) have used cells from fetal or embryonic sources. For example, fetal rat parietal bones are used to generate osteoblast enriched cultures in which parathyroid hormone and prostaglandin E_2 increase IGF-I mRNA abundance (McCarthy *et al.*, 1989, 1990, 1991). cortisol inhibits IGF-I expression in these cells. Cortisol inhibits IGF-I gene expression in oesteoblast cultures from fetal rat bone (McCarthy *et al.*, 1990)

Tissue specific factors also regulate IGF gene expression in the fetus. IGF-II mRNA in fetal sheep lungs is reduced when lung growth is limited by spinal cord transection and abolition of fetal breathing movements (Harding *et al.*, 1993), or by draining of fetal lung liquid (Hooper *et al.*, 1993). Conversely, IGF-II mRNA increases when lung growth is increased by tracheal obstruction (Hooper *et al.*, 1993). The mechanism through which IGF-II expression in fetal lung is regulated by lung distension in these studies is not certain.

1.3.4 *IGF binding proteins in the fetus*

As in postnatal life, the IGFs are present in fetal blood and tissues complexed to IGFBPs. In rats, humans, pigs, rhesus monkeys and sheep, the concentrations of various IGFBPs in fetal plasma differs from that observed postnatally (Orlowski *et al.*, 1990; Crystal &Giudice , 1991; Liu *et al.*, 1991; Lee *et al.*, 1993; Carr *et al.*, 1995). In fetal sheep and human, IGFBPs 1 to 4 can be detected in blood plasma (Crystal & Giudice , 1991; McLellan *et al.*, 1992; Fant *et al.*, 1993; Delhanty & Han, 1993; Carr *et al.*, 1995). Circulating concentrations of IGFBP-1 and -2 are high from mid to late gestation, and decrease after birth in these species (Drop *et al.*, 1984; Delhanty & Han, 1993; Carr *et al.*, 1995). In fetal sheep, plasma levels of IGFBP-3 and 4 increase from 45 days gestation to term (Carr *et al.*, 1995). In contrast with other species, such as the rat and pig, significant concentrations of IGFBP-3 are detected in the blood of fetal sheep and humans (Crystal & Giudice 1991; Delhanty & Han, 1993; Fant *et al.*, 1993). In fetal rats and pigs, IGFBP-2 is the predominant serum IGFBP and IGFBP-3 levels in fetal rat plasma are very low to undetectable (Orlowski *et al.*, 1990; Lee *et al.*, 1993).

Many fetal tissues produce IGFBPs. IGFBPs 1-6 mRNAs are present in fetal rat tissues from at least mid-gestation onwards, and expression of the IGFBPs is tissue and cell type specific (Wood *et al.*, 1990; Cerro *et al.*, 1993; Schuller *et al.*, 1993). In late gestation, the highest levels of expression of IGFBP-1 and 2 are observed in fetal rat liver, and hepatic expression of IGFBP-1 and -2 decreases after birth (Ooi *et al.*, 1990; Orlowski *et al.*, 1990). Liver is the major site of expression of IGFBP-1 in the human and sheep fetus (Phillips *et al.*, 1991; Pannier *et al.*, 1994). However, localisation studies of IGFBP-1 peptide in the human fetus indicate that the protein is present in numerous fetal tissues, with a cellular distribution resembling that of IGF-I (Hill *et al.*, 1989). In early to mid gestation, IGFBP-2 is expressed in all fetal tissues examined in the sheep fetus but is reduced in most tissues in late gestation (Delhanty & Han, 1993; Carr *et al.*, 1995). IGF-II mRNA is most abundant in liver, kidney and choroid plexus of fetal sheep in early gestation and these tissues continue to express IGF-II in late gestation (Delhanty & Han, 1993; Carr *et al.*, 1995). This pattern of expression parallels that of IGF-II in fetal sheep tissues (Delhanty & Han, 1993). In the human fetus, IGFBP-3 mRNA is detected in spleen, liver, kidney and brain (Pannier *et al.*, 1994). IGFBPs-4, 5 and 6

are expressed in a number of human fetal tissues, including kidney, muscle, heart, skin, lung, intestine and liver (Delhanty et al., 1993; Pannier *et al.*, 1994). In fetal sheep, IGFBP-4 mRNA is also detected in a number of tissues, including liver, kidney, lung and heart (Carr *et al.*, 1995). IGFBPs 1-4 are also present in the placenta (Zhou & Bondy, 1992; Hill *et al.*, 1993). The abundance of IGFBP-2 mRNA is higher than that of the other IGFBPs in rat and human placenta (Zhou & Bondy, 1992; Hill *et al.*, 1993). In the ovine placenta IGFBP-2 mRNA is localised to the maternal synctium in mid-gestation and to the mononuclear cells of the trophoblast tissue from mid-gestation to term (Bassett & Challis, 1992).

Plasma levels of IGFBP-1 and hepatic IGFBP-1 mRNA abundance are increased in fetal rats following maternal starvation or uterine artery ligation (Straus *et al.*, 1991b; Price *et al.*, 1992a; Unterman *et al.*, 1993). Similarly, plasma IGFBP-1 and hepatic IGFBP-1 mRNA are increased in fetal sheep by 72 hours maternal fasting or 24 hours reduction of uterine blood flow (McLellan *et al.*, 1992; Osborn *et al.*, 1992). Effects of maternal fasting on IGFBP-1 production are reversed by refeeding and prevented by glucose infusion during fasting (Osborn *et al.*, 1992) suggesting that nutrient supply and/or insulin may regulate IGFBP-1 production in the fetus. In contrast, an increase in fetal catecholamine concentrations may be responsible for increased IGFBP-1 expression in hypoxic fetal sheep (Hooper *et al.*, 1994). Maternal starvation does not alter IGFBP-2 mRNA abundance in fetal rat and sheep liver (Straus *et al.*, 1991b; Osborn *et al.*, 1992). However others report an increase in concentrations of IGFBP-2 in fetal sheep plasma following maternal starvation, which cannot be restored to normal by intra-fetal infusion of glucose or insulin, suggesting that other nutrients may regulate IGFBP-2 (Gallaher *et al.*, 1994). In contrast, in fetal sheep, plasma IGFBP-2 and IGFBP-2 mRNA abundance in liver and kidney are reduced, when uterine blood flow, and thus fetal oxygen supply, is restricted (McLellan *et al.*, 1992).

1.3.5 *IGF receptors in the fetus*

Type 1 and type 2 IGF receptors are present in multiple fetal tissues. Messenger RNA for the type 2 IGF receptor is detected at the two cell stage in mouse embryos, while the type 1 IGF receptor is expressed at the eight cell stage (Rappolee *et al.*, 1992). *In situ* hybridisation studies

indicate that type 1 IGF receptor mRNA is widely distributed in embryonic tissues of the rat (Bondy *et al.*, 1990). In late gestation, type 1 receptor mRNA is detected by solution hybridisation assay in a number of fetal rat tissues including liver, brain, muscle, kidney, heart and lung (Werner *et al.*, 1989). Expression of the type 1 receptor is also abundant in rat and in human placenta (Zhou & Bondy, 1992). Type 1 receptor mRNA abundance decreases after birth in all rat tissues examined. Expression of the type 1 IGF receptor is also observed in a number of tissues of the fetal pig, including liver, muscle, lung and kidney (Lee *et al.*, 1993).

Type 2 receptor protein and mRNA are abundant in the fetus (Sklar *et al.*, 1989, 1992; Funk *et al.*, 1992; reviewed by Nissley *et al.*, 1993). Messenger RNA and and type 2 receptor protein are detected in the extra-embryonic membranes of the rat at day 7 of gestation. High levels of expression are evident in the developing heart and blood vessels, skeletal muscle and perichondrium from mid gestation in the rat embryo (Senior *et al.*, 1990). In late gestation fetal rats, type 2 receptor mRNA and protein are highest in heart followed by limb, muscle, lung, intestine, kidney, liver and brain (Sklar *et al.*, 1989,1992). Extraction of protein from whole rat embryos in early to mid gestation indicated that the type 2 receptor accounted for 0.1 to 0.4% of total protein (Sklar *et al.*, 1989). Type 2 receptor mRNA is abundant in rat placenta throughout pregnancy (Zhou & Bondy, 1992). Abundance of the type 2 IGF receptor mRNA and protein in rat tissues decreases rapidly after birth (Sklar *et al.*, 1989, 1992; Senior *et al.*, 1990). Explants of fetal rat tissues release the soluble form of the type 2 receptor into culture medium (Bobek *et al.*, 1992) and abundance of the soluble receptor in rat blood is developmentally regulated (Kiess *et al.*, 1987).

Binding studies indicate that the type 2 IGF receptor is present in tissues of the human and sheep fetus (Owens *et al.*, 1980; Sara *et al.*, 1983; Owens *et al.*, 1985). In fetal sheep binding of IGF-II to fetal liver and lung preparations increases with gestation, while binding to fetal heart and placenta falls (Owens *et al.*, 1980). Binding to fetal sheep liver preparations was highest at all ages studied (Owens *et al.*, 1980). In sheep, soluble type 2 receptor is abundant in fetal blood and is reported to carry around 40 to 50% of circulating IGF-II (Gelato *et al.*, 1989). Type 2 receptor protein is also present in extracts of human fetal tissues, with the highest abundance found in the heart, thymus and kidney (Funk *et al.*, 1992). In contrast to

fetal rats, type 2 receptor protein concentrations are low in human fetal muscle and no developmental pattern of type 2 receptor abundance is evident in human fetal tissues between 23 weeks of gestation and 24 months postnatal age. Type 2 receptor mRNA is found in human placenta (Zhou & Bondy, 1992).

1.3.6 *Biological actions of the IGFs in the fetus*

The IGFs stimulate cellular proliferation, protein synthesis, glycogen synthesis and differentiation of a wide variety of fetal cells in culture, including fibroblasts, cartilage, myoblasts, chondrocytes and osteoblasts, glial cells and hepatocytes (reviewed in Sara & Carlsson-Skwirut, 1988; Han & Hill, 1992).Many of the cell types in which IGFs are reported to influence cellular differentiation (1.2.4) are fetal and embryonic in nature. For example, the IGFs enhance the terminal differentiation of muscle cells to form myotubes (Florini *et al.*, 1991) and in several species, including human, cow and sheep, the majority of skeletal muscle differentiation occurs *in utero*. Differentiation and maturation of organs is of particular importance during fetal development and the IGFs influence cellular differentiation of several major organs including bone, cartilage, adrenal glands and nervous tissue (see Han & Hill, 1992). Mitogenic and metabolic actions of the IGFs are also described in placenta. The IGFs stimulate proliferation of human placental cells *in vitro* (Fant *et al.*, 1986, Ohlsson *et al.*, 1989). IGF-I increases the uptake of amino acids by human cytotrophoblast (Ohlsson *et al.*, 1989) and both IGFs stimulate 3β-hydroxysteroid dehydrogenase activity in human cytotrophoblasts (Nestler, 1989). In the mouse placenta, IGF-I stimulates the proliferation and migration of ectoplacental cells while IGF-II induces the transformation of these cells into trophoblastic giant cells *in vitro* (Kanai-Azuma *et al.*, 1993).

The expression of the IGFs, their binding proteins and receptors in many fetal and embryonic tissues and their biological actions on fetal cells in culture are indirect evidence for a role for the IGFs as regulators of fetal growth. Direct evidence for this role has now been provided by the demonstration that null mutations of the IGF-I, IGF-II or type 1 receptor gene retard the growth of fetal mice (DeChiara *et al.*, 1990; Liu *et al.*, 1993, Powell-Braxton *et al.*, 1993). Mice deficient in IGF-I or IGF-II weigh 60% of normal wild type litter mates at birth. Powell-

Braxton *et al* (1993) report that 95% of IGF-I deficient mice die shortly after birth while Liu et al. (1993) observe a 10 to 68% survival rate depending on the genetic background. IGF-II deficient mice, although growth retarded are viable (De Chiara *et al.*, 1990). Type 1 receptor deficient fetuses are severely growth retarded (45% normal size) and die at birth of respiratory failure (Liu *et al.*, 1993). The phenotype of type 1 receptor mutants is identical to that of mutants lacking both the receptor and the IGF-I ligand, consistent with the actions of IGF-I being exerted through the type 1 receptor. Muscle hypoplasia, developmental delays in ossification, a thinning of the epidermis and abnormal cell numbers in regions of the central nervous system are observed in type 1 deficient fetuses (Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993).

Mice lacking both IGF-I and -II, or both IGF-II and type 1 receptor genes are more severely growth-retarded (30% normal size) than those lacking the type 1 receptor alone (45% normal size). This suggests that some IGF-II actions *in utero* must be mediated through a receptor other than the type 1 receptor (Liu *et al.*, 1993; Baker *et al.*, 1993). Placental size is also reduced in IGF-II deficient mice, but not in those in lacking IGF-I or type 1 receptor genes (De Chiara *et al.*, 1990; Baker *et al.*, 1993) suggesting that IGF-II also influences placental growth via an alternative receptor. Crossing mice carrying the T^{hp} chromosomal deletion, which incorporates the type 2 IGF receptor gene locus, with type 1 receptor deficient mice produces fetuses with normal birthweight and variable survival (Baker *et al.*, 1993). Therefore, actions of IGF-II exerted through the type 2 receptor cannot account for the severely growth retarded phenotype of IGF-II, type 1 IGF receptor deficient double mutants (Baker *et al.*, 1993).

The IGF-II gene and type 2 IGF / mannose 6 phosphate receptor gene are parentally imprinted in the mouse (DeChiara *et al.*, 1990; 1991; Barlow *et al.*, 1991). Only the paternal allelle of the IGF-II gene is expressed in embryonic mouse tissues, with the exception of choroid plexus and leptomeninges where both allelles are active (DeChiara *et al.*, 1991). In contrast, mouse embryos express the type 2 receptor gene from the maternal chromosome only (Barlow *et al.*, 1991). Based on the observation of reciprocal imprinting of these genes in mice, Haig and Graham (1991) suggest that the principal function of the type 2 IGF receptor *in utero* is to regulate the turnover of paternally produced IGF-II and limit fetal growth. In support of this

view, disruption of the type 2 IGF receptor produces mutants which are 30% larger than their wild-type litter mates and die *in utero* (Wang *et al.*, 1994). In contrast, mice lacking both the IGF-II and type 2 receptor gene are viable at birth (Wang *et al.*, 1994), suggesting that the lethal phenotype of type 2 receptor deficient mice may be due to an increased abundance of IGF-II ligand. The T^{hp} chromosomal deletion is lethal when transmitted through the maternal germline. Mice carrying this mutation are rescued when they also carry a null mutation of the IGF-II gene (Filson *et al.*, 1993). However, imprinting of the type 2 receptor gene is species specific and both maternal and paternal allelles are expressed in human fetal tissues (Kalscheuer *et al.*, 1993).

The high abundance of IGF-II in fetal tissues and blood, compared to IGF-I and to postnatal life, suggested that IGF-II may be a more important growth factor for fetal growth. However, gene knockout studies clearly indicate that both IGFs are necessary for normal fetal growth. Production of IGF proteins by multiple fetal tissues in the embryo and fetus suggests that autocrine and paracrine actions of the IGFs are important *in utero*. However, circulating levels of IGF-I have been shown to correlate positively with birthweight (Lassarre *et al.*, 1991; Fant *et al.*, 1993; 1.3.7), and blood levels of the IGFs are regulated by factors known to regulate fetal growth, such as substrate supply (1.3.3). Furthermore, intravenous infusion of IGF-I into late gestation fetal sheep promotes the growth of major fetal organs (Lok *et al.*, 1994). Acute infusion of IGF-I into the fetal sheep alters placental lactate production and enhances feto-placental glucose and amino acid uptake (Harding *et al.*, 1994), suggesting that IGF-I in fetal blood may influence fetal growth by regulation of placental function. Therefore a role for circulating IGF-I in the regulation of growth *in utero* is suggested.

1.3.7 *IGFs and IGFBPs in intra-uterine growth retardation*

In support of a role for the IGFs in regulating growth *in utero*, concentrations of IGF-I are reduced in the blood of growth retarded human fetuses in late gestation (Lassarre *et al.*, 1991) and correlate positively with fetal size or birthweight (Gluckman & Brinsmead, 1976; Lassarre *et al.*, 1991; Fant *et al.*, 1993). Fetal plasma levels of IGF-I are reduced when intrauterine growth is retarded experimentally by restriction of maternal supply of nutrients or oxygen

(1.3.3; Bassett *et al.*, 1990; Jones *et al.*, 1990; Straus *et al.*, 1991b; Unterman *et al.*, 1993). Thus a role for IGF-I in the aetiology of intra-uterine growth retardation is suggested.

In contrast the concentration of IGF-II, measured in cord blood throughout late gestation and at birth, is not related to fetal size (Lassarre *et al.*, 1991; Fant *et al.*, 1993). However, Fant *et al.* (1993) report a positive association between IGF-II in human cord serum and fetal ponderal index. In addition, IGF-II is reported to correlate with infant birthweight when measured in cord blood from normal term and preterm infants (Bennett *et al.*, 1983). Thus in human intra-uterine growth retardation, IGF-II appears to be unchanged or reduced. Fetal plasma levels of IGF-II are also decreased (Straus *et al.*, 1991b) or unchanged (Davenport *et al.*, 1990; Jones *et al.*, 1990; Kampman *et al.*, 1993) in growth-retarded fetal rats and pigs. However, elevated levels of plasma IGF-II and hepatic IGF-II mRNA have been reported in fetal sheep and rats made growth-retarded by restriction of maternal nutrient supply alone, or in combination with restricted oxygen (Jones *et al.*, 1988; Price *et al.*, 1992a; Townsend *et al.*, 1992; Tapanainen *et al.*, 1994; 1.3.3) or by maternal dexamethasone treatment (Price *et al.*, 1992b).

Intra-uterine growth retardation is also associated with altered concentrations of IGFBPs in fetal blood. Concentrations of IGFBP-3 are reduced in growth-retarded human infants (Crystal & Giudice, 1991), and correlate positively with infant birthweight (Fant *et al.*, 1993). Concentrations of IGFBP-1 are increased in the blood of growth retarded human infants (Crystal & Giudice, 1991) and IGFBP-1 in umbilical cord blood correlates negatively with birthweight (Wang *et al.*, 1991). This suggests that IGFBP-1 may function to inhibit the actions of the IGFs when the supply of substrates to the fetus is limited. Increased concentrations of IGFBP-1 are also observed in the blood of growth-retarded fetal rats and sheep (Straus *et al.*, 1991b; Osborn *et al.*, 1992; McLellan *et al.*, 1992; Unterman *et al.*, 1993). In contrast, plasma levels of IGFBP-2 or hepatic IGFBP-2 mRNA is increase (Gallaher *et al.*, 1994), decrease (McLellan et al., 1992) or remain unchanged (Osborn *et al.*, 1992) when fetal growth is limited by short term restriction of substrate supply, as observed for IGF-II.

1.4 ANIMAL MODELS OF INTRAUTERINE GROWTH RETARDATION

Studies investigating the roles and regulation of the IGFs in the fetus have used animal models of intra-uterine growth retardation. A number of animal models of intra-uterine growth retardation have been developed and have been recently reviewed (Owens *et al.*, 1989a; Harding & Charlton, 1991; Robinson *et al.*, 1994). In general, growth of the fetus can be experimentally restricted by altering maternal substrate availability, by interference with the uteroplacental or umbilical circulation, or by restriction of placental growth (Owens *et al.*, 1989a). In small species, such as the rat, high fetal number, a short gestation period (21 days) and a natural variation in fetal size relating to the position in the uterine horn can be confounding factors. In contrast, the sheep has a relatively long gestation of around 150 days, singleton pregnancies are common and the sheep can tolerate fetal surgery for the insertion of permanently indwelling vascular catheters in late gestation.

Intra-uterine growth retardation, associated with restricted placental size in sheep has been extensively studied (Alexander, 1964; Robinson *et al.*, 1979; Harding *et al.*, 1985; Owens *et al.*, 1986, 1987a,b,c). The ovine placenta consists of individual placentomes or cotyledons. Surgical removal of endometrial caruncles, the potential placental implantation sites, from the non-pregnant uterus of the ewe, limits the number of cotyledons which are formed in a subsequent pregnancy and reduces placental size (Alexander, 1964). In late gestation, fetal weight is related to the degree of placental restriction achieved (Alexander, 1964; Harding *et al.*, 1985; Owens *et al.*, 1986, 1988). Fetal growth retardation associated with restricted placental size is asymmetric (Robinson *et al.*, 1979; Harding *et al.*, 1985; Owens *et al.*, 1989a). The weight of the brain is spared relative to body weight, while the weights of liver and spleen are disproportionately reduced. Increases in the weight of the adrenal and kidneys as a fraction of body weight are also reported, while other tissues, such as lung and heart, decrease in proportion with body weight (Harding *et al.*, 1985). An increase in the ratio of fetal to placental weight is also observed (Robinson *et al.*, 1979; Harding *et al.*, 1985).

The growth-retarded fetal sheep produced by restriction of placental growth is chronically hypoxaemic and hypoglycaemic in late gestation (Harding *et al.*, 1985; Owens *et al.*, 1987a,b).

Oxygen and glucose delivery to and consumption by the gravid uterus and fetus are reduced with decreasing placental weight (Owens *et al.*, 1987a,b). However, oxygen and glucose consumption per kg of fetus or placenta is not different from control animals, reflecting increased extraction. Since the weight of the fetus is disproportionately maintained relative to placental weight this indicates a redistribution of these substrates to the fetus (Owens *et al.*, 1987a,b; Owens *et al.*, 1989). In addition, production of lactate is increased in the small placenta and a greater proportion of the lactate is released into the umbilical circulation (Owens *et al.*, 1987a). Plasma concentrations of alanine and the branched chain amino acids are also increased in the small fetus, however the normal flux of amino acids from the mother to the fetus reversed in growth-retarded fetal sheep, suggesting that breakdown of fetal muscle protein stores may occur (Owens *et al.*, 1989a).

A number of hormones are altered in the blood of growth-retarded fetal sheep. Concentrations of insulin, thyroid hormones and prolactin are reduced, and concentration of noradrenaline, adrenaline and cortisol increased in growth-retarded fetal sheep (Robinson *et al.*, 1980; Harding *et al.*, 1985; Robinson *et al.*, 1994). Increased blood levels of cortisol in small fetal sheep are associated with a premature prepartum rise in cortisol, with cortisol levels increased in the blood of fetal sheep with restricted placental size from approximately 120 days gestation (Robinson *et al.*, 1980).

In addition, many of the metabolic changes observed in the blood of growth-retarded fetal sheep have also been detected in samples collected from growth-retarded human infants, via cordocentesis. The growth retarded human infant is hypoxaemic, hypoglycaemic and has elevated concentrations of blood lactate (Soothill *et al.*, 1987; Economides *et al.*, 1990, see Robinson *et al.*, 1994). Growth-retarded human infants have low blood concentrations of most amino acids, particularly the branched chain amino acids (Cetin *et al.*, 1988; 1990). In addition, the normal positive umbilical veno-arterial difference is not observed in growth retarded human fetuses, and for some amino acids this difference is negative.

1.5 AIMS OF THIS STUDY

The general aim of this thesis was to examine the relationship between placental supply of substrates, growth and circulating levels and tissue production of the insulin-like growth factors in the fetal sheep.

At the commencement of these studies, tissue expression of the IGFs had not been examined in the sheep fetus. Therefore, no information was available as to the factors regulating expression of the IGF genes in different tissues, the likely sources of circulating IGFs and the relationship of local production of the IGF peptides to tissue growth in the fetal sheep. Supply of substrates to the fetus was indicated as a potential regulator of circulating concentrations of IGF-I, and possibly IGF-II, in the fetus. Placental size is a major determinant of growth of the fetus, due to its pivotal role as the organ which transfers essential substrates between mother, and fetus and restriction of placental size limits fetal growth by reducing the supply of both oxygen and nutrients to the fetus.

Therefore the following *hypotheses* were proposed:

(1) IGF-I and -II proteins are produced by fetal tissues and the placenta in sheep.

(2) Several tissues release IGF proteins into the blood of fetal sheep.

(3) Restriction of the supply of oxygen and nutrients to the fetus alters production of the IGFs by fetal tissues.

(4) Alterations in the circulating levels or tissue abundance of IGFs mediate the effects of restricted substrate supply on growth of the fetal sheep.

The *specific aims* of these studies were

- To measure IGF-I and -II proteins in the blood of fetal sheep with variable restriction of placental development.
- To measure IGF-I and -II mRNAs in tissues from fetal sheep with variable restriction of placental development.

- To determine the relationships between circulating concentrations of the IGFs, tissue expression of the IGFs and fetal growth.
- To identify potential sources of circulating IGFs in fetal blood.
- To examine factor(s) regulating production of the IGFs in the sheep fetus.

In the course of these studies, examination of the potential sources of IGF proteins in fetal blood suggested that several tissues, in particular liver and skeletal muscle, may release IGF-I into fetal blood. Negative feedback regulation of IGF-I and -II production by IGF-I was then described in adult rats.

Therefore, an additional *hypothesis* that negative feedback regulation could regulate production of IGF-I in the sheep fetus was tested. The *specific aim* was to determine whether infusion of IGF-I into fetal sheep regulated production of IGF-I and -II in fetal liver and skeletal muscle, putative sources of circulating IGFs in the fetus.

CHAPTER 2

CONCENTRATIONS OF INSULIN-LIKE GROWTH FACTORS-I AND -II IN FETAL BLOOD: RELATIONSHIP TO FETAL GROWTH AND SUBSTRATE SUPPLY IN SHEEP.

2.1 INTRODUCTION

Direct evidence that the IGFs are necessary for normal fetal growth and development has been provided by the observation that inactivation of the genes for IGF-I, IGF-II and their receptors retards the growth of fetal mice (De Chiara *et al.*, 1990; Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993; Wang *et al.*, 1994; 1.3.6). In the fetus with the normal genetic potential to both produce and respond to IGF-I and -II, the principal role of these growth factors may be to mediate the effects of external influences on fetal growth, particularly that of placental supply of substrates. However, little is known about the regulation of IGF expression and action before birth, and of the relative importance of systemic (endocrine) and local (autocrine/paracrine) mechanisms of IGF action in prenatal life. However, birthweight in human infants correlates positively with concentrations of IGF-I, and in some cases IGF-II, in cord blood (Bennett *et al.*, 1983; Gluckman *et al.*, 1983; Fant *et al.*, 1993), suggesting a role for circulating IGFs in regulating the growth of the human fetus.

Placental weight is the major influence on birthweight in humans and impaired placental delivery of essential fetal substrates is strongly implicated in human fetal growth retardation (Owens & Robinson, 1988; 1.1, 1.4). Placental size is a major determinant of fetal growth in all species and experimental restriction of placental size in sheep retards fetal growth by limiting the supply of oxygen and nutrients to the fetus (Robinson *et al.*, 1979; Harding *et al.*, 1985, Owens *et al.*, 1988; 1.4). A previous report indicates that the concentration of IGF-I is reduced in the blood of fetal sheep made growth-retarded by restriction of placental size, while the concentration of IGF-II increases (Jones *et al.*, 1988). However, the metabolic status of these animals was not documented and the relationship of circulating IGFs in the fetus to supply of oxygen or nutrients is unknown.

Therefore the ***specific aim*** of this study was to examine the relationship between placental supply of substrates, fetal growth and the circulating concentrations of the IGFs in fetal sheep.

The ontogeny of IGF-I and -II proteins and fetal blood pO_2 and glucose was determined in fetal sheep with normal and restricted placental development in late gestation.

2.2. MATERIALS AND METHODS

2.2.1 SURGICAL PROCEDURES

Removal of endometrial caruncles

Surgical removal of endometrial caruncles from the uterus of non-pregnant Border-Leicester / Merino crossbred ewes was performed to restrict placental growth and consequently retard fetal growth as previously described (Robinson *et al.*, 1979, Owens *et al.*, 1986). The ewe was anaesthetised by intravenous injection of 0.75 g of sodium thiopentane (Pentothal Sodium, Abbott). Anaesthesia was maintained using halothane (Fluothane, ICI) in oxygen. The abdomen was clipped and scrubbed. A low midline incision was made and the uterus was exposed. Each horn of the uterus was opened along the antimesometrial border from the cervix to near the uterotubal junction. Most visible endometrial caruncles were excised using a scalpel blade. Bleeding was controlled by application of pressure and clotted blood was removed before closing the incision. The uterine incision was closed in a single layer with continuous suture using 3-0 chromic catgut (Johnson & Johnson). The uterus was swabbed to remove any remaining blood and the abdominal incision was closed in two layers (Vicryl, Metric 4, Johnson & Johnson). Antibiotics (1 gram streptomycin and 300 mg benzylpenicillin, Commonwealth Serum Laboratories) were administered by intramuscular injection to the ewe prior to surgery. The ewes were returned to Mortlock Research Station, Mintaro, South Australia one week after surgery and were allowed at least two months recovery before mating.

Ewes, from which endometrial caruncles had been removed ('caruncle' ewes), were maintained with a flock of unoperated control ewes. Rams harnessed with marking raddles were used for mating. Pregnancy was confirmed by a lack of return to oestrus and by ultrasound examination at approximately 60 days of pregnancy.

In the experiments described in this chapter, six 'caruncle' ewes, in which an average of 84 $\pm$ 6 (mean $\pm$ SEM) caruncles had been removed from the uterus, were studied.

Insertion of vascular catheters

The six 'caruncle' ewes and ten previously unoperated pregnant ewes were maintained under normal grazing conditions until 100 days of gestation. At this time they were moved to the Institute of Medical and Veterinary Science animal facility, Adelaide, SA and were housed in individual metabolism cages under conditions of controlled temperature and lighting. Lucerne hay, oats, alfalfa pellets and water were provided *ad libitum* daily.

At 110 days gestation, vascular catheters were implanted in the sixteen ewes and their fetuses as previously described (Owens *et al.*, 1986). Pregnant ewes were anaesthetised using 1 g of sodium thiopentane and anaesthesia was maintained using halothane in oxygen and nitrous oxide. All procedures were carried out under strict aseptic and antiseptic conditions. The abdomen and flank were clipped and surgically scrubbed. A low midline incision was made, avoiding the previous scar in ewes where endometrial caruncles had been removed. The pregnant uterus was exposed. All catheters were passed into the abdominal cavity through a stab wound in the flank. An incision was made in the uterus and the fetal hindlimb was exposed. Polyvinyl catheters (Dural Plastics) were implanted in the fetal tarsal vein (SV 70, 1.0 mm ID, 1.5 mm OD) and fetal femoral artery (SV55, 0.8 mm ID, 1.2 mm OD) and advanced into the inferior vena cava and abdominal aorta respectively. The uterine incision was closed (Mersilk 2/0) and a second incision was made over the position of the fetal abdomen. An umbilical vein was exposed through an incision in the skin of the abdomen near the point of entry of the umbilical cord. A purse string suture (Mersilk 5/0, Johnson & Johnson) was placed in the wall of the vein. A silastic catheter (0.02 inches ID, 0.037 inches OD, Dow Corning Medical Products) sleeved onto a polyvinyl catheter (SV37, 0.5 mm ID, 1.0 mm OD) was inserted into a small incision made in the centre of the pursestring. The catheter was advanced approximately 3 cm to lie in the common umbilical vein. The purse string suture was closed and a small amount of cyanoacrylate (Supaglue, Selley) was applied to the surface of the vein at the point of entry of the catheter. The uterus was closed as before. A further catheter (SV70) was placed into a uterine vein and advanced approximately 20-25 cm to lie in the utero-ovarian vein. The uterus was returned to the abdomen and the abdominal incision was closed in two layers

using 4/0 Mersilk. Catheters (SV 102, 1.4 mm ID, 1.9 mm OD) were also placed in a maternal carotid artery and jugular vein. The ewe was returned to the metabolism cage and monitored until standing, which was generally within 1-2 hours of surgery. Catheters were flushed with 50 U/ml heparin in saline sealed with 3 way taps, stoppered, covered, wrapped in plastic and attached to the ewes back. Antibiotics were administered to the pregnant ewe at the time of surgery and for the following three days as described (2.2.1). The fetus received 300 mg benzylpenicillin intravenously once daily for three days following surgery.

2.2.2 SAMPLE COLLECTION.

Catheter patency was maintained by flushing with heparinised saline (50 U/ml) on alternate days commencing the day following surgery. Patency of the umbilical vein catheter was maintained by continuous infusion of heparin saline (250 U/ml) at a rate of 0.2 ml/hr using a Braun Perfusor VI pump. Animals were allowed a minimum of seven days recovery from surgery before experimental studies commenced.

At 120 ± 1 and again at 127 ± 1 (mean ± SEM) days of pregnancy in each ewe, two blood samples were collected from the fetal femoral artery (2.75 ml), fetal umbilical vein (2.75 ml), maternal carotid artery (10 ml) and maternal utero-ovarian vein (10 ml), one at 1030 and the other at 1045 hours.

Blood samples were collected into heparinised syringes. Sealed syringes were placed on ice until measurement of blood gases and pH. Remaining blood was centrifuged at 2 000 *x g* for 20 min at 4°C (J-6B centrifuge, Beckman Instruments). Plasma was recovered and stored at -20°C.

The ewe and fetus were sacrificed by intravenous maternal overdose of sodium pentobarbitone (Lethobarb, Arnolds of Reading) at 130 ± 1 (mean ± SEM) days of gestation. The fetus was dried and its weight recorded. Individual fetal organs were dissected and weighed. Placental weight was determined as the sum of the weights of all placentomes. Fetal weights were adjusted to 130 days of gestation, using growth curves for control and chronically catheterised fetal sheep established previously in this laboratory.

2.2.3 BLOOD GASES AND METABOLITES

Blood gases (pO_2, pCO_2, pH) were measured using a Radiometer ABL330 blood gas analyser and oxygen saturation and haemoglobin concentration were measured using a Radiometer OSM2 hemoximeter. Blood glucose concentrations were measured in deproteinised blood using glucose oxidase (Sigma Chemical Co.) as previously described (Owens *et al.*, 1987).

2.2.4 IGF-I AND IGF-II CONCENTRATIONS IN FETAL PLASMA

IGF-I and -II concentrations were measured in fetal arterial blood plasma by radioimmunoassay (RIA) and radioreceptor assay (RRA) respectively (Francis *et al.*, 1989b; Owens *et al.*, 1990). Prior to analysis, IGFs were dissociated and separated from their binding proteins in plasma by size exclusion high performance liquid chromatography (HPLC) under acid conditions (Scott & Baxter, 1986; Owens *et al.*, 1990), to remove binding protein artefacts from the IGF assays (Mesiano *et al.*, 1988).

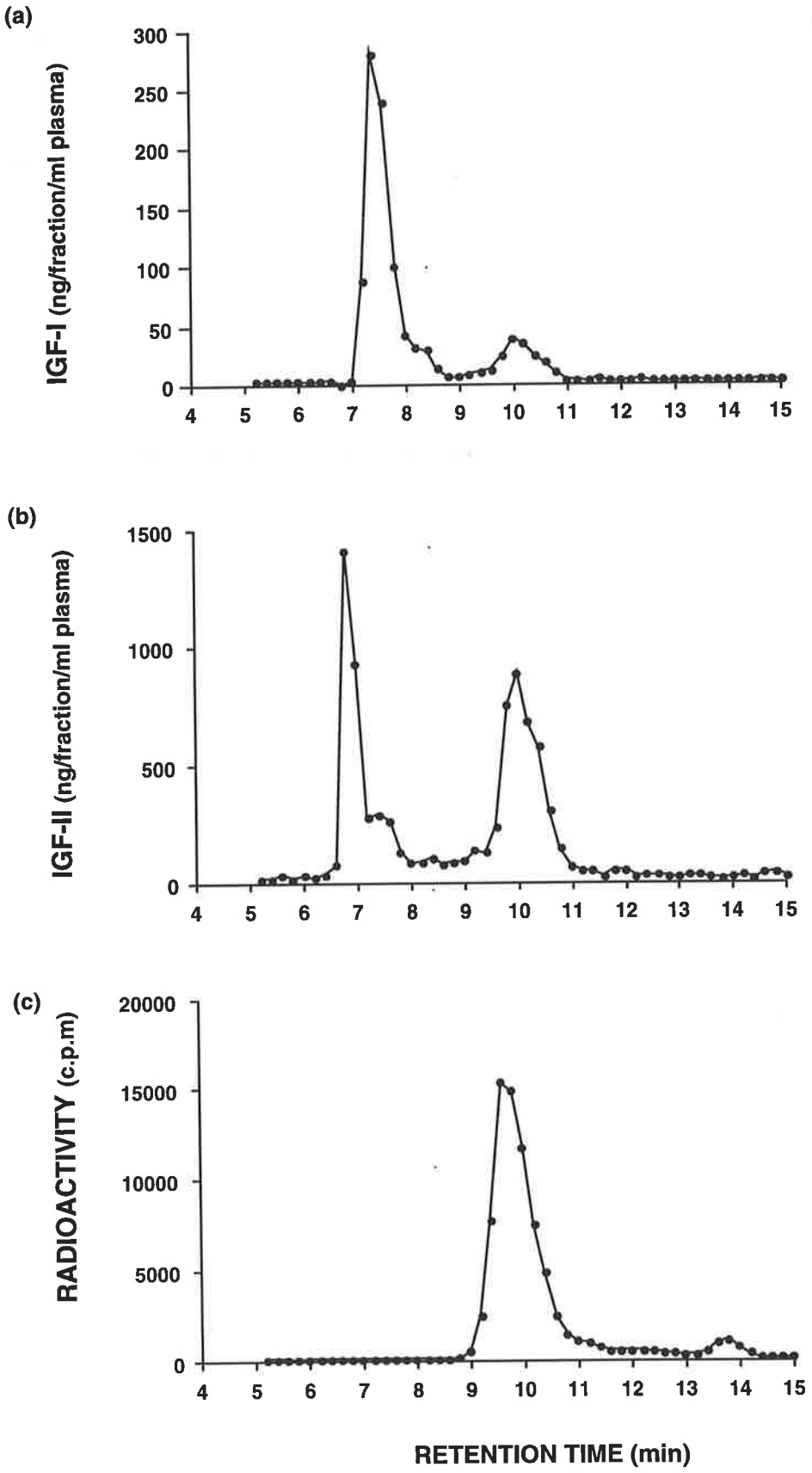
Extraction of IGFs from fetal plasma by size exclusion chromatography under acid conditions.

Fetal sheep plasma was acidified by dilution to a final concentration of between 15 and 30% (v/v) in a solution to achieve the same final concentration as acidic chromatography mobile phase (200 mM acetic acid, 50 mM trimethylamine, 5 ml/l Tween-20, pH 2.8). Diluted plasma samples were mixed with an equal volume of Freon (1,1,2-trichloro-1,2,2-trifluoroethane) centrifuged at 10 000 x *g* for 10 min and the upper aqueous phase recovered. Defatted plasma was clarified by centrifugation through a microfilter containing a 0.45 µm cellulose acetate filter membrane (Cat No 8740, Alltech Associates Inc.). Between 200 and 350 µl of the acidified, defatted, filtered plasma (containing between 30 and 40 µl of fetal sheep plasma) was injected onto a Protein-Pak 125 HPLC column (Waters /Millipore) using an automatic injector (ICI AS 2000, ICI Instruments). Samples were eluted at 1 ml/min in acidic chromatography mobile phase (pH 2.8).

To calibrate the autocollection program for the fraction collector, fractions of 0.2 ml were initially collected between 6 and 12 min after injection of plasma samples into the size exclusion liquid chromatography system. IGF-I and -II content was measured in all fractions after neutralisation with 0.6 volumes of 0.4 M Tris-base. Two peaks of activity were detected in these assays (Figure 2.1). The first peak of activity eluting between 6.0 and 8.5 min after injection, contains IGF binding proteins. The IGFBPs, separated from the IGF ligands under the acid conditions, are able to bind [^{125}I]-IGF-I and -II (data not shown). This inhibits association of the radioligand with the antibody or receptor in the assay and appears as activity in competitive radioligand binding assays. The second peak of activity, eluting between 9.0 and 11.0 min, corresponds to the position at which [^{125}I]-IGF-I or -II elutes from the column, and contains IGF-I and IGF-II that has been dissociated from binding proteins by the acid conditions. For routine analysis, the eluate corresponding to the IGF region was collected as a 2 ml pool and replicate subsamples were assayed for IGF-I and -II. ^{125}I-IGF-I was injected prior to and following chromatography of each series of samples, to confirm the elution position of the IGF proteins. Recovery of [^{125}I]-IGF-I through the size exclusion HPLC system was 96 ± 1% (n=29).

FIGURE 2.1 Acid size exclusion chromatography of fetal sheep plasma IGFs.

Samples were chromatographed on a Protein Pak 125 column at 1 ml/min in 0.1M acetic acid, 0.05 M triethylamine, 5% Tween-20, pH 2.8.
(a) Chromatography of fetal sheep plasma with subsequent addition of neutralised fractions to IGF-I radioimmunoassay,
(b) as for (a) but with neutralised fractions added to the IGF-II radioreceptor assay.
IGF content is expressed as ng per fraction corrected to the equivalent of chromatographing 1 ml of plasma.
(c) Chromatography of ^{125}I-labelled recombinant human IGF-I and measurement of the radioactivity content of the fractions.


(a)
IGF-I (ng/fraction/ml plasma)
300
250
200
150
100
50
0
4 5 6 7 8 9 10 11 12 13 14 15
(b)
IGF-II (ng/fraction/ml plasma)
1500
1000
500
0
4 5 6 7 8 9 10 11 12 13 14 15
(c)
RADIOACTIVITY (c.p.m)
20000
15000
10000
5000
0
4 5 6 7 8 9 10 11 12 13 14 15
RETENTION TIME (min)

Radioimmunoassay of IGF-I

Recombinant human IGF-I (Gropep Pty. Ltd.) was used as the assay standard and as radioligand, following iodination using chloramine-T and Na ^{125}I (Amersham Australia Pty. Ltd.) to a specific activity of 60-80 Ci/g (Francis *et al.*, 1986). Radiolabelled IGF-I was separated from unincorporated label by chromatography through a Sephadex G50 size exclusion column in a buffer of 0.05 M sodium phosphate, 0.15 M NaCl pH 6.5, containing 0.25% (w/v) bovine serum albumin (RIA grade, Sigma Chemical Co.). Labelled IGFs were stored at -20°C. Antiserum (PM87) to recombinant human IGF-I was raised in rabbit.

Subsamples (50 µl) of the IGF containing column eluate (equivalent to 7.5 to 10 µl of plasma) were neutralised by addition of 30 µl of 0.4 M Tris base. Neutralised aliquots were combined with 200 µl IGF-I assay buffer (30 mM NaH_2PO_4, 10 mM EDTA, 0.02% (w/v) sodium azide, 0.05% (v/v) Tween 20 at pH 7.5), 50 µl [^{125}I]-IGF-I (approximately 20 000 cpm) in assay buffer, and 50 µl of rabbit anti-IGF-I antiserum in assay buffer (final dilution in the assay incubate, 1:10 000). Standards (3.9 to 2000 ρg in 200 µl IGF-RIA buffer) were mixed with 30 µl 0.4 M Tris base, 50 µl acid column mobile phase and [^{125}I]-IGF-I and anti-IGF-I antiserum were added as for the samples. All tubes were incubated at 4°C for 16 h. Fifty µl of goat anti-rabbit γ-globulin solution in assay buffer and 10 µl of 5% (v/v) normal rabbit serum were added. The tubes were incubated at 4°C for 30 min, followed by addition of 1 ml of ice-cold 5.5% (w/v) polyethylene glycol 6000 in 150 mM NaCl, and centrifugation at 4°C, 3 500 *x g* for 20 min (J6B Centrifuge, Beckman Instruments). Supernatants were aspirated and the radioactivity in the pellet was measured (1261 MultiGamma gamma counter, Pharmacia/LKB, Wallac Oy). IGF concentrations were determined using the 1224 RiaCalc /LM data management program (Wallac Oy).

IGF-I was measured in triplicate aliquots from each plasma injection. The cross-reaction of ovine IGF-II in the assay is less than 1% (Francis *et al.*, 1989b). The average minimal detectable amount of IGF-I, estimated by the 1224 RiaCalc/ LM program (Wallac Oy), was 25 pg/tube This corresponds to a concentration of 25 to 34 ng IGF-I/ml of plasma chromatographed. The intra- and inter- assay co-efficients of variation, assessed by repeat analysis of a reference adult sheep plasma sample containing 330 ng IGF-I/ml, were 3.2% and 12.4% respectively.

Radioreceptor assay of IGF-II

Recombinant human IGF-II (donated by Dr. T. L. Jeatran, Lilly Research Laboratories) was used as a standard and as radioligand following iodination as described for IGF-I. Ovine placental membranes were prepared as described by Baxter & De Mellow (1986).

As for the IGF-I assay, 50 µl subsamples of the column fractions (equivalent to 7.5 to 10 µl of plasma) were neutralised by addition of 30 µl 0.4 M Tris base. Samples were combined with 200 µl RRA buffer (10 mM Tris, 0.5% (w/v) bovine serum albumin, 10 mM $CaCl_2$, pH 7.4), 50 µl [^{125}I]-IGF-II (approximately 20 000 cpm) and 100 µl ovine placental membranes in assay buffer. Standards (9 to 5000 ρg, in assay buffer) with 50 µl mobile phase and 30 µl of neutralising solution added were set up as for the unknowns. All tubes were incubated for 16 h at 4°C, and 1 ml of 10 mM Tris, 100 mM $CaCl_2$, pH 7.4 buffer containing 0.1% (w/v) bovine serum albumin was added. The tubes were centrifuged and the radioactivity in the pellet measured as for the IGF-I assay.

IGF-II was measured in quadruplicate aliquots from each plasma injection. Cross reaction of ovine IGF-I in the IGF-II assay is less than 1% (Francis *et al.,* 1989b). The average minimal detectable concentration of IGF-II in plasma, estimated by the 1224 RiaCalc /LM program (Wallac Oy), was 60 ng/ml. This corresponds to a plasma concentration of 60 to 79 ng IGF-II/ml of plasma chromatographed. The intra- and inter-assay co-efficients of variation for the IGF-II assays, assessed by repeat analysis of a reference fetal sheep plasma sample containing 460 ng/ml IGF-II, were 4.3% and 16.6% respectively.

2.2.5 STATISTICS

All results are expressed as mean ± SEM. Treatment effects were analysed by analysis of variance (ANOVA) with one between factor (size) and one within factor (age) using the SuperANOVA program (Abacus Concepts Inc.). Specific comparisons were compared by one way ANOVA and Bonferroni/Dunn tests. Differences with gestational age were assessed using repeated measures ANOVA (SuperANOVA program). Relationships between variables were tested using simple and partial correlation analysis and multiple regression analysis (Statview SE + Graphics, Abacus Concepts Inc. and SAS/STAT software). Results were considered significant at the 5% level.

2.3 RESULTS

2.3.1 *Fetal growth, blood gases and metabolites*

Placental weight ranged from 102 g to 477 g at 130 ± 1 days of gestation. Fetal weight ranged from 1480 g to 3550 g and correlated positively with placental size (r=0.81, P< 0.0005, n= 15) (Figure 2.2). Fetal growth retardation was asymmetric. When fetal tissue weights were expressed as a fraction of fetal body weight, fractional fetal liver weight decreased (r=0.52, P< 0.05) whereas fractional fetal brain weight increased (r=-0.73, P< 0.005) with decreasing placental weight (Figure 2.2).

Fetal arterial blood pO_2 (r=0.51, P< 0.05, n=16) and blood glucose (r=0.57, P< 0.02, n= 16) at 120 days gestation were positively associated with placental weight (at 130 days gestation). At 127 days gestation, blood pO_2 (r=0.63, P< 0.02, n= 15), but not blood glucose, correlated positively with placental weight. Fetal weight (at 130 days gestation) was positively associated with arterial blood pO_2 (r= 0.63, P< 0.02, n=14) and glucose (r= 0.57, P < 0.03, n= 14) at 127 days gestation only.

2.3.2 *Plasma IGF-I and -II concentrations*

Concentrations of IGF proteins measured at two time points within each animal on each day varied from their mean value by 5.8% (IGF-I) and 8.8% (IGF-II). The average of these two measurements per fetus was used for all analyses.

Concentrations of IGF-I protein in fetal blood at 120 days gestation (r= 0.87, P< 0.0001, n=16) and at 127 days gestation (r= 0.75, P< 0.001, n=15) correlated positively with placental weight (Figure 2.3a). IGF-I concentrations in fetal plasma at 120 days gestation (r= 0.93, P< 0.0001, n=15) and 127 days gestation (r= 0.84, P< 0.0002, n=14) also correlated positively with fetal weight. Concentrations of IGF-II at 120 days of pregnancy were not related to fetal weight or placental weight. At 127 days gestation, however, fetal plasma IGF-II was positively associated with both placental weight (r= 0.58, P< 0.02, n=15) and fetal weight (r= 0.60, P< 0.02, n=14).

Fetal plasma IGF-I was also positively related to fetal blood glucose (120 days, r= 0.59, P< 0.02; 127 days, r= 0.72, P< 0.005) (Figure 2.4a) and fetal arterial pO_2 (120 days, r= 0.58, P< 0.02; 127 days, r= 0.67, P< 0.01) (Figure 2.4b). Plasma concentrations of IGF-II correlated with fetal blood glucose concentrations (r= 0.51, P< 0.05) (Figure 2.4c) and arterial blood pO_2 (r= 0.51, P< 0.05) (Figure 2.4d) at 127, but not 120, days gestation. No associations were observed between fetal blood pH and IGF-I or -II protein concentrations.

Concentrations of IGF-I in fetal blood at 120 and at 127 days gestation correlated positively with the weights of individual fetal tissues (measured at 130 days gestation), including liver (120 days, r= 0.73, 127 days, r= 0.84), kidney (120 days, r= 0.74; 127 days, r= 0.82), lungs (120 days, r= 0.68, 127 days, r= 0.69), heart (120 days, r= 0.83, 127 days, r= 0.80), and brain (120 days, r= 0.73, 127 days, r= 0.69) (all P< 0.01).

At 127 days gestation plasma IGF-II concentrations also correlated positively with the weights of individual fetal tissues (measured at 130 days gestation) including liver (r= 0.57), kidney (r= 0.79), lungs (r= 0.60), heart (r= 0.77) and brain (r= 0.60) (all P < 0.05).

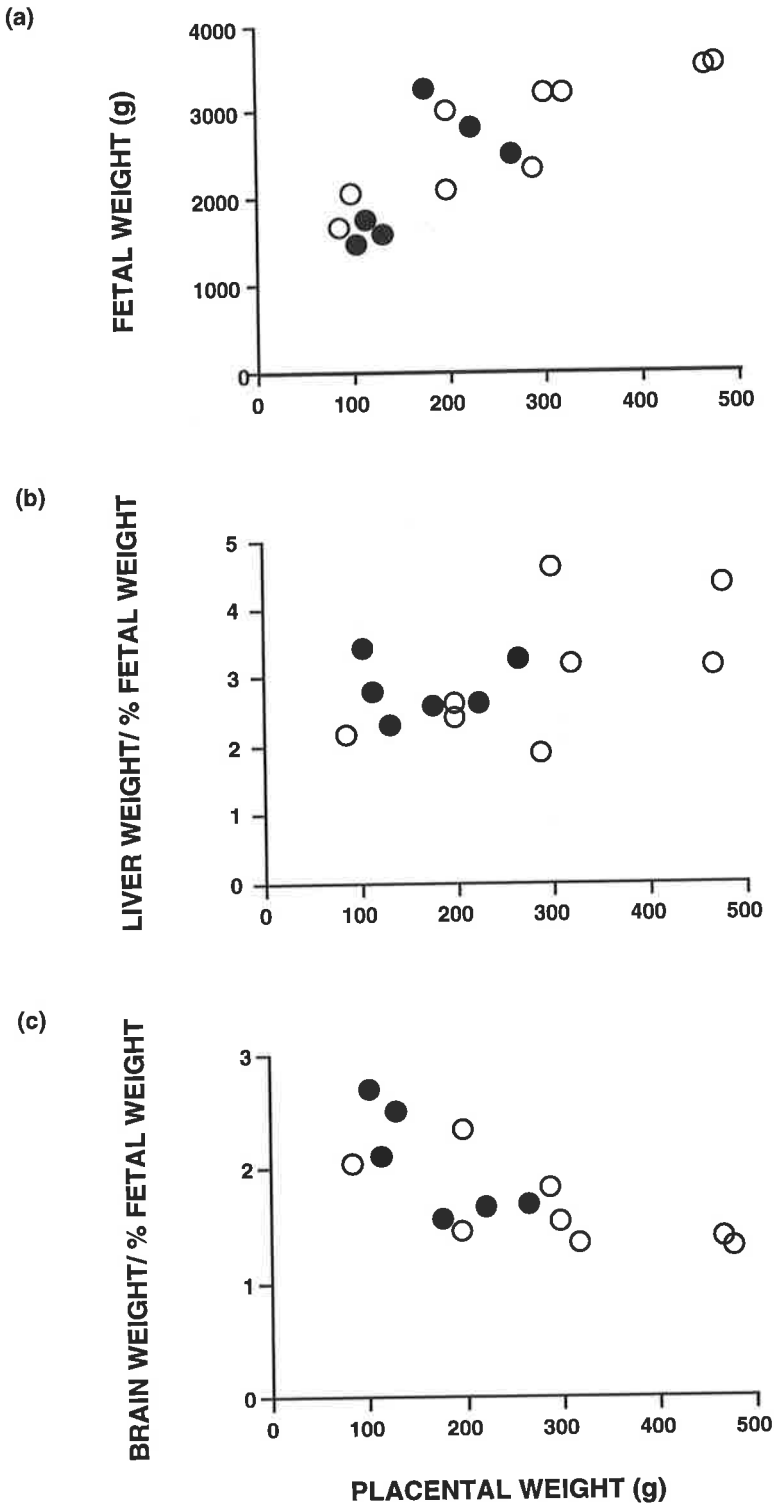


FIGURE 2.2 Effect of restriction of placental size on fetal growth at 130 days gestation.

Relationship between (a) Fetal weight (r= 0.81, P< 0.0005 , n=15), (b) Liver weight as a percentage of fetal weight (r= 0.52, P< 0.05, n=14), (c) Brain weight as a percentage of fetal weight (r= -0.73, P< 0.003, n=14) and placental weight measured at 130 days gestation in fetal sheep from ewes with normal (○, n=9) and restricted (●, n=6) placental development.

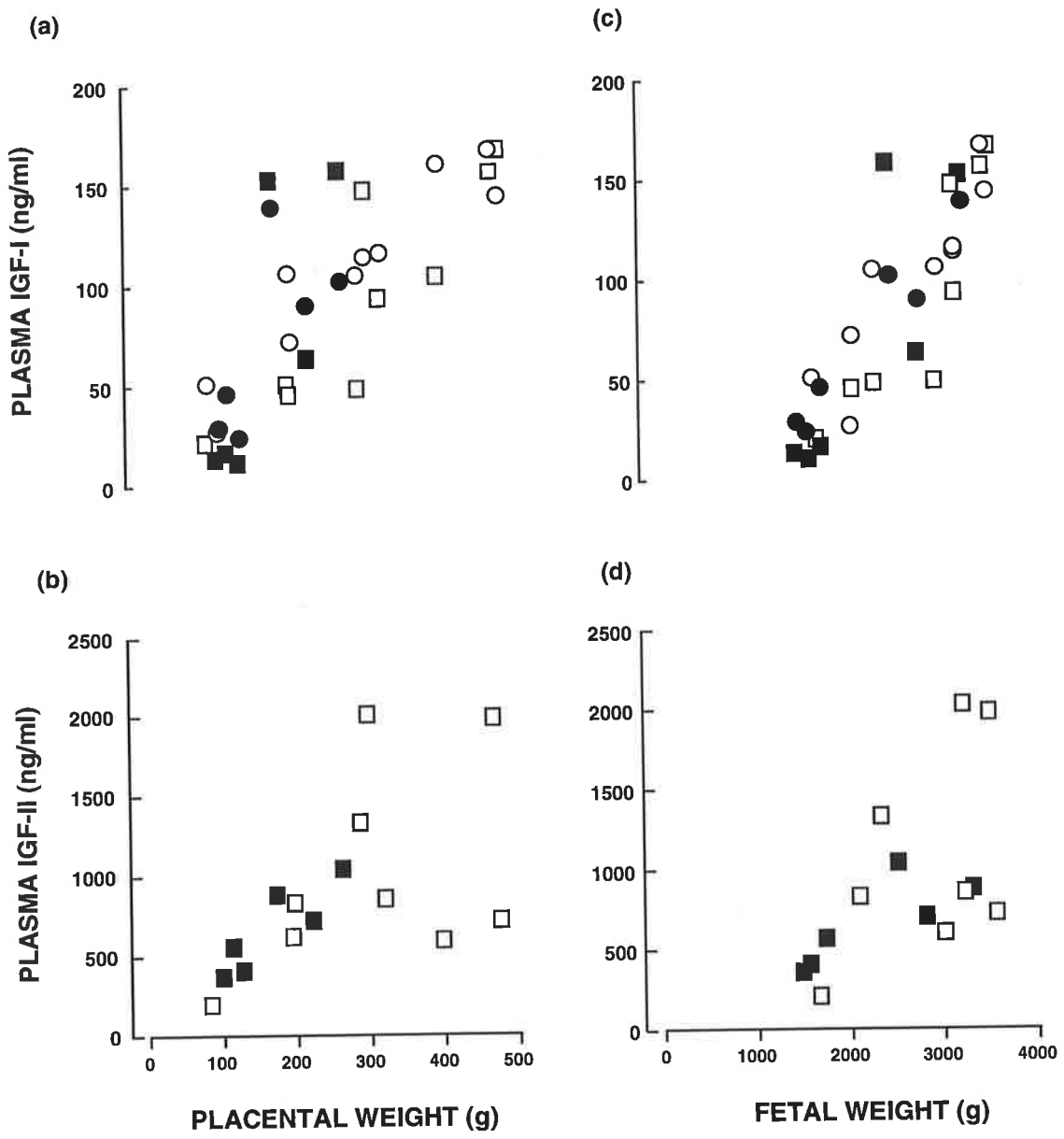


FIGURE 2.3 Relationship between the concentration of IGFs in fetal blood and fetal growth.

Correlations between **(a)** concentrations of IGF-I in fetal plasma at 120 days (r= 0.87, P< 0.0001, n=15, circles) and 127 days (r= 0.75, P< 0.001, n=15, squares) and placental weight measured at 130 days gestation in fetal sheep with normal (open symbols) or restricted (closed symbols) placental development. **(b)** concentrations of IGF-II in fetal plasma at 127 days and placental weight measured at 130 days gestation (r=0.58, P< 0.02, n= 15) in fetal sheep from ewes with normal (open symbols) and restricted (closed symbols) placental development .

Correlations between **(c)** concentrations of IGF-I in fetal plasma at 120 days (r= 0.93, P< 0.0001, n= 15, circles) and 127 days (r= 0.84, P< 0.0002, n=14, squares) and fetal weight measured at 130 days gestation in fetal sheep with normal (open symbols) or restricted (closed symbols) placental development. **(d)** concentrations of IGF-II in fetal plasma at 127 days and fetal weight measured at 130 days gestation (r= 0.60, P< 0.02, n= 14) in fetal sheep with normal (open symbols) or restricted (closed symbols) placental development.

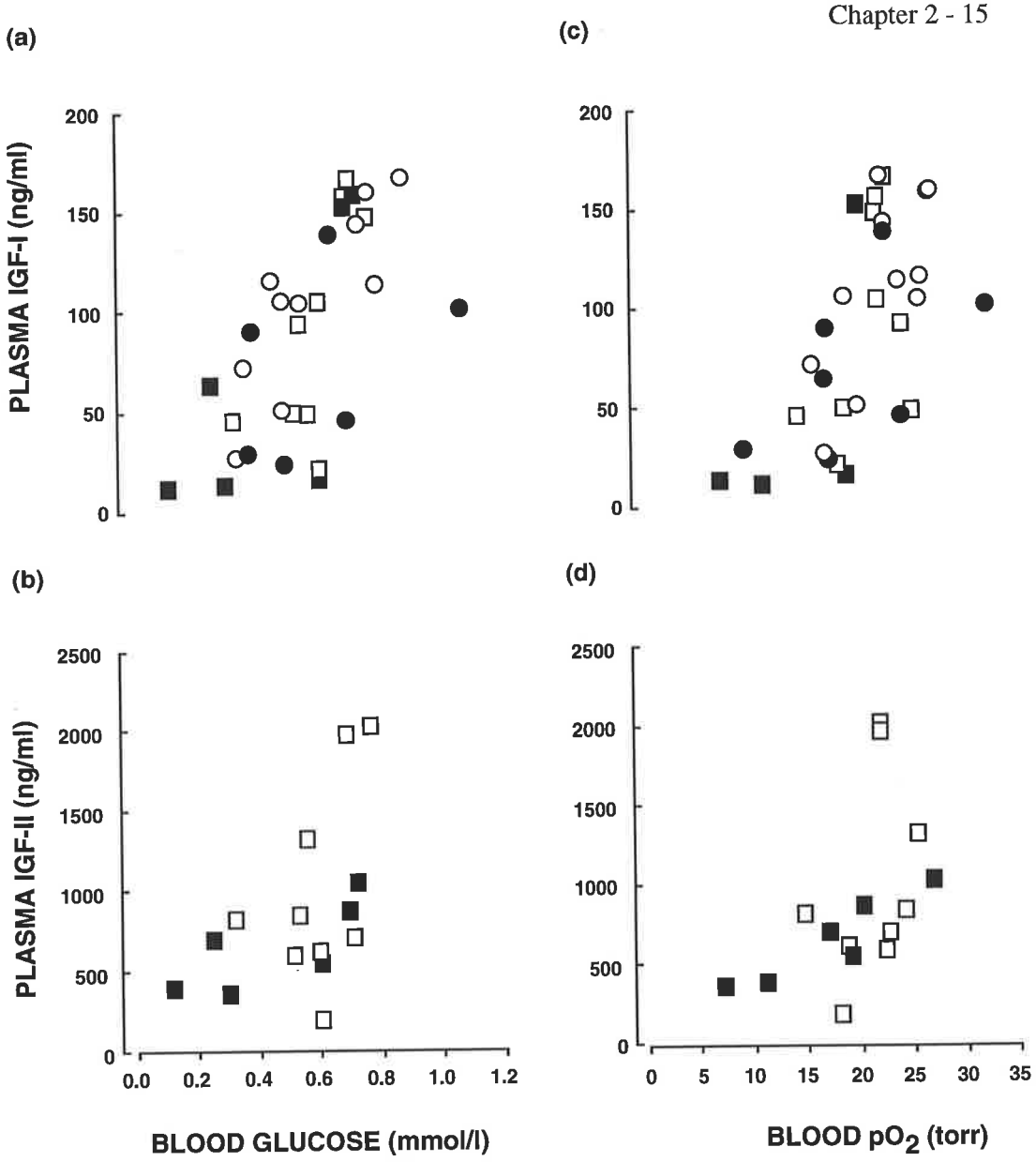


FIGURE 2.4 Relationship between the concentration of IGFs in fetal blood and and fetal blood glucose and pO_2.

Correlations between **(a)** concentrations of IGF-I in fetal plasma at 120 days (r= 0.59, P< 0.02, n= 16, circles) and 127 days (r= 0.72, P< 0.005, n= 15, squares) and fetal blood glucose concentrations and **(b)** concentrations of IGF-II in fetal plasma at 127 days (r= 0.51, P< 0.05, n= 15) and fetal blood glucose concentrations in fetal sheep from ewes with normal (open symbols) or restricted (closed symbols) placental development.

Correlations between **(c)** concentrations of IGF-I in fetal plasma at 120 days (r= 0.58, P< 0.02, n= 15, circles) and 127 days (r= 0.67, P< 0.01, n=14, squares) and fetal blood pO_2 and **(d)** concentrations of IGF-II in fetal plasma at 127 days (r= 0.51, P< 0.05, n= 14) and fetal blood pO_2 in fetal sheep from ewes with normal (open symbols) or restricted (closed symbols) placental development.

2.3.3 *Plasma IGFs, blood gases and metabolites at 120 and 127 days gestation.*

To determine whether the associations observed between plasma IGF-II, fetal weight and substrate supply related to changes in the supply of substrates with advancing gestation, the fetal sheep in this study were divided into three groups according to size (Table 2.1). Based on the weight of normal catheterised singleton fetal sheep at 130 days gestation in this flock (3230 ± 190 g, mean ± SD, n=17), the fetuses were classified as 'normal' size (weights within 2 SD of mean control weight or > 2850 g), moderately growth-retarded or 'small' fetal sheep (weights between 2SD and 6SD below mean control weight or 2850 to 2090 g) and severely growth-retarded or 'very small' (weights more than 6SD below mean control weight or < 2090 g). According to this division, control ewes carried five 'normal size', three 'small' and two 'very small' fetal sheep, while ewes from which endometrial caruncles has been removed carried one 'normal', two 'small' and three 'very small' fetal sheep.

Blood pO_2 and glucose were not significantly different between the three fetal size groups at 120 days gestation (Table 2.2). At 127 days gestation blood pO_2 was reduced in 'very small' fetal sheep, when compared to 'normal size' and to 'small' fetuses and blood glucose concentrations were lower in 'very small' fetal sheep when compared to fetal sheep of 'normal size' (Table 2.2). In addition, blood pO_2 decreased with increasing gestational age in 'very small' fetal sheep (Table 2.2). Fetal blood pCO_2 increased with gestational age in 'very small' fetal sheep and was increased when compared to 'normal' size fetuses at 127 days gestation (Table 2.2). Haemoglobin concentrations in fetal blood were reduced in the blood of 'very small' fetal sheep when compared to 'small' fetal sheep.

Concentrations of IGF-I were lower in 'small' and 'very small' fetal sheep at 120 and 127 days gestation when compared to 'normal' size (Table 2.3). At 120 days gestation, no differences in plasma IGF-II were evident between groups. However, by 127 days gestation in 'very small' fetal sheep, IGF-II concentrations were lower than those of 'normal' size fetuses ($P< 0.05$, Table 2.3). The ratio of IGF-I:IGF-II in fetal plasma decreased with fetal weight ($P= 0.04$), such that at 120 and 127 days gestation the ratio of IGF-I:IGF-II was reduced in 'very small' fetal sheep when compared to 'normal' size fetuses.

TABLE 2.1 Morphometry of fetal sheep from ewes with normal and restricted placental development, divided into three groups according to fetal size.

	NORMAL (6)	SMALL (5)	VERY SMALL (5)
Fetal weight (g)	3344 ± 75	2545 ± 163 ***	1708 ± 100 *** #
Placental weight (g)	356 ± 47	233 ± 18 *	105 ± 7 *** #
Number of cotyledons	57.5 ± 9	33.4 ± 4	32.6 ± 9
F:P ratio	10.8 ± 2	11.2 ± 1	16.6 ± 2 * #
Liver weight (g)	115 ± 12	65 ± 7 **	43 ± 4 ***
Brain weight (g)	48 ± 1	45 ± 1	38 ± 1 *** #
Brain weight/ Fetal weight	0.014 ± 0.0005	0.018 ± 0.001	0.023 ± 0.002*** #
Brain weight/ Liver weight	0.42 ± 0.05	0.72 ± 0.09 *	0.89 ± 0.08 **

Values are mean ± SEM (number of animals). *** $P < 0.0005$, ** $P < 0.005$, * $P < 0.05$ compared to control. One way analysis of variance (ANOVA) and Bonferroni Dunn. # $P < 0.05$, significantly different from 'small'. F:P ratio is the ratio of fetal to placental weight. All measurements were made at 130 ± 1 (mean ± SEM) days gestation.

TABLE 2.2 Glucose concentrations, pO_2 and pH of arterial blood from fetal sheep with normal and restricted placental development, divided according to fetal size.

	NORMAL (6)	SMALL (5)	VERY SMALL (5)
Glucose (mmol/l)			
120 days	0.71 ± 0.06	0.56 ± 0.13	0.47 ± 0.06
127 days	0.66 ± 0.03	0.47 ± 0.09	0.40 ± 0.12 *
pO_2 (torr)			
120 days	24.2 ± 0.8	22.1 ± 3.1	17.6 ± 2.4
127 days	22.2 ± 0.5 †	20.5 ± 2.4	13.7 ± 2.9 * # †
pH			
120 days	7.32 ± 0.01	7.30 ± 0.02	7.32 ± 0.02
127 days	7.32 ± 0.01	7.31 ± 0.02	7.32 ± 0.01
pCO_2			
120 days	54.8 ± 1.3	57.4 ± 3.4	55.9 ± 2.2
127 days	54.2 ± 1.4	56.1 ± 2.4	62.5 ± 2.4 * #
Hb (g/dl)			
120 days	10.9 ± 0.2	12.4 ± 0.7	11.4 ± 0.6
127 days	10.9 ± 0.4	12.3 ± 1.1	9.4 ± 1.0 #

Values are mean ± SEM (number of animals). ** $P < 0.0005$, * $P < 0.05$ compared to normal. One way analysis of variance (ANOVA) and Bonferroni Dunn. # $P < 0.05$, significantly different from 'small'. † $P < 0.05$, significantly different from 120 days gestation. Measurements were made at 120 ± 1 and 127 ± 1 (mean ± SEM) days gestation.

TABLE 2.3 Concentrations of IGF proteins in arterial blood from fetal sheep with normal and restricted placental development, divided according to size.

	NORMAL (6)	SMALL (5)	VERY SMALL (5)
IGF-I (ng/ml)			
120 days	140 ± 9	95 ± 6 **	35 ± 5 ** #
127 days	137 ± 12	73 ± 22 *	16 ± 2 ** #
IGF-II (ng/ml)			
120 days	709 ± 188	1023 ± 240	671 ± 142
127 days	1181 ± 265 †	910 ± 128	385 ± 74 *
IGF-I/IGF-II			
120 days	0.27 ± 0.08	0.11 ± 0.02	0.06 ± 0.02 *
127 days	0.14 ± 0.03 †	0.08 ± 0.02	0.05 ± 0.02 *

Values are mean ± SEM (number of animals). ** $P < 0.0005$, * $P < 0.05$ compared to normal. One way analysis of variance (ANOVA) and Bonferroni Dunn. # $P < 0.05$, significantly different from 'small'. † $P < 0.05$, significantly different from 120 days gestation. Measurements were made at 120 ± 1 and 127 ± 1 (mean ± SEM) days gestation.

2.4 DISCUSSION

In the present study, intra-uterine growth retardation in sheep was accompanied by low blood pO_2 and glucose, by decreased concentrations of IGF-I in fetal blood, and in late gestation, by reduced blood levels of IGF-II. Placental size correlated positively with fetal weight and with the concentration of substrates in fetal blood, as reported previously (Harding *et al.*, 1985; Owens *et al.*, 1986). Restriction of placental size retards fetal growth by limiting the supply of oxygen and nutrients to the fetus and is associated with chronic hypoxaemia and hypoglycaemia (Robinson *et al.*, 1979; Harding *et al.*, 1985; Owens *et al.*, 1987a,b,c). A proportion of the growth-retarded fetal sheep, in this study, were carried by normal unoperated ewes. However, the associations observed between placental size and fetal weight, and between placental size and fetal blood pO_2 and glucose suggests that this growth retardation may also be, at least in part, a consequence of a reduction in the placental supply of substrates to the fetus.

Direct evidence that IGF-I is essential for normal growth and development *in utero* in mice is provided by the observation that null mutation of the IGF-I gene retards fetal growth and increases the rate of perinatal mortality (Liu *et al.*, 1993; Powell-Braxton *et al.*, 1993). Positive associations observed between plasma IGF-I and the weight of fetal sheep (Jones *et al.*, 1988), rats (Unterman *et al.*, 1993) and guinea pigs (Jones *et al.*, 1988) also suggest that IGF-I is an important influence on intra-uterine growth in several species. In the human fetus, concentrations of IGF-I measured in cord serum at birth correlate positively with both placental weight and birthweight (Gluckman *et al.*, 1983, Bennett *et al.*, 1983; Fant *et al.*, 1993), suggesting that IGF-I may also be involved in the aetiology of intra-uterine growth retardation, resulting from placental dysfunction, in the human fetus. In support of this view, regression analysis indicated that the concentration of IGF-I in fetal blood at 120 and 127 days gestation could potentially account for 87% (r^2=0.87) and 71% (r^2=0.71) respectively of the variation in fetal weight at 130 days gestation, in the present study of sheep. This evidence is consistent with an endocrine role for IGF-I in fetal sheep.

The positive relationships observed between plasma IGF-I and fetal blood glucose and pO_2 suggest that the low circulating level of IGF-I in growth-retarded fetal sheep may be a consequence of the limitation of the supply of oxygen and nutrients to the fetus. In support of this view, restriction of the supply of substrates to the fetal rat by maternal starvation (Davenport *et al.*, 1990; Bernstein *et al.*, 1991; Straus *et al.*, 1991b) or by uterine artery ligation (Vileisis & D'Ercole, 1986; Unterman *et al.*, 1993) reduces fetal blood levels of IGF-I. In fetal sheep, acute restriction of oxygen or nutrients lowers plasma IGF-I, indicating that oxygen and nutrient supply can independently regulate circulating concentrations of IGF-I, at least in the short term (Iwamoto *et al.*, 1992; Oliver *et al.*, 1993). Maternal starvation in sheep reduces the concentration of IGF-I in fetal plasma and intrafetal infusion of glucose, but not mixed amino acids, restores fetal plasma IGF-I to control levels in these animals (Oliver *et al.*, 1993). Thus, glucose appears to be the major nutrient regulating fetal plasma IGF-I. Concentrations of amino acids in the blood of growth-retarded fetal sheep are generally normal or higher than normal (Robinson *et al.*, 1979; Owens *et al.*, 1989a). Therefore, glucose may also be the major nutrient limiting IGF-I production in the current study. Since restriction of placental size retards fetal growth by limiting placental supply of oxygen and nutrients to the fetus (Harding *et al.*, 1985; Owens *et al.*, 1987a,b,c; 1.4), the results of the present study suggest that the effect of limited substrate supply on fetal growth may be mediated, in part, through nutritional regulation of the concentration of IGF-I in fetal blood.

A role for IGF-II as a determinant of prenatal growth is also indicated by gene knockout studies in mice (DeChiara *et al.*, 1990; Baker *et al.*, 1993). The positive association observed between the concentration of IGF-II in fetal blood at 127 days gestation and fetal size suggests that IGF-II may also be involved in the regulation of fetal growth in sheep. However, concentrations of IGF-II in fetal blood at 120 days gestation were not altered in growth-retarded fetal sheep, suggesting that any endocrine role of IGF-II in the regulation of fetal growth occurs later in gestation than is the case for IGF-I. At 127 days gestation, but not at 120 days gestation, 'very small' fetal sheep were hypoxaemic and hypoglycaemic and had reduced blood levels of IGF-II. These results are consistent with regulation of

circulating IGF-II in the sheep fetus by substrate supply, but at more advanced or severe stages of restriction than occurs with IGF-I. No distinction could be made between the effect of oxygen or glucose on plasma IGF-II using partial correlation analysis. However, in contrast to fetal blood glucose, a significant decrease in fetal blood pO_2 occurs between 120 and 127 days gestation in severely growth-retarded fetal sheep, concomitant with a fall in blood levels of IGF-II. Therefore, the decrease in plasma IGF-II concentrations in growth-retarded fetal sheep may be related to increasing fetal hypoxaemia.

Expression of the IGF-II gene in fetal tissues and the concentrations of IGF-II protein in fetal blood decrease as gestation proceeds in sheep (Delhanty & Han, 1993; O'Mahoney *et al.*, 1991b; Carr *et al.*, 1994) and recent studies suggest a role for cortisol in the regulation of IGF-II production in the sheep fetus. Increasing the concentration of cortisol in the blood of late gestation fetal sheep, by direct cortisol infusion, reduces hepatic IGF-II mRNA (Li *et al.*, 1993), suggesting that the normal ontogenic decrease in IGF-II gene expression in late gestation may be regulated by the prepartum cortisol surge that is characteristic of fetal ontogeny in this species (Bassett & Thorburn, 1969; Silver, 1990). Blood cortisol concentrations increase prematurely in growth-retarded fetal sheep in late gestation (Robinson *et al.*, 1980). Chronic fetal hypoxaemia alone accelerates the ontogenic increase in circulating cortisol concentrations in fetal sheep (Jacobs, 1987). Therefore, the premature decrease in blood levels of IGF-II in severely growth-retarded fetal sheep may be due, at least in part, to an earlier and greater increase in fetal blood cortisol associated with the increasing severity of the hypoxaemia, and possibly hypoglycaemia, which occurs with advancing gestation.

The results of the current study contrast with a previous report in which increased concentrations of IGF-II were detected in the blood of growth-retarded fetal sheep between 127 and 141 days of gestation (Jones *et al.*, 1988). Growth-retarded fetal sheep in the study of Jones *et al* (1988) weighed approximately 68% of normal size controls. In the present study, 'small' fetuses weighed 76% and 'very small' fetuses weighed only 51% of controls. If 'very small' fetal sheep are excluded from the correlation analyses at 120 days gestation in the present study, a tendency towards a negative correlation is observed between fetal size

and plasma IGF-II (r = -0.48, P = 0.16), particularly when the influence of plasma IGF-I is controlled for using partial correlation analysis (r = -0.81, P = 0.008). Therefore, the difference between these studies may relate to the degree of growth retardation achieved. In man, a positive association has been reported between plasma IGF-II in cord blood serum and infant birthweight (Bennett *et al.*, 1983) or fetal ponderal index (Fant *et al.*, 1993). However, others report no association between cord blood levels of IGF-II and birthweight in a large population of fetuses in which the majority of fetuses fell within the normal weight range (Gluckman *et al.*, 1983). Similarly IGF-II is not reduced in the blood of small for gestational age infants between 33 weeks and term (Lassarre *et al.*, 1991). Differences in the range of fetal weights examined and the gestational ages at which samples were collected may explain the conflicting results in these studies.

Restriction of nutrient supply also has variable effects on plasma IGF-II in the fetus of other mammalian species. Maternal starvation in guinea pigs or rats, which will produce fetal hypoglycaemia, results in unchanged or decreased concentrations of IGF-II in fetal plasma (Davenport *et al.*, 1990; Jones *et al.*, 1990; Straus *et al.*, 1991b). In contrast, uterine artery ligation, which will produce both hypoxaemia and hypoglycaemia, is associated with unchanged or with increased blood levels of IGF-II in these species (Jones *et al.*, 1988, 1990; Unterman *et al.*, 1993). Increased blood levels of IGF-II are also reported in hypoxaemic fetal rats (Tapanainen *et al.*, 1994). Thus, in the rodent fetus, limitation of nutrient supply alone and hence glucose, tends to reduce blood levels of IGF-II, while restriction of oxygen or concomitant restriction of oxygen and nutrients increases plasma IGF-II in late gestation. This suggests that pertubations which reduce glucose or nutrient supply will reduce plasma IGF-II, while limiting oxygen supply will increase plasma IGF-II in fetal rats and guinea pigs. Uterine artery ligation reduces blood cortisol concentrations while maternal starvation tends to increase blood cortisol in the fetal guinea pig (Jones *et al.*, 1990). This diffential response of cortisol to restriction of various substrates in the fetal guinea pig is the opposite of that of fetal sheep. Nevertheless, as in fetal sheep (Li *et al.*, 1993), increased fetal plasma cortisol is associated with a reduction in plasma IGF-II in the guinea pig (Jones *et al.*, 1990). Therefore, the nature of the substrates which are limited and

the precise hormonal response to that limitation may be an important determinant of the response of plasma IGF-II to restriction in the fetus.

2.5 SUMMARY

- Intra-uterine growth retardation in sheep was associated with reduced fetal blood glucose and pO_2 and reduced circulating concentrations of IGF-I, and later in gestation, lower IGF-II.

- IGF-I concentrations in fetal blood, at 120 and 127 days gestation, and IGF-II protein concentrations in fetal blood at 127 days gestation, correlated positively with placental weight and fetal weight, measured at 130 days gestation.

- IGF-I protein concentrations in blood correlated positively with fetal blood pO_2 and blood glucose. Positive associations were observed between the concentration of IGF-II in fetal plasma and fetal blood pO_2 and blood glucose at 127 days, but not 120 days, gestation.

- The results of this study suggest that circulating IGF-I and IGF-II may mediate, in part, the influence of placental substrate supply on fetal growth, but that their roles vary with developmental stage.

CHAPTER 3

IGF-I AND -II GENE EXPRESSION IN FETAL TISSUES: RELATIONSHIP TO CIRCULATING IGFS AND FETAL GROWTH IN SHEEP.

3.1 INTRODUCTION

Retarded fetal growth in sheep was accompanied by chronic fetal hypoxaemia and hypoglycaemia and reduced circulating concentrations of IGF-I and IGF-II in late gestation in the preceding study (Chapter 2). The positive relationship between circulating IGFs and fetal blood pO_2 and glucose suggested that reduced supply of substrates to the fetus decreased production of the IGFs in the fetus. In all species studied, including the sheep, many fetal tissues and the placenta contain IGF-I and -II mRNAs (Han *et al.*, 1988, Lund *et al.*, 1986, O'Mahoney *et al.*, 1991, Dickson *et al.*, 1991, 1.3.2). In adult sheep and rats, the liver contains the highest concentration of IGF-I mRNA (Murphy *et al.*, 1987a; Dickson *et al.*, 1991; Pell *et al.*, 1993) and appears to be the major source of IGF-I protein in blood (Emler & Schalch, 1987, Pell *et al.*, 1993). Liver is also a principal site of IGF-II synthesis after birth in sheep (O'Mahoney *et al.*, 1991; Delhanty & Han, 1993). In contrast to postnatal life, a number of fetal tissues contain IGF-I and -II mRNAs at levels equivalent to or greater than that of fetal liver (Han *et al.*, 1988, Lund *et al.*, 1986, Dickson *et al.*, 1991; O'Mahoney *et al.*, 1991; Delhanty & Han, 1993). The abundance of IGF-II mRNA in most fetal tissues and the abundance of IGF-I mRNA in a number of extrahepatic tissues is high compared to that observed in postnatal life (Dickson *et al.*, 1991; O'Mahoney *et al.*, 1991; Delhanty & Han, 1993). Therefore, changes in the concentration of IGF proteins in fetal blood may be the result of altered production at one or more tissue sites.

The abundance of IGF-I protein and mRNA in liver and the concentration of IGF-I protein in blood are reduced in fetal rats made growth retarded by restriction of maternal nutrient supply (Vileisis & D'Ercole, 1986; Straus *et al.,* 1991b; Bernstein *et al.,* 1991), suggesting that liver is a major source of circulating IGF-I in the fetal rat. In contrast, the amount of IGF-I protein and mRNA in fetal lung is not altered when growth of the rat fetus, and thus fetal lung growth, is restricted (Vileisis & D'Ercole, 1986; Davenport *et al.*, 1990). This implies that any influence of IGF-I on growth of the fetal lung, when maternal substrate supply is limited in the rat, is related to decreased hepatic production of IGF-I and reduced circulating concentrations of IGF-I, rather than changes in locally expressed IGF-I. Expression of IGF-I

in tissues other than liver and lung, that also produce IGF-I, has not been studied in growth-retarded fetal rats.

In fetal sheep, restriction of uterine blood flow for 24 hours reduces the supply of oxygen to the fetus and reduces IGF-I mRNA abundance in fetal liver, but does not alter IGF-I protein levels in blood or IGF-I mRNA in lung, muscle, thymus or kidney (McLellan *et al.*, 1992). This suggests that liver may not be the principal source of circulating IGF-I in the sheep fetus. However, in contrast to short term oxygen restriction, chronic restriction of nutrient and oxygen supply to the fetus, as reported in the preceding study (Chapter 2), is associated with reduced plasma IGF-I concentrations. Expression of IGF-I in fetal sheep tissues has not been previously examined when supply of oxygen and nutrients to the fetus is chronically limited.

In contrast to IGF-I, IGF-II protein levels in the blood of growth-retarded fetal sheep were not altered at 120 days gestation (Chapter 2). In the rat, IGF-II mRNA abundance in fetal liver is not changed by 48 to 72 hours maternal starvation (Davenport *et al.*, 1990; Straus *et al.*, 1991b). However, hepatic IGF-II mRNA in fetal rats is reported to increase following uterine artery ligation (Price *et al.*, 1992a). In fetal sheep, IGF-II mRNA is not altered by 24 hours of hypoxia (McLellan *et al.*, 1992). However, hepatic IGF-II mRNA, measured at 135 days gestation in the sheep fetus, is increased by chronic maternal undernutrition from early pregnancy and by insulin-induced maternal hypoglycaemia from mid-pregnancy (Townsend *et al.*, 1992a,b) while fetal kidney IGF-II mRNA is reduced at 135 days gestation following infusion of insulin into the pregnant ewe from mid-pregnancy (Townsend *et al.*, 1992a). Chronic maternal undernutrition and insulin-induced maternal hypoglycaemia reduce fetal weight and lower fetal plasma glucose and insulin (Townsend *et al.*, 1992a,b). This suggests that nutrient supply regulates IGF-II expression in the sheep fetus and in a tissue specific manner. It is possible therefore, that local expression of IGF-II may be altered in tissues of fetal sheep with restricted placental size. The effect of chronic restriction of nutrient supply on IGF-II gene expression in extrahepatic tissues in the sheep fetus, apart from the kidney (Townsend *et al.*, 1992a), has not been determined previously. In addition, the effect of

chronically restricting both oxygen and nutrient supply on IGF-II gene expression in fetal tissues is not known.

Therefore the ***specific aims*** of the studies in this chapter were

(1) to determine whether altered circulating concentrations of IGF-I protein detected at 120 days gestation in the blood of fetal sheep following variable restriction of placental size (Chapter 2) are accompanied by changes in synthesis of IGF-I at the level of gene expression in liver and/or other major fetal tissues.

(2) to determine whether IGF-II gene expression was altered in liver and/or other major fetal tissues, at 120 days gestation following variable restriction of placental size.

(3) to determine the potential source(s) of IGF proteins in fetal blood.

(4) to identify potential regulators of IGF gene expression in the late gestation sheep fetus.

Therefore, blood and tissues were collected from a further group of normal and growth-retarded fetal sheep at 121 days gestation, and IGF proteins in fetal blood and IGF mRNAs in fetal tissues were measured.

3.2 MATERIALS AND METHODS

3.2.1 SURGICAL PROCEDURES

Removal of endometrial caruncles

Endometrial caruncles were removed from the uterus of non pregnant Border-Leicester / Merino crossbred ewes as described in Section 2.2.1. In the experiments described in this chapter 12 ewes which had undergone this surgical procedure were studied. These animals came from a group of 38 pregnant ewes from which endometrial caruncles had been removed. The twelve 'caruncle' ewes studied in this chapter were chosen at random and had an average of 61 ± 3 (10) (mean ± SEM (SD)) caruncles removed and 16 ± 0.6 (2) (mean ±

SEM (SD)) visible caruncles remaining in the non-pregnant uterus following surgery. All animals were mated as described in Section 2.2.1.

Insertion of vascular catheters

Vascular catheters were implanted into the twelve 'caruncle' ewes and their fetuses and a further eight normal unoperated ewes and their fetuses at 109 ± 1 days gestation, as described (2.2.1) except that polyvinyl tubing with a double lumen (DV8, 0.64 mm ID, 1.5 mm OD, Dural Plastics) was inserted in the fetal tarsal vein. In addition, 28 cm of silastic tubing (0.3 inches I.D., 0.65 inches O.D., Dow Corning Medical Products) was sleeved onto the maternal utero-ovarian vein catheter and inserted into a uterine vein as described.

Catheter patency was maintained by flushing with heparinised saline (50 U/ml) on alternate days, commencing the day after surgery. Patency of the umbilical vein, utero-ovarian vein and double lumen tarsal vein catheters was maintained by continuous infusion of heparinised saline as described (2.2.2), except that the concentration of the heparin infused into the fetal vessels was reduced to 50 U/ ml of saline.

3.2.2 COLLECTION OF BLOOD AND TISSUE.

At 121 ± 2 days gestation blood samples (1.5 ml from the fetal femoral artery and umbilical vein) were collected at hourly intervals commencing at 0730 h. The volume of the final sample collected at 1530 h from the fetal femoral artery was increased to 5 ml. Blood samples were collected into heparinised syringes and processed as previously described (2.2.2). Metabolites, hormones and IGF proteins were measured in blood plasma collected from the fetal femoral artery at 1530 h, unless otherwise specified.

Immediately following collection of the final blood samples at 121 ± 2 days gestation, the ewe and fetus were sacrificed by intravenous overdose of sodium pentobarbitone (Lethobarb, Arnolds of Reading). The uterus and contents were rapidly removed and weighed. The fetus was dried with a towel to remove excess moisture, before its weight was recorded. Fetal tissues were rapidly dissected, blotted and weighed. Individual placentomes were dissected,

and weighed. Placental weight was defined as the sum of weights of the individual placentomes. Tissues pieces of 1 to 2 g from the fetal liver, right lung, right kidney, quadriceps muscle, cerebrum, cerebellum and heart were immediately snap frozen in liquid nitrogen. Placental cotyledons of various sizes were snap frozen whole in liquid nitrogen. During the dissection and weighing of fetal and placental tissues, care was taken to avoid ribonuclease contamination. Working surfaces and instruments were cleaned regularly with alcohol. To minimise RNA degradation, five people were routinely involved in the collection of fetal tissues, so that samples were removed and frozen as rapidly as possible. Tissue samples were stored at -70°C until extraction of total RNA.

3.2.3 BLOOD GASES AND METABOLITES

Blood gases were measured as described in Section 2.2.3. The blood gas results reported in this chapter are the mean results of analysis of the eight blood samples collected throughout the eight hours of sampling on day 121 ± 2 of gestation.

Glucose concentrations were measured in arterial blood plasma by enzymatic analysis using hexokinase and glucose 6 phosphate dehydrogenase and automated analysis (Cobas Mira, Glucose HK Uni kit, Roche Diagnostics Systems). All samples were analysed in duplicate within one assay.

3.2.4 PLASMA IGF-I AND -II RADIOIMMUNOASSAYS.

Fetal arterial plasma samples were subjected to acid gel chromatography as described in Section 2.2.4, except that the pH of the acid chromatography mobile phase was reduced to 2.5. The lower pH was used because dissociation of IGF-II from the binding proteins in fetal sheep plasma is only complete when pH is 2.8 or less (K.J. Quinn & P.C Owens, unpublished observations). Therefore, pH 2.5 was used to improve reproducibility. The recovery of [^{125}I]-IGF-I following chromatography was 96 ± 4% (n=6).

IGF-I protein concentrations were measured by the same procedures as described in Section 2.2.4, except that the original antiserum (PM87) to IGF-I was no longer available and an

alternative primary antiserum (MAC 89/1), also raised in rabbits against recombinant human IGF-I, was used. Recombinant human IGF-I (GroPep Pty Ltd) was used as a standard in the assay and was iodinated using [^{125}I]-NaI (Amersham Australia Pty Ltd.) to a specific activity of 86 Ci/g for use as a radioligand. Subsamples (100 µl) of the IGF containing column eluate were neutralised by addition of 60 µl of 0.4 M Tris base. With rabbit anti hIGF-I (MAC 89/1 at 1/60 000 final dilution) and hIGF-I as standard and radioligand, the cross reaction of IGF-II (human) is <1%. Ovine IGF-II, which was not available for direct assay calibration, differs from human IGF-II in only 4 of the 67 amino acids (Francis *et al.,* 1989b). Immune complexes were precipitated as previously described, except that a sheep antiserum against rabbit γ-globulin (Silenus) and rabbit immunoglobulin (Dako) were added. All samples in this chapter were analysed in triplicate within a single assay. The within assay variation was 5%. The minimum detectable concentration was 9 ng/ml plasma.

Concentrations of IGF-II in fetal blood were measured in these samples by radioimmunoassay, in contrast to the radioreceptor assay described previously (2.2.4). A mouse monoclonal antibody (S1-F2) to rat IGF-II was generously provided by Dr. K. Nishikawa, Kanaza Medical University, Ishikawa, Japan. Recombinant human IGF-II (Gropep Pty Ltd.) was used as standard and radioligand following iodination to a specific activity of 76 Ci/g. This preparation of recombinant human IGF-II was equipotent with the preparation used as a standard in the IGF-II assays performed in Chapter 2.

The IGF-II RIA was set up as for the IGF-I RIA (Section 2.2.4). Aliquots of 50 µl of the acid column fractions were neutralised by addition of 30 µl of 0.4 M Tris base. This was combined with 200 µl RIA buffer (2.2.4), 50 µl of IGF-II antibody (1 ng) in assay buffer and 50 µl [^{125}I]-IGF-II in assay buffer. Tubes were processed as for the IGF-I RIA (Section 3.2.4) except that a sheep antiserum against mouse γ-globulin (Silenus) and mouse serum were added to precipitate the complexes.

With mouse anti-rIGF-II IgG S1-F2 at 1 ng/tube and hIGF-II as standard and radioligand, the crossreaction of IGF-I (human) is 2.5%. Ovine IGF-I, which was not available for assay calibration, differs from human IGF-I in only one of the 70 amino acids (Francis *et al.,*

1989b). All samples in this chapter were analysed in triplicate within a single assay. The within assay variation was 6%. The minimum detectable concentration of IGF-II was 35 ng/ml plasma.

Because IGF-II was measured by receptor assay in the previous study (Chapter 2) and by immunoassay in Chapters 3 and 4, a comparison of the two methods was made. The concentration of IGF-II protein was measured by RRA following acid gel chromatography at pH 2.8 and by RIA following acid-gel chromatography at pH 2.5 in arterial plasma from the twelve fetal sheep in which IGF mRNAs were measured in all six fetal tissues. A positive association (r= 0.96, y= 1.24x + 126, P<0.0001, n=12) was observed between the results obtained by the two assay methods. Values obtained from the RIA were higher than those obtained using the RRA assay. This may be due to a slight increase in the amount of IGF-II dissociated from the IGF binding proteins with the use of pH 2.5 for size exclusion chromatography. Alternatively, the reactivity of hIGF-II and oIGF-II towards the type 2 IGF receptor and the antibody (S1-F2) to rat IGF-II may be different. In addition, it is also possible that the RIA detects species of IGF-II in fetal blood, which do not react with the type 2 IGF receptor used in the radioreceptor assay.

3.2.5 MEASUREMENT OF IGF-I AND -II mRNAS IN FETAL TISSUES

Extraction of total RNA from fetal tissues and placental cotyledons

RNA extraction

Total RNA was extracted from fetal sheep tissues and placental cotyledons using the method of Chomczynski & Sacchi (1987). RNA was extracted from fetal liver samples from all twenty fetal sheep in the study, and from quadriceps muscle, heart, lung, kidney, cerebellum and a placental cotyledon from a subset of twelve fetuses, consisting of four fetal sheep with normal placental growth and eight with restricted placental growth.

All chemicals used for RNA analysis, unless otherwise specified, were purchased from Sigma Chemical Company. All solvents (chloroform, isoamyl alcohol, propan-2-ol) were purchased from Ajax Chemicals. Guanidine thiocyanate was obtained from Fluka Chemika-

Biochemika. Phenol (Wako Pure Chemical Industries Ltd) was equilibrated with Tris-Cl pH 8.0, as described by Sambrook *et al.*, 1989.

Fetal tissues were homogenised using an Ultra-Turrax T25 homogeniser (Janke & Kunkel) with an S25 8G dispersing tool. Before use the tool was cleaned thoroughly by washing with sterile water and homogenising 1 M sodium hydroxide for 30 seconds at $^1/_2$ speed, and 15-20 ml fresh sterile water four times for 1 min. Immediately before use the tool was rinsed in 5 ml of the denaturing solution in which tissues were to be homogenised.

Frozen tissue (approximately 1 g) was removed from -70°C storage into preweighed 50 ml centrifuge tubes (Oak Ridge tubes, Nalgene Company). Denaturing solution (4M guanidinium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol, Chomczynski & Sacchi, 1987) was added to a volume of 10 ml per gram of tissue. The tissue was homogenised at $^1/_2$ to $^3/_4$ speed for a total of 5 to 7 min, in 1 min bursts with the tube placed on ice for 20 seconds between bursts. The same tissue from six animals was extracted at one time. Homogenised tissues were maintained on ice, while subsequent tissues were prepared.

One ml of 2 M sodium acetate, pH 4.0; 10 ml buffer saturated phenol and 2 ml chloroform/ isoamyl alcohol (49:1) per gram of tissue were added sequentially to the homogenised tissue with mixing by inversion between each addition. The final solution was shaken for 15 seconds and incubated on ice for 20 min. Samples were centrifuged at 4°C, 10 000 x *g* for 20 min (Damon, IEC centrifuges). The upper aqueous phase was removed to a fresh tube and an equal volume of isopropanol was added with thorough mixing. Samples were incubated overnight at -20°C to precipitate the RNA. The RNA was pelleted by centrifugation as before. The supernatant was decanted and excess solution was removed by aspiration.

The pellet was redissolved in 3 ml of denaturing solution per gram of original tissue. Tissues with high glycogen content, including liver and muscle, often required gentle heating (60°C water bath) and vigorous vortexing. An equal volume of isopropanol was added and solutions were mixed and stored at -20°C for at least 1 h to reprecipitate the RNA. The RNA was pelleted as before and the supernatant discarded. To remove salt residues, 5 ml of 70%

ethanol was added with brief vortexing. The pellet was resedimented and the ethanol removed by careful aspiration. The RNA pellet was allowed to dry at room temperature and was redissolved in 1 ml of sterile water. For tissues with a higher RNA yield, this volume was increased to 2 ml.

To further remove glycogen and smaller transfer RNAs and ribosomal RNAs, a modified method of Cathala *et al.* (1983) was applied to RNA extracts from all tissues. The RNA solution was divided into 200 µl aliquots and 1 ml (5 volumes) of 4 M lithium chloride (LiCl) was added. The solutions were mixed and RNA was precipitated overnight at 4°C. RNA was pelleted by centrifugation for 30 min, 10 000 x *g*, 4°C (Eppendorf, 5412 centrifuge). The supernatant was discarded and 500 µl 3 M LiCl was added. The tube was vortexed, the RNA pelleted as previously and the supernatant discarded. Salt residues were removed by addition of 500 µl 70% ethanol, brief vortexing and centrifuging. The supernatant was removed by aspiration and the pellet was airdried. RNA was redissolved in 250 µl sterile water and stored at -70°C following addition of $^1/_{25}$ th volume of 5M NaCl and 2.5 volumes of ethanol. Small aliquots (~20 µl) of the RNA solution were removed before ethanol addition for determination of RNA concentration and integrity.

RNA was extracted from placental cotyledons by the same methods. The median cotyledon weight for placentae of the four control ewes was 7.7 g. The weights of the cotyledon chosen for extraction ranged from 3.84 g to 10.72 g with a mean weight of 6.97 g. Before extraction whole frozen cotyledons were placed on dry ice and sliced with a sterile scalpel blade along the radius to the centre so that a section of approximately 1.0 g to 1.5 g, containing all regions of the cotyledon, in similar proportions to the whole cotyledon, was removed for extraction of RNA.

Integrity and concentration of RNA

The absorbance at 260 and 280 nm of a 1:250 dilution of the RNA solution (4 µl RNA in 1 ml sterile water) was determined (Shimadzu, UV-120-02 spectrophotometer). From the absorbance at 260 nm the concentration of RNA in the solution was calculated (40 µg/ml RNA has an A_{260} of 1.0, (Davis *et al.*, 1986). The ratio of A_{260} / A_{280} was determined.

Ratios between 1.8 and 2.0 indicated that RNA solutions were free of phenol and protein contamination.

The integrity of the RNA was assessed following electrophoresis through a 1% agarose / 1 x Tris-borate EDTA (TBE, 0.089 M Tris borate, 0.089 M boric acid, 0.002 M EDTA) mini gel (5cm x 7.5 cm) at 50 mA for 35-45 min in sterile 1 x TBE buffer. The gel was stained in 0.5 μg/ml ethidium bromide for 30 min and visualised under ultraviolet light (UltraLum) The presence of distinct 28S and 18S ribosomal RNA bands and a lack of degraded RNA was confirmed for each sample.

Prior to analysis, subsamples of RNA of known amount were prepared. RNA, stored at -70°C, was precipitated by centrifugation for 30 min at 4°C. The pellet was airdried and redissolved in sterile water. The concentration of RNA was calculated from the spectrophotometric analysis (A_{260}) of 1:100 solutions of the RNA (10 μl in 1 ml sterile water) in triplicate. Aliquots of RNA from a single tissue from all animals were prepared at the same time to avoid variation in the spectrophotometric analysis, and stored at -70°C in screw cap tubes with the addition of 2.5 volumes of ethanol and $^1/_{25}$th volume of 5M sodium chloride until analysis (see page 3-15).

Assay control RNA

A pool of total RNA extracted from livers of normal fetal sheep was prepared as an assay control and stored at -70°C in 50 or 25 μg aliquots, in ethanol and sodium chloride as described above.

Ovine IGF-I and -II DNA

Ovine IGF-I and -II DNA cloned into the Bluescript vector (Stratagene Ltd.) for the generation of antisense IGF-I and -II RNAs were provided by Dr. R. Stewart Gilmour, Department of Cellular Physiology, Babraham, Cambridge, UK. The isolation of IGF-I and -II clones from a sheep genomic DNA library and the preparation of these Bluescript vectors has been described (Dickson *et al.*, 1991; Saunders *et al.*, 1991; Li *et al.*, 1993).

DNA containing 189 bases of sheep genomic intron 3 sequence and 83 bases of exon 4 of the sheep IGF-I gene, cloned into the *Eco*R1-*Hind*III sites in the polylinker region of the Bluescript vector, was used to generate a 404 base pair [^{32}P] labelled antisense IGF-I RNA, following linearisation of the vector with *Hind*III (Dickson *et al.*, 1991, Saunders *et al.*, 1991, Pell *et al.*, 1993). The antisense RNA consists of 272 bases complementary to 189 bases of genomic ovine intron 3, 83 bases of exon 4 and 132 bases of vector DNA sequence. This exon was originally designated exon 3 of the sheep IGF-I gene (Dickson *et al.*, 1991), but represents exon 4 according to the revised nomenclature for the IGF-I gene (Holthuizen *et al.*, 1991).

Exon 8 of the sheep IGF-II gene was isolated from an ovine genomic library and amplified by polymerase chain reaction using primers complementary to sequences of the flanking introns (Li *et al.*, 1993). The amplified 267 base fragment was cloned into the *Eco*RI - *Hind*III sites in the polylinker region of the Bluescript vector. Linearisation of the Bluescript vector with *Hind*III produced a template for transcription of a 325 base [^{32}P] labelled antisense IGF-II RNA, consisting of 165 bases complementary to exon 8, 94 bases complementary to introns 7 and 8, and 66 bases complementary to vector DNA sequence (Li *et al.* 1993). Exon 8, the first coding exon of the ovine IGF-II gene, was originally designated exon 4 by Li *et al.* (1993). However, following characterisation of the complete ovine IGF-II gene this exon was designated exon 8 by Ohlsen *et al.* (1994). The full length of ovine exon 8 is 165 nucleotides (Dr. Juan Li, Department of Cellular Physiology, Babraham, Cambridge, UK, personal communication).

Production of antisense RNA probes

Linearisation of Bluescript vectors

The Bluescript vector was linearised by addition of approximately 5-10 µg of vector DNA to 10 µl restriction enzyme buffer (incubation buffer B, 10 mM Tris-Cl, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM mercaptoethanol, pH 8.0, Boehringer Mannheim), 2µl *Hind*III (12 U/µl Boehringer Mannheim) and sterile water to a volume of 100 µl, followed by incubation at

37°C for a minimum of 2 h. The reaction was stopped by addition of 100 µl sterile water and extraction with an equal volume of phenol/chloroform/isoamyl alcohol (50:48:2) (Sambrook *et al.*, 1989). Approximately 170 µl of the upper phase was removed to a fresh tube and the DNA was precipitated on ice for 2 h by addition of $^1/_{10}$ th volume of 3M sodium acetate, pH 5.2 and 2 volumes of ethanol. The DNA was pelleted by centrifugation for 30 min at 10 000 *x g*, 4°C. The pellet was airdried, redissolved in 30 µl TE buffer (10 mM Tris-Cl pH 7.4, 1 mM EDTA pH 8.0) and stored at -70°C.

In vitro transcription of antisense RNA

In vitro transcription of the linearised DNA template, to produce an antisense IGF-I or -II RNA was performed using a Message Maker *in vitro* transcription Kit (Bresatec Ltd.). Transcription was initiated at the T_7 RNA polymerase site and incorporated [α–^{32}P] uridine 5'-triphosphate ([^{32}P]-UTP) (Bresatec Ltd.).

Briefly, 5 to 6 µls linearised template DNA was combined in a sterile screw cap tube with 2 µl Nucleotide Buffer Cocktail (400 mM Tris-HCl, pH 7.6, 60 mM $MgCl_2$, 5 mM ATP, 5 mM CTP, 5 mM GTP, 150 uM UTP, 1 mg/ml BSA), 2 µl 100 mM dithiothreitol and 1 µl human placental ribonuclease inhibitor. Approximately 50-60 µCi [^{32}P]-UTP of specific activity 3000 Ci / mmol, 10 µCi/µl was added. The volume of the reaction was increased to 18 µl by addition of sterile water and 2 µl T_7 RNA polymerase (2 U/µl) was added. The tube and contents were incubated at 37°C for 1 h. The DNA template was removed by addition of 1 µl of ribonuclease free DNase (10 U/µl) and incubation at 37°C for a further 10 min. All solutions were provided in the Message Maker Kit.

The reaction was stopped by addition of 5 µl 200 mM EDTA. Sterile water (270 µl) and yeast tRNA (5 mg/ml, 4 µl) were added. Two 1 µl aliquots of the solution were removed and spotted onto GF/C glass microfibre filters (Whatman International Ltd.) for assessment of the percentage of [^{32}P]-UTP incorporated and calculation of the specific activity of the riboprobe. The antisense RNA solution was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (50:48:2) (Sambrook *et al.*, 1989) and 270 µl of the

aqueous phase was recovered. Antisense RNA transcripts were precipitated by addition of 15 µl 4M NaCl and 750 µl ethanol and incubation at -70°C overnight. The antisense RNA was pelleted by centrifugation at 10 000 *x g* for 30 min at 4°C. The pellet was air dried and redissolved in 100 µl sterile water. Ethanol (250 µl) and 5 M NaCl (4 µl) were added and the RNA was precipitated at -70°C for 1 h and pelleted as before. The RNA pellet was redissolved in 100 µl sterile water and the ethanol precipitation process repeated.

Following the third ethanol precipitation the antisense RNA was dissolved in 10-25 µl of sterile water, depending on the calculated specific activity (Section 3.2.5). Antisense RNA probes were used immediately in a ribonuclease protection assay, or were stored at -70°C with the addition of 2.5 volumes of ethanol for up to ten days. For use in subsequent assays the riboprobe was precipitated by addition of $^1/_{25}$ th volume of 5M NaCl, incubation at -70°C for 1 h and centrifugation at 4°C, 10 000 *x g* for 30 min.

Calculation of specific activity of antisense RNA

The proportion of [^{32}P]-UTP incorporated into antisense RNA was determined following trichloroacetic acid precipitation (TCA) of the antisense RNA onto glass fibre filters as described in Sambrook *et al.* (1989). One of the glass fibre filters prepared above was washed with TCA. Both the unwashed and washed filters were counted, using Cerenkov counting in the tritium channel of the β counter (Tri-carb, 1900TR, Packard Instrument Co.). The efficiency of this method of counting, using this counter, was determined as 25%.

An approximate specific activity for the riboprobe was calculated by -

1. *Calculating the proportion of [^{32}P]-UTP incorporated into RNA*

$$\% \text{ incorporated} = \frac{\text{cpm in washed filter}}{\text{cpm in unwashed filter}} \times 100$$

2. *Calculating the total amount of UTP incorporated into RNA (nmoles incorporated),*

Total UTP incorporated (nmole) = total UTP in reaction (nmole) x % incorporated.

3. *Calculating the amount of RNA synthesized in nanograms*

The assumption was made that all ribonucleotides were incorporated into the antisense RNA at an equivalent rate to that of [^{32}P]-UTP. The average molecular weight of each the nucleotides is 330 ng/nmole, therefore,

RNA synthesised (ngs) = 1320 x nmoles UTP incorporated.

4. *Calculating the total cpm incorporated into the RNA*

Total cpm incorporated = cpm of washed filter x 300

(1 µl counted from a total volume of 300 µl).

5. *Calculating the specific activity of the antisense RNA*

$$\text{Specific activity} = \frac{\text{total cpm incorporated (cpm)}}{\text{amount of RNA synthesised (ng)}}$$

The specific activities of the antisense IGF RNAs ranged from 4 x 10^8 to 7 x 10^8 cpm/µg.

Solution hybridisation / ribonuclease protection assays

Samples to be analysed had been stored as an ethanol suspension (2.5 volumes of ethanol, $^1/_{25}$ th volume of 5M NaCl) at -70°C (see page 3-11). Immediately prior to ribonuclease protection assays, samples were precipitated by centrifugation for 30 min at 10 000 *x g*, 4°C. Pellets were airdried and redissolved in 30 µl of hybridisation buffer (80% (vol/vol) formamide, 40 mM PIPES (pH 6.4), 400 mM NaCl and 1 mM EDTA). Approximately 300-600 ρg (1-2 µl) of radiolabelled antisense IGF-I or -II RNA was added. For determination of IGF-I mRNA content 50 µg of total RNA from fetal tissues and 75 µg total RNA from placenta was analysed. For measurement of IGF-II mRNA 25 µg total RNA was analysed from all tissues. For negative controls in the assay, two aliquots of 20 µg yeast tRNA (Boehringer Mannheim) were precipitated, redissolved in hybridisation buffer and riboprobe was added as for the samples. Samples and negative controls were mixed, incubated at 70°C for 7 min and hybridised in solution at 45°C overnight.

Following hybridisation, single stranded RNA was digested by addition of 270 µl of a mixture of ribonuclease A (40 µg/ml, approximately 0.1 units/µg, Cat no. R-5125, Sigma Chemical Co.) and ribonuclease T_1 (2 µg/ml, approximately 500 units/µg, Cat no. R-8251,

Sigma Chemical Co.) in 300 mM NaCl, 10 mM Tris-Cl (pH 7.6) and 5mM EDTA for 1 h at 30°C. RNase digestion buffer (270 µl) containing no ribonuclease was added to one of the yeast tRNA tubes. Five µl of 10 mg/ml proteinase K (Boehringer Mannheim) and 10 µl 20% SDS (w/v) were added and the samples were incubated for 15 min at 37°C. Samples were extracted with phenol/ chloroform/ isoamyl alcohol (Sambrook *et al.,* 1989) and 270 µl of the upper aqueous phase was transferred to a fresh tube. Undigested double stranded RNA was precipitated by addition of 4 µl yeast tRNA (5 mg/ml) and 675 µl ethanol and incubation at -70°C for 1 h. The resulting pellet was air-dried and redissolved in 5 µl of gel loading buffer (80% formamide (v/v), 10 mM EDTA, 1 mg/ml xylene cyanol, 1 mg/ml bromophenol blue).

The protected fragments were separated on a 33 cm x 41 cm x 0.25 mm thick 7.2% acrylamide / 8 M urea / 1 x TBE sequencing gel (IGF-I mRNA assays) or a 0.25 mm 8% acrylamide / 8 M urea / 1 x TBE sequencing gel (IGF-II mRNA assays) at 60-65 watts for $1^1/_2$ to 2 h in 1 x TBE electrophoresis buffer. Molecular weight markers (*Msp*1 digested pBr322, Clontech), end labelled using T_4 polynucleotide kinase (DNA 5'-End labelling Kit, Boehringer Mannheim) and [γ-^{32}P]-ATP (Bresatec Ltd.) were electrophoresed along with samples. Following electrophoresis, the gel was washed with 400 ml 10% acetic acid (v/v) for 15 min, rinsed with 1 l of 20% ethanol (v/v) and dried onto filter paper under vacuum at 80°C.

Dried gels were exposed to Fuji RX X-ray film with intensifying screens (Cronex Hi-Plus, Du Pont) at -70°C. Exposure times for IGF-I assays ranged from 4 days (liver, muscle) to 4 weeks (placenta), while IGF-II assays required exposure times of only 6 to 18 h.

RNA from a single tissue from all twelve fetuses was analysed within one assay. All assays were repeated at least once. The pooled control fetal liver RNA was included in quadruplicate in every assay. Fifty µg of control liver RNA was analysed in IGF-I mRNA assays of lung, liver and skeletal muscle. For tissues with lower abundance of IGF-I mRNA (placenta, heart, kidney) and for all IGF-II assays, four 25 µg aliquots of control liver RNA were analysed. Assays including all twenty liver RNA samples were also performed for IGF-I and -II. These assays were repeated once.

Densitometry

Autoradiographs were scanned using the Molecular Dynamics 300A scanning densitometer and the Image Quant program. A rectangle of constant size was defined and used to analyse the intensity of the protected fragments in each sample. This process was repeated three times for each autoradiograph and the results were averaged.

The intensity of the protected fragment measured for each experimental sample was expressed as a fraction of the mean intensity of the within assay control RNA replicates. This value (intensity of protected fragment divided by intensity of the assay control) was used to compare results from different assays. The results presented are the mean of two assays for each tissue. IGF-I mRNA measurements are expressed relative to 50 µg control RNA. For those assays where 25 µg assay control RNA was analysed (kidney, heart), final results were divided by two, so that the abundance could be compared between tissues. For placenta, where 75 µg total RNA from experimental samples was analysed and compared to 25 µg assay control RNA, the final result was divided by three. The within assay coefficient of variation for analysis of control RNA ranged from 7% to 26% for IGF-I mRNA (n= 12 assays) and 8% to 24% for IGF-II (n= 12 assays).

3.2.6 INSULIN RADIOIMMUNOASSAY

Insulin concentrations were measured in fetal plasma by radioimmunoassay. A guinea pig antibody raised against ovine insulin (Ab GP2 21/7/86) was provided by Dr. R. Newman, CSIRO Division of Animal Production, Prospect, NSW. Ovine insulin (Sigma Chemical Co.) was used as the standard. Porcine insulin (Eli Lilly Co.) was used as the radioligand following iodination to 70 Ci/g with chloramine-T.

Fetal arterial plasma samples (100 µl) were mixed with 200 µl Insulin RIA buffer (50 mM NaH_2PO_4, 0.1% [w/v] NaN_3, 10 mg/ml bovine serum albumin, pH 7.4). Fetal sheep plasma (100 µl) which had been stripped of insulin by treatment with activated charcoal was added to insulin standards (0.975 to 500 ρg) in 200 µl assay buffer. Anti-insulin antiserum in 50 µl

assay buffer (final dilution in assay incubate, 1:400 000) was added to all standards and unknowns. Tubes were incubated for 92-96 h at 4°C. Fifty µl [^{125}I]-insulin (approximately 20 000 cpm) in assay buffer was added and the incubation was continued overnight at 4°C. A goat anti-guinea pig immunoglobulin-coated cellulose suspension (50 µl) (Sac-Cell, IDS) was added. Following a 30 min incubation at room temperature, 1 ml of water was added and the samples were centrifuged at 2 000 rpm, 4°C for 10 min (J6B centrifuge, Beckman). Supernatants were aspirated and the radioactivity in the pellet was measured. All samples were analysed in duplicate. The within assay coefficient of variation, assessed by analysis of a reference plasma sample, was 3.8 % (n= 6). The minimum detectable concentration of insulin was 24.4 ρg/ml plasma.

3.2.7 IGF BINDING PROTEINS

Western ligand blotting

The molecular forms of IGF binding proteins (IGFBPs) in arterial blood plasma, from the twelve fetal sheep in which IGF mRNAs were analysed in six tissues, were examined by Western ligand blot analysis using previously described methods (Hossenlopp *et al.*, 1986). Six µl of fetal arterial plasma was mixed with 54 µl of SDS loading buffer (3.02% Tris, 8% SDS, 0.004% bromophenol blue (w/v), 20% glycerol (v/v)) and heated at 65°C for 15 min. Twenty µl of the sample/buffer mix (2 µl fetal sheep plasma) was subjected to discontinuous SDS-polyacrylamide gel electrophoresis through a 4% stacking gel and 10% separating gel (Laemmli, 1970) at 12 mA for 20 h. Molecular size markers (^{14}C-labelled Rainbow Protein Molecular Weight markers, 14-200 kDa, Amersham Australia Pty. Ltd.) were co-electrophoresed with samples. Proteins were transferred onto 0.45 µM nitrocellulose membrane (Schleicher and Scheull) by electroblotting (Transfor TE42 unit, Hoeffer Scientific Instruments) at 300 mA for 3 h in Tris/glycine buffer (0.2 M Tris, 0.15 M glycine, 20% ethanol (v/v)). Membranes were washed at room temperature in saline buffer (0.15 M NaCl, 0.1 M Tris, pH 7.4) containing 1% Triton X-100 (v/v) for 30 min, 1% bovine serum albumin (w/v) for 2 h and 0.1% Tween-20 (v/v) for 15 min. The membranes were then incubated in saline buffer containing 1% bovine serum albumin (w/v), 0.1% Tween-20 (v/v) and [^{125}I]

labelled IGF-II (approximately 500 000 cpm) for 4 h. The nitrocellulose was washed four times for 20 min in saline buffer containing 0.1% Tween-20, airdried, and exposed to Fuji-RX X-ray film at -70°C with intensifying screens (Cronex Hi-Plus, Du Pont) for 7 days.

IGFBP-3 radioimmunoassay

Plasma ovine IGFBP-3 was measured using a radioimmunoassay developed as previously described for porcine IGFBP-3 (Walton & Etherton, 1989). Purified ovine IGFBP-3 (Carr *et al.*, 1994) was used to generate a rabbit polyclonal antibody with a titre of 2,400. The RIA was performed as described by Walton and Etherton (1989) with the exception that radiolabelled IGF-I/BP-3 complex was crosslinked as described by Baxter and Martin (1986). This assay exhibited parallel displacement with ovine plasma and ovine IGFBP-3 with an ED_{50} of approximately 1 ng per assay tube (300 µl). Furthermore, the assay was specific for oIGFBP-3, exhibiting no cross-reactivity with IGF-I, IGF-II, oIGFBP-4, hIGFBP-3, pIGFBP-3 or human, rat, porcine and bovine plasma. The RIA had a within assay coefficient of variation of 6% (for 6 assays) and a between assay co-efficient of variation of 13%.

3.2.8 STATISTICS

All results are expressed as mean ± SEM with the number of animals in parentheses. Treatment effects were analysed using one way analysis of variance (ANOVA) and the means compared by Bonferroni/Dunn tests using the SuperANOVA (Abacus Concepts Inc.) program. Relationships between variables were tested using simple and partial correlation analysis and multiple regression analysis (Statview SE + Graphics, Abacus Concepts Inc. and SAS/STAT software). Results were considered significant at the 5% level.

3.3 RESULTS

3.3.1 IGF-I AND -II GENE EXPRESSION IN FETAL SHEEP TISSUES

IGF gene expression in six fetal tissues (liver, lung, skeletal muscle, heart, kidney and placenta), plasma IGF proteins and metabolites (3.3.1) were characterised in four normal fetal sheep and eight fetal sheep with restricted placental growth. In addition, hepatic IGF gene expression, plasma IGF proteins and metabolites were studied in an additional four normal fetuses and four fetuses with restricted placental size (3.3.2).

Fetal growth

Placental weight ranged from 619 g to 187 g (n=12) at 121 days gestation. Fetal weight ranged from 2908 g to 1627 g and correlated positively with placental size (r=0.89, P= 0.0001) (Figure 3.1) as expected. Restriction of placental size was associated with asymmetrical fetal growth retardation, as observed in the preceding study (2.3.1). The weights of fetal liver, kidney and heart were reduced in fetuses with restricted placental size (Figure 3.2a). Brain weight and lung weight were not different between control and restricted groups (Figure 3.2a). When fetal tissue weights were expressed relative to fetal weight, the relative weights of the liver, lung, kidney and heart were not affected by restriction (Figure 3.2b). Brain weight was disproportionately maintained relative to fetal body weight (P< 0.02, Table 3.1, Figure 3.2b) and to liver weight (P< 0.005, Table 3.1), as observed previously (Chapter 2).

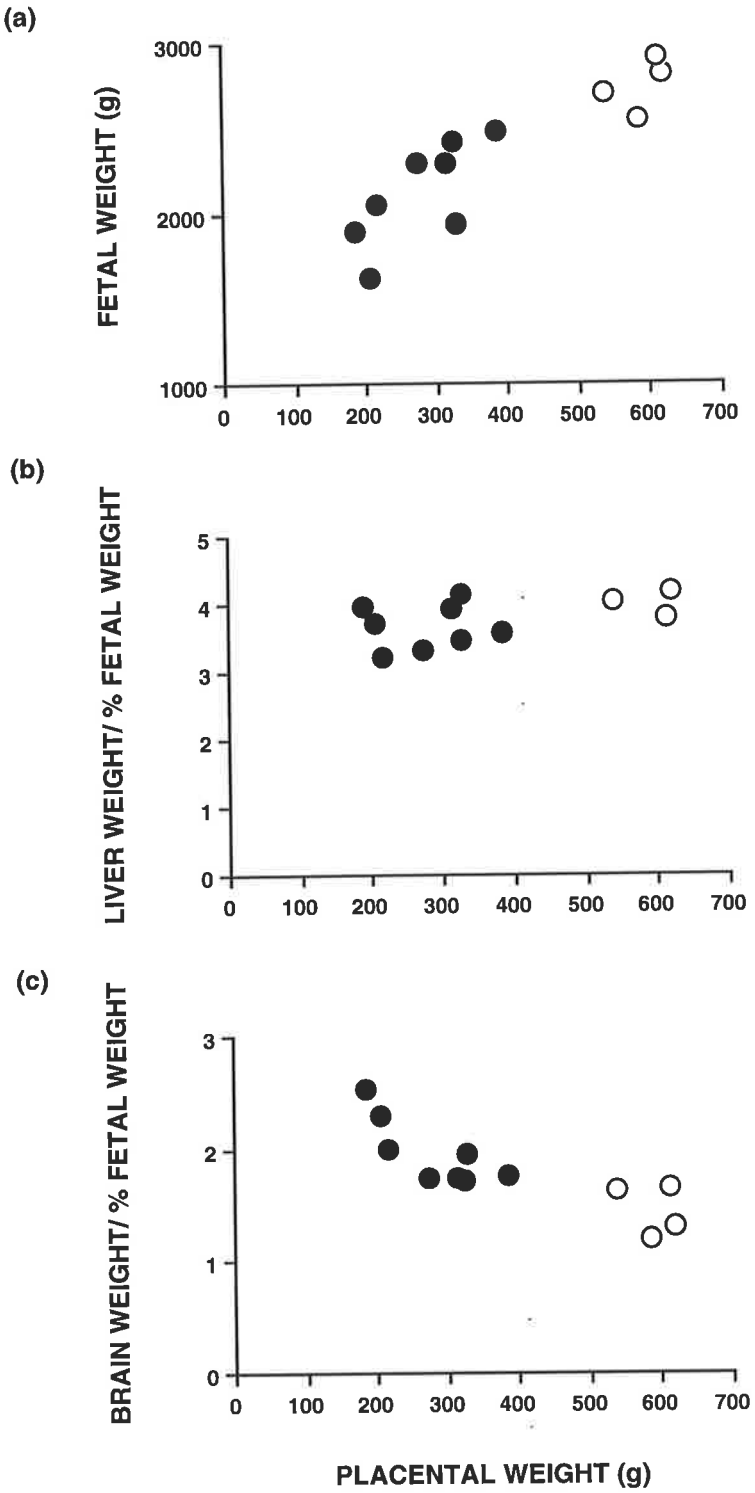


FIGURE 3.1 Effect of restriction of placental size on fetal growth at 121 days gestation.

Relationship between (a) Fetal weight (r=0.89, $P < 0.0001$, n= 12),
(b) Liver weight as a percentage of fetal weight (r=0.46, NS, n= 12),
(c) Brain weight as a percentage of fetal weight (r=0.80, $P < 0.003$, n= 12)

and placental weight measured at 121 days gestation in fetal sheep from ewes with normal (○ , n=4) or restricted (● , n=8) placental development.

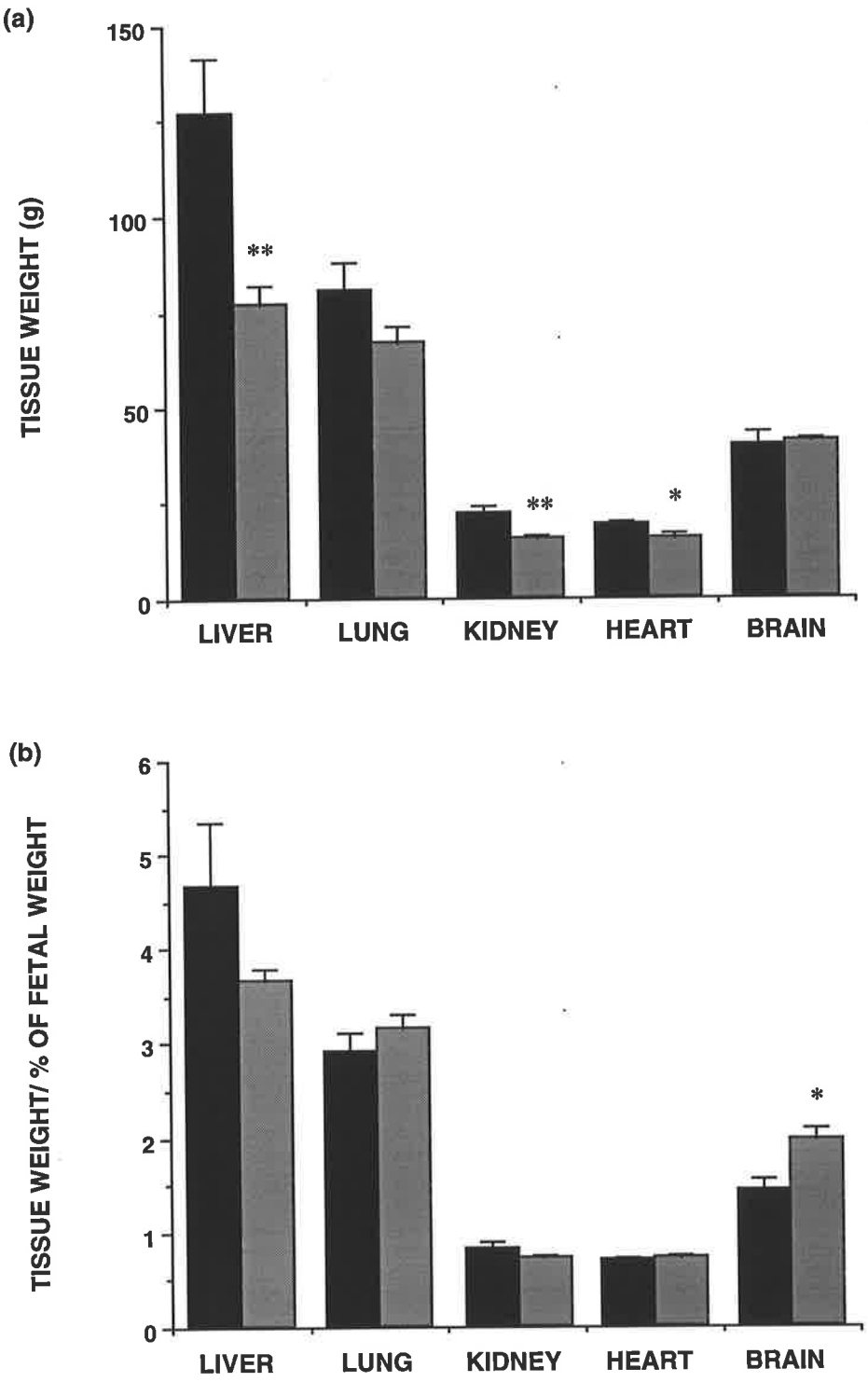


FIGURE 3.2 Effect of restriction of placental growth on fetal tissue growth at 121 days gestation.

(a) Tissue weights (g) in normal fetal sheep (solid bars, n=4) and those with restricted placental size (hatched bars, n=8).

(b) Tissue weights expressed as a percentage of total fetal body weight (g%). The groups are the same as for (a)

Values are mean ± SEM. * $P < 0.05$, ** $P < 0.005$ compared to normal size

TABLE 3.1 Morphometry of fetal sheep with normal and restricted placental development.

	NORMAL (4)	PLACENTAL RESTRICTION (8)
Fetal weight (g)	2743 ± 78	2121 ± 104 **
Placental weight (g)	588.8 ± 18	279.3 ± 25 ***
Number of cotyledons formed	72 ± 4	28 ± 2 ***
Fetal/placental weight ratio	4.7 ± 0.1	7.9 ± 0.5 **
Liver weight (g)	127.0 ± 15	77.4 ± 4 **
Brain weight (g)	39.8 ± 4	40.8 ± 0.9
Brain weight/ fetal weight	0.014 ± 0.001	0.02 ± 0.001 *
Brain weight/ liver weight	0.331 ± 0.06	0.539 ± 0.03 **
Lung weight (g)	80.5 ± 0.8	66.7 ± 4
Heart weight (g)	19.2 ± 0.8	15.7 ± 0.9 *
Kidney weight (total) (g)	22.3 ± 1.9	15.4 ± 0.8 **

Values are mean ± SEM, *** $P< 0.0001$, ** $P< 0.005$, * $P<0.05$, compared to normal. One way analysis of variance (ANOVA) and Bonferroni Dunn. All measurements were made at 121 ± 2 days gestation.

Blood gases and metabolites

Fetal arterial blood pO_2, pCO_2 and haemoglobin and arterial plasma glucose concentrations were not different between groups (Table 3.2). Glucose concentrations in fetal sheep plasma were higher than those measured in deproteinised blood in the previous study, even when corrected for haematocrit (data not shown). This may be due to improved condition and hence body energy stores of the pregnant ewes used in these experiments, however this was not assessed. Blood pH was slightly lower in fetuses with restricted placental size ($P< 0.01$). The pO_2 of umbilical venous and fetal arterial blood ($r= 0.61$, $P< 0.04$) correlated positively with placental weight. No association was observed between placental weight and fetal plasma glucose ($r= 0.49$, NS). Fetal weight was positively associated with umbilical venous blood pO_2 ($r= 0.61$, $P< 0.03$) and arterial plasma glucose ($r= 0.57$, $P< 0.05$), but was not related to the pO_2 of fetal arterial blood ($r= 0.39$, NS).

TABLE 3.2 Blood gases and plasma glucose concentrations in fetal sheep with normal and restricted placental development.

	NORMAL (4)	PLACENTAL RESTRICTION (8)
pO_2, arterial (torr)	21.2 ± 2.5	16.6 ± 1.1
pO_2, venous (torr)	33.0 ± 3.1	25.3 ± 2.2
pCO_2, arterial (torr)	52.4 ± 0.7	55.9 ± 1.3
Haemoglobin (g/dl)	13.0 ± 2.9	9.2 ± 0.5
pH, arterial	7.34 ± 0.001	7.32 ± 0.01 *
Plasma glucose, arterial (mmol/l)	1.8 ± 0.3	1.4 ± 0.4

Values are mean ± SEM, * $P<0.05$, compared to normal. One way analysis of variance (ANOVA) and Bonferroni Dunn. All measurements were made at 121 ± 2 days gestation.

Plasma IGF-I and IGF-II

The IGF-I protein concentration, measured in arterial blood plasma from the twelve fetal sheep in which mRNA for IGF-I and -II was analysed in six fetal tissues, ranged from 63 ng/ml to 176 ng/ml. Weak associations were observed between plasma IGF-I and placental weight (r=0.56, P= 0.057, Figure 3.3b) and fetal weight (r=0.60, P< 0.04, Figure 3.3a). A positive association was observed between plasma IGF-I and the concentration of glucose in fetal plasma (r=0.80, P< 0.002, (data not shown)) as seen in Chapter 2. In contrast to the preceding study, no association was observed between IGF-I protein concentration and fetal arterial blood pO_2 (r=0.43, NS). However, this may relate to the smaller number of animals studied here (see 3.3.2). Plasma IGF-I protein was positively correlated with fetal arterial blood pH (r= 0.58, P< 0.05).

Fetal plasma IGF-I also correlated positively with the weights of individual fetal tissues, including kidney weight (r= 0.76, P< 0.004, n=12) and liver weight (r= 0.74, P < 0.01, n=12), but was not related to lung weight or heart weight at 121 ± 2 days gestation.

IGF-II protein in fetal blood plasma ranged from 423 ng/ml to 931 ng/ml. Plasma IGF-II was not related to fetal weight, placental weight (Figure 3.3c,d), fetal plasma glucose concentration or the pO_2 of fetal blood (not shown) as expected (Chapter 2).

TABLE 3.3 Concentrations of IGF proteins in arterial plasma from fetal sheep with normal and restricted placental development.

	NORMAL (4)	PLACENTAL RESTRICTION (8)
Plasma IGF-I (ng/ml)	164 ± 10	120 ± 12 *
Plasma IGF-II (ng/ml)	664 ± 71	699 ± 61

Values are mean ± SEM, * P< 0.05, compared to normal. One way analysis of variance (ANOVA) and Bonferroni Dunn. All measurements were made at 121 ± 2 days gestation.

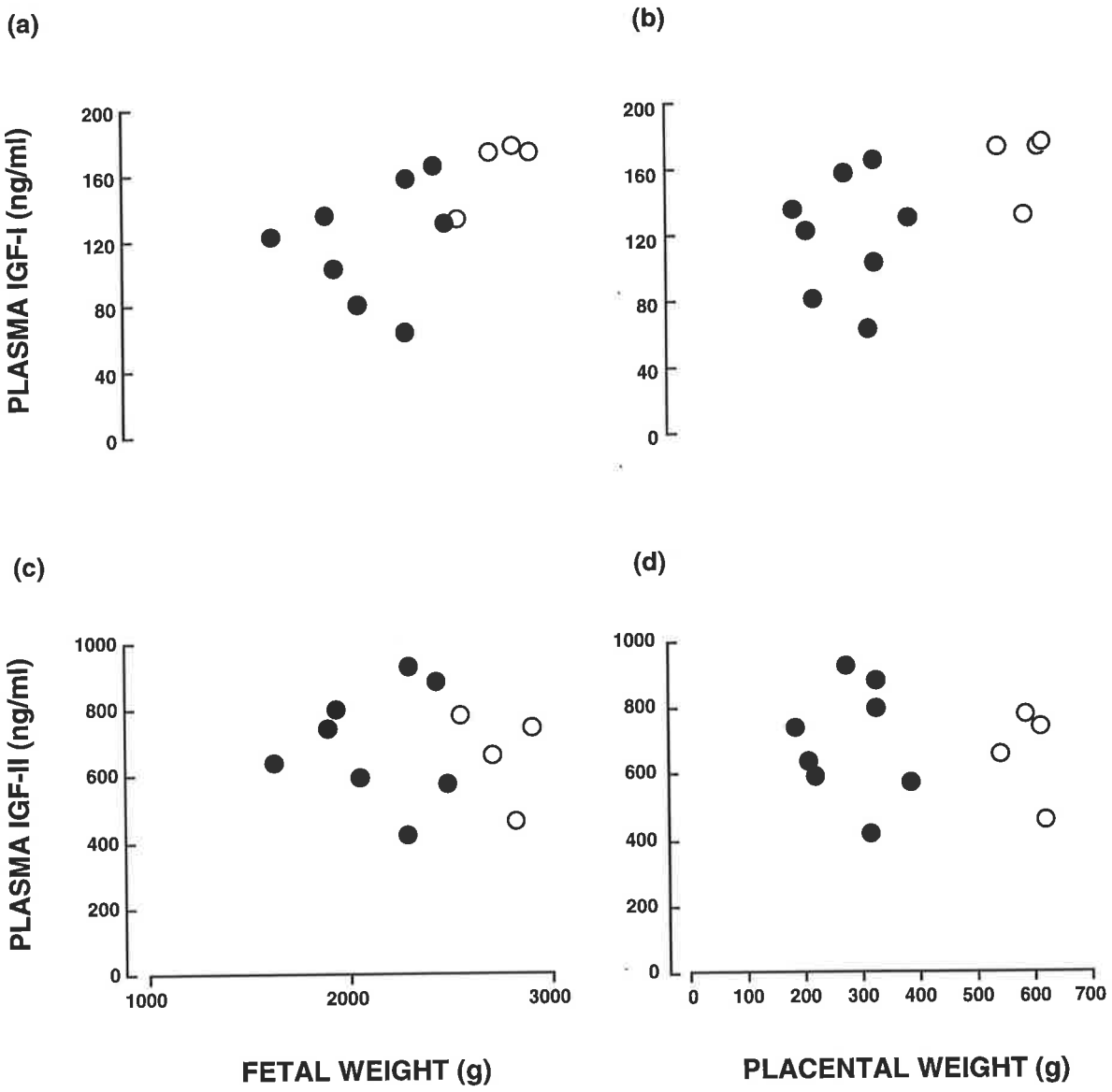


FIGURE 3.3 Relationship between circulating IGFs and fetal and placental growth.

Correlations of IGF-I protein concentrations in fetal arterial plasma with
(a) Fetal weight (r= 0.6, P = 0.04, n= 12),
(b) Placental weight (r= 0.56, P = 0.06, n= 12).

Correlations of IGF-II protein concentrations in fetal arterial plasma with
(c) Fetal weight (NS),
(d) Placental weight (NS)
in fetal sheep with normal (○ ,n=4) and restricted (●, n=8) placental development.

Messenger RNAs for IGF-I and -II in fetal sheep tissues

A representative ribonuclease protection assay of IGF-I mRNA in total RNA from fetal sheep skeletal muscle is shown in Figure 3.4. As previously reported, sheep RNA protected two major fragments of labelled antisense IGF-I RNA from ribonuclease digestion (Saunders *et al.*, 1991). By comparison to the migration of DNA molecular weight markers, the apparent size of these fragments was 85 and 81 bases. The region complementary to coding region of exon 4 of the sheep IGF-I gene in the antisense IGF-I RNA consists of 83 bases. Therefore, the 85 and 81 base fragments are likely to represent slight underdigestion and overdigestion respectively of the predicted protected fragment under the ribonuclease digestion conditions used. For scanning densitometry, both fragments protected from ribonuclease digestion were scanned and the results compared. The scanned intensity of the 85 and 81 base bands was positively correlated. Values obtained for the 85 base band alone were used for all calculations.

Figure 3.5 shows a representative ribonuclease protection assay of IGF-II mRNA in total RNA from fetal sheep kidney. Sheep RNA protected a number of fragments of labelled antisense IGF-II RNA from ribonuclease digestion as previously reported (Li *et al.*, 1993). Major fragments of approximately 163-165 and 160-161 bases, estimated by comparison to the migration of DNA molecular weight markers, were protected from ribonuclease digestion (Figure 3.5). A minor band was also observed at 170 bases. In most assays the size of the major protected fragment was estimated as 164 bases. The antisense RNA region is complementary to the entire sequence of exon 8 of the ovine IGF-II gene, which consists of 165 bases (Dr. Juan Li, Department of Cellular Physiology, Babraham, Cambridge, personal communication). In addition, the antisense RNA contains sequence complementary to the 5' and 3' flanking introns. Exon 8 of the ovine IGF-II gene represents the first coding exon according to the nomenclature of Ohlsen *et al.* (1994). At least three different exons can be spliced to exon 8 of the IGF-II gene to produce IGF-II mRNAs containing alternative 5' untranslated regions (Ohlsen *et al.*, 1994). Therefore, the multiple protected fragments, observed in ribonuclease protection assays using this antisense RNA probe, may be due at least in part, to the variation in sequence found in the alternative IGF-II mRNA forms

immediately preceding the 5' end of the double stranded protected fragment. The intensity of the 164 base band was determined by densitometric scanning. In addition, all bands in the region were scanned using a rectangle of constant size. These results were found to correlate and the results for the 164 base band alone were used in all calculations in this chapter.

IGF-I and -II mRNA was analysed by ribonuclease protection assay across a range of concentrations of total RNA from normal fetal sheep liver (Figure 3.6). Approximately 500 ρg of antisense IGF-I RNA or 700 ρg of antisense IGF-II RNA were used in the assay. The scanned intensity of IGF-I, expressed in relative densitometric units, increased linearly between 5 and 100 μg of total liver RNA (r=0.989, P< 0.0002). The intensity of IGF-II mRNA increased between 5 and 75 μg of total liver RNA (r= 0.998, P< 0.0001). For further ribonuclease protection assays of IGF-I mRNA, 50 μg of total RNA was analysed from most tissues. For all further ribonuclease protection assays of IGF-II mRNA, 25 μg of total RNA was analysed. Approximately 300 ρg of antisense IGF-I riboprobe or 500 ρg of antisense IGF-II riboprobe was used for all subsequent assays.

The scanned intensity of IGF-II mRNA in 5 μg total liver RNA was four times higher than that of IGF-I mRNA in 75 μg of the same fetal liver RNA preparation when assayed, electrophoresed and exposed to X-ray film under identical conditions. Since the specific activities of the antisense IGF-I and -II RNAs were not the same, direct comparisons between the abundance of IGF-I and -II mRNA are not possible. However, the protected antisense IGF-I RNA contains approximately 13 U residues, and the protected antisense IGF-II RNA contains approximately 40 U residues. Assuming [^{32}P]-UTP is incorporated into each riboprobe at similar efficiencies during T_7 polymerase transcription, the abundance of IGF-II mRNA in 5 μg of liver RNA can be estimated to be 1.3 times higher than that of IGF-I mRNA in 75 μg fetal liver. Thus, the relative abundance of IGF-II mRNA in total RNA from fetal sheep liver is approximately 20 times greater than that of IGF-I.

Preliminary experiments were performed to determine whether IGF-I and -II were expressed at 121 days gestation in the fetal sheep tissues chosen for the study. IGF-I mRNA was present in all tissues examined; fetal liver, skeletal muscle, lung, kidney, heart, cerebellum

and placenta. IGF-II mRNA was present in liver, skeletal muscle, lung, kidney, heart and placenta. No IGF-II mRNA could be detected in total RNA from fetal sheep cerebellum, using the ribonuclease protection assay. Figure 3.7 shows the relative intensities of the labelled fragments protected in (a) IGF-I mRNA and (b) IGF-II mRNA assays of total RNA from a range of fetal sheep tissues at 121 days gestation. Brain (cerebellum) was excluded from further assays as IGF-II mRNA could not be detected, and IGF-I mRNA abundance was equivalent to or less than that observed in kidney and heart, making quantitative analysis difficult. The relative abundance of IGF mRNA in different tissues (liver, kidney, lung, skeletal muscle, heart, placenta) was expressed relative to a fetal liver RNA sample included in quadruplicate in every assay as a reference control (Figures 3.4, 3.5, 3.10).

Quadriceps muscle and liver demonstrated the highest relative abundance of IGF-I mRNA in normal fetal sheep, followed by lung, kidney, heart and placenta (Figure 3.8a). The highest relative amount of IGF-II mRNA was detected in kidney, followed by lung, liver ≈ muscle, placenta and heart (Figure 3.8b). Estimates of the total amount of IGF-I or -II mRNA in tissues from normal fetal sheep were made by multiplying the weight of the tissue by the relative abundance of (a) IGF-I or (b) IGF-II mRNA in that tissue (Figure 3.9). These calculations give approximations only as they do not take into account variations in the concentration of total RNA per gram of tissue, as this data was not available for all tissues. Furthermore, muscle weights were not collected, thus skeletal muscle has been omitted from Figure 3.9. However, these estimates suggest that of the tissues studied the liver contains the most IGF-I mRNA in normal fetal sheep followed by placenta, lung, kidney and heart, while the most IGF-II mRNA is contained in placenta, followed by lung, liver, kidney and heart.

Based on the estimation that the abundance of IGF-II mRNA in total RNA from normal fetal sheep liver is approximately twenty times that of IGF-I mRNA (see page 3-28), estimates were also made of the relative abundance of IGF-I and -II mRNAs in fetal tissues using the data presented in Figure 3.8. These estimates suggest that the relative abundance of IGF-II mRNA is 190-times that of IGF-I mRNA within fetal kidney, 85-times that of IGF-I mRNA within fetal lung, 80-times that of IGF-I mRNA within placenta, 25-times that of IGF-I mRNA within fetal heart and 17-times that of IGF-I mRNA within fetal skeletal muscle.

FIGURE 3.4 Autoradiograph of electrophoresis of ribonuclease protected products of solution hybridisation of IGF-I mRNA in 50 μg total RNA extracted from quadriceps muscle of fetal sheep with normal and restricted placental development at 121 days gestation.

Products of 85 and 81 bases were protected from ribonuclease digestion. M= ^{32}P labelled DNA molecular weight markers. Control liver RNA= 50 μg of total RNA extracted from normal fetal sheep liver. P= undigested, labelled antisense IGF-I RNA. Y= yeast tRNA, showing complete ribonuclease digestion of the unhybridised antisense IGF-I RNA. The autoradiograph was exposed to X-ray film for 4 days at -70°C with intensifying screens. An enlargement of the same figure indicating normal (n=4) and restricted (n=8) fetal sheep is shown in Figure 3.10.

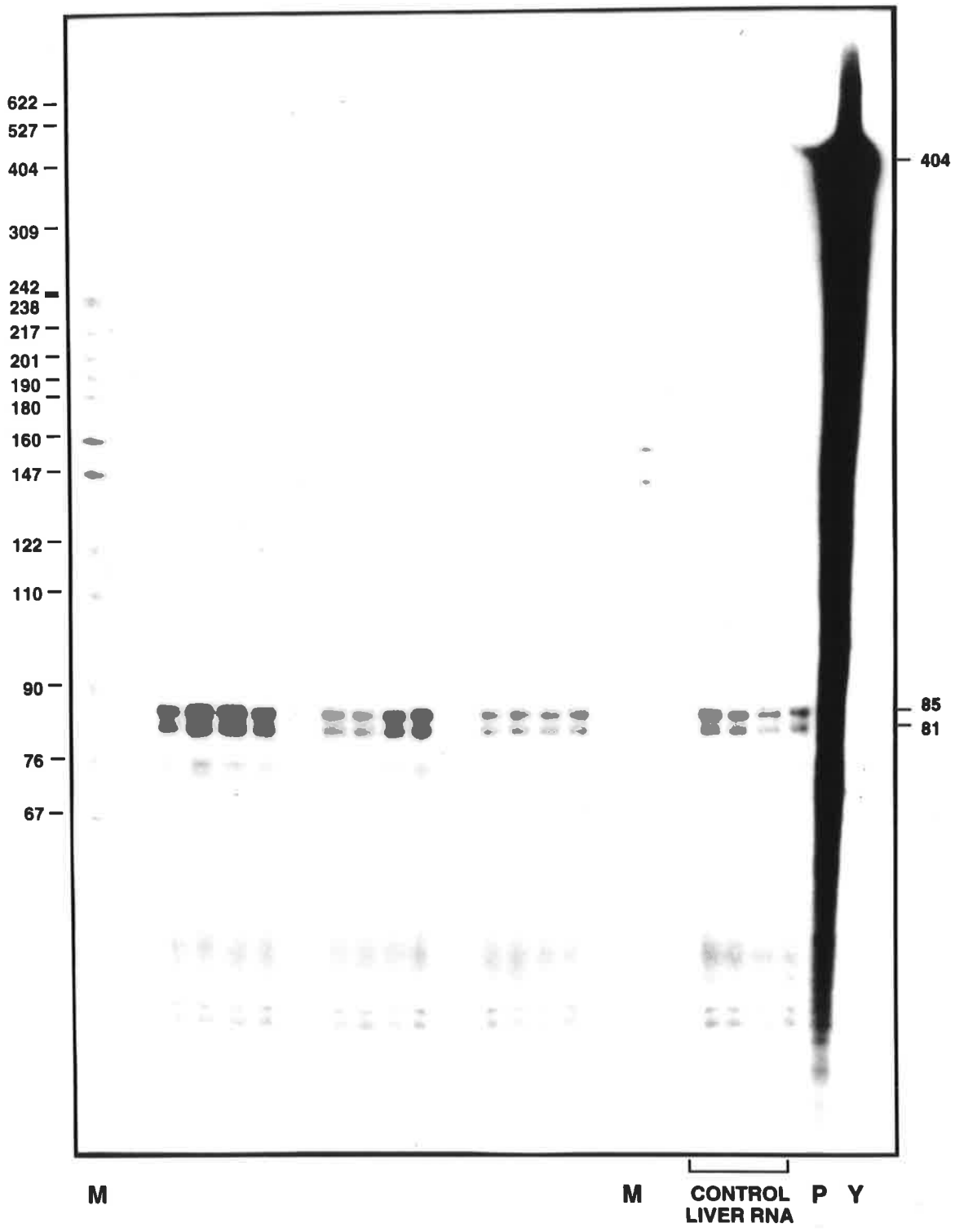

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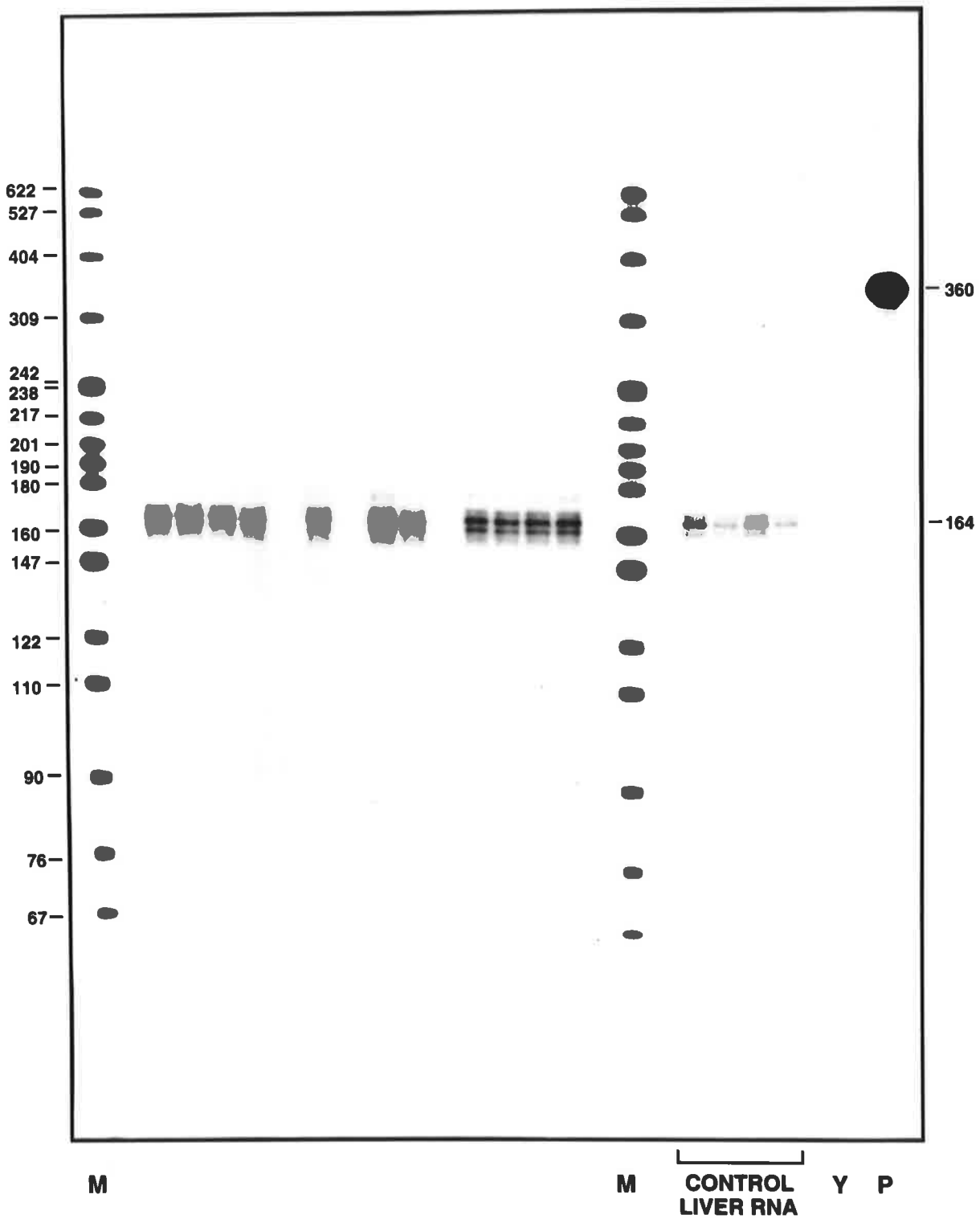
FIGURE 3.5 Autoradiograph of electrophoresis of ribonuclease protected products of solution hybridisation of IGF-II mRNA in 25 μg total RNA extracted from the kidney of fetal sheep with normal and restricted placental development at 121 days gestation.

M= ^{32}P labelled DNA molecular weight markers. Control liver RNA= 25 μg of total RNA extracted from normal fetal sheep liver. P= undigested, labelled antisense IGF-II RNA. Y= yeast tRNA, showing complete ribonuclease digestion of the unhybridised antisense IGF-II RNA. Products between 170 and 160 bases were protected, with the major protected product at 164 bases. The autoradiograph was exposed to X-ray film for 15 hours at room temperature. An enlargement of the same figure indicating normal (n=4) and restricted (n=7) fetal sheep is shown in Figure 3.10.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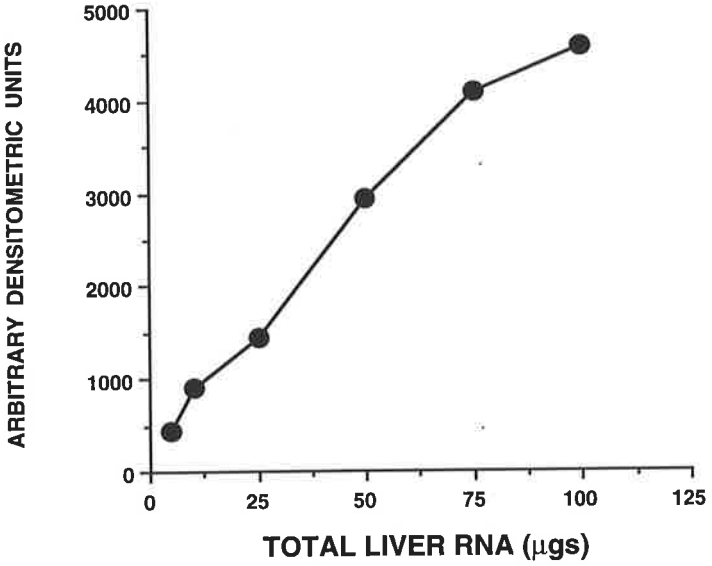
(a) IGF-I mRNA



(b) IGF-II mRNA

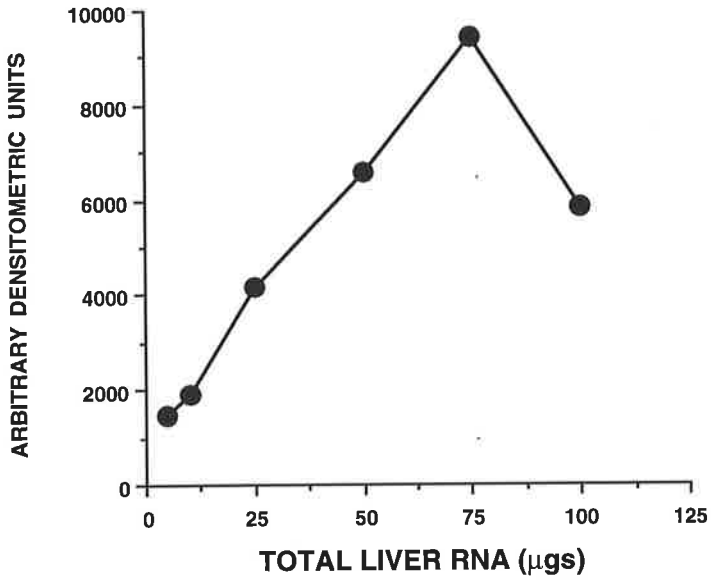


FIGURE 3.6 Scanned intensity of ribonuclease protected products of solution hybridisation of IGF-I and -II mRNA in various amounts of normal fetal sheep liver total RNA.

Scanned intensity of ribonuclease protected products of solution hybridisation of
(a) IGF-I mRNA ($r= 0.99$, $P< 0.0002$, $y= 0.45x + 406$) and
(b) IGF-II mRNA ($r=0.97$, $P< 0.99$, $P< 0.0001$, $y=113x + 959$, excluding 100 µg)
in various amounts (5, 10, 25, 50, 75, 100 µg) of total RNA from normal fetal sheep liver.

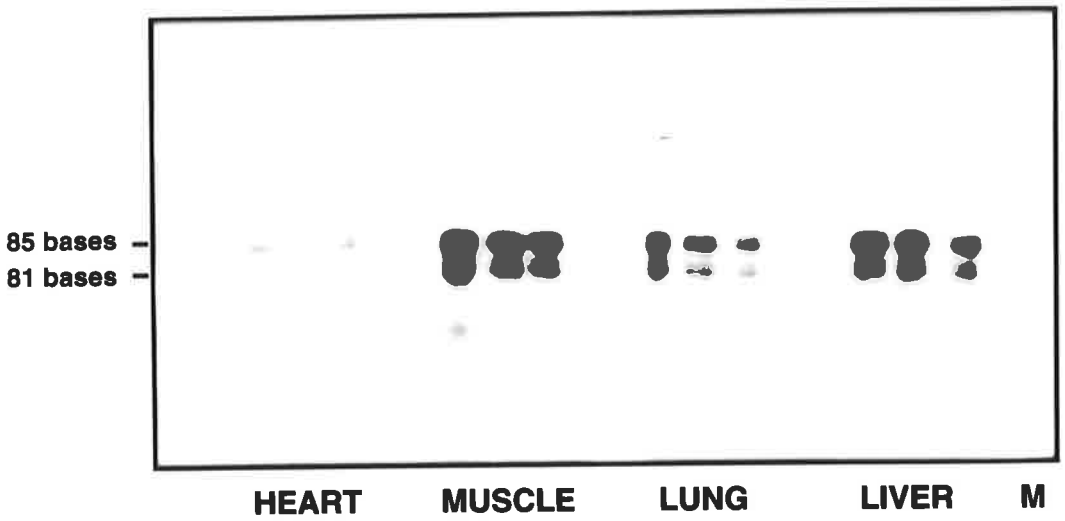
The exposure time for the autoradiograph scanned for (a) was 10 days at -70^{o}C with intensifying screens. The exposure time for the autoradiograph scanned for (b) was 48 hours at room temperature. Values are expressed in arbitrary densitometric units only. The relative abundance of IGF-II mRNA is approximately 20 times greater than that of IGF-I mRNA (see page 3-28).

FIGURE 3.7 Autoradiograph of electrophoresis of ribonuclease protected products of solution hybridisation of IGF-I and -II mRNA in fetal sheep tissues at 121 days gestation.

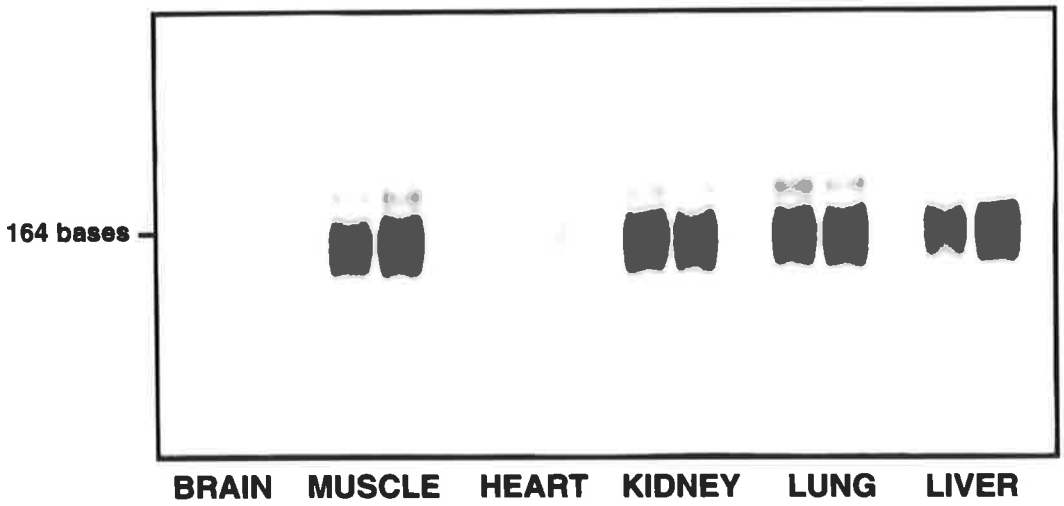
Autoradiograph of electrophoresis of ribonuclease protected products of solution hybridisation of (a) IGF-I mRNA in 50 μg total RNA extracted from fetal sheep tissues at 121 days gestation and
(b) IGF-II mRNA in 25 μg total RNA extracted from fetal sheep tissues at 121 days gestation.

The exposure time for the autoradiograph in (a) was 3 days at -70°C with intensifying screens. The exposure time for the autoradiograph in (b) was 15 hours at -70°C with intensifying screens.

(a) IGF-I mRNA



(b) IGF-II mRNA



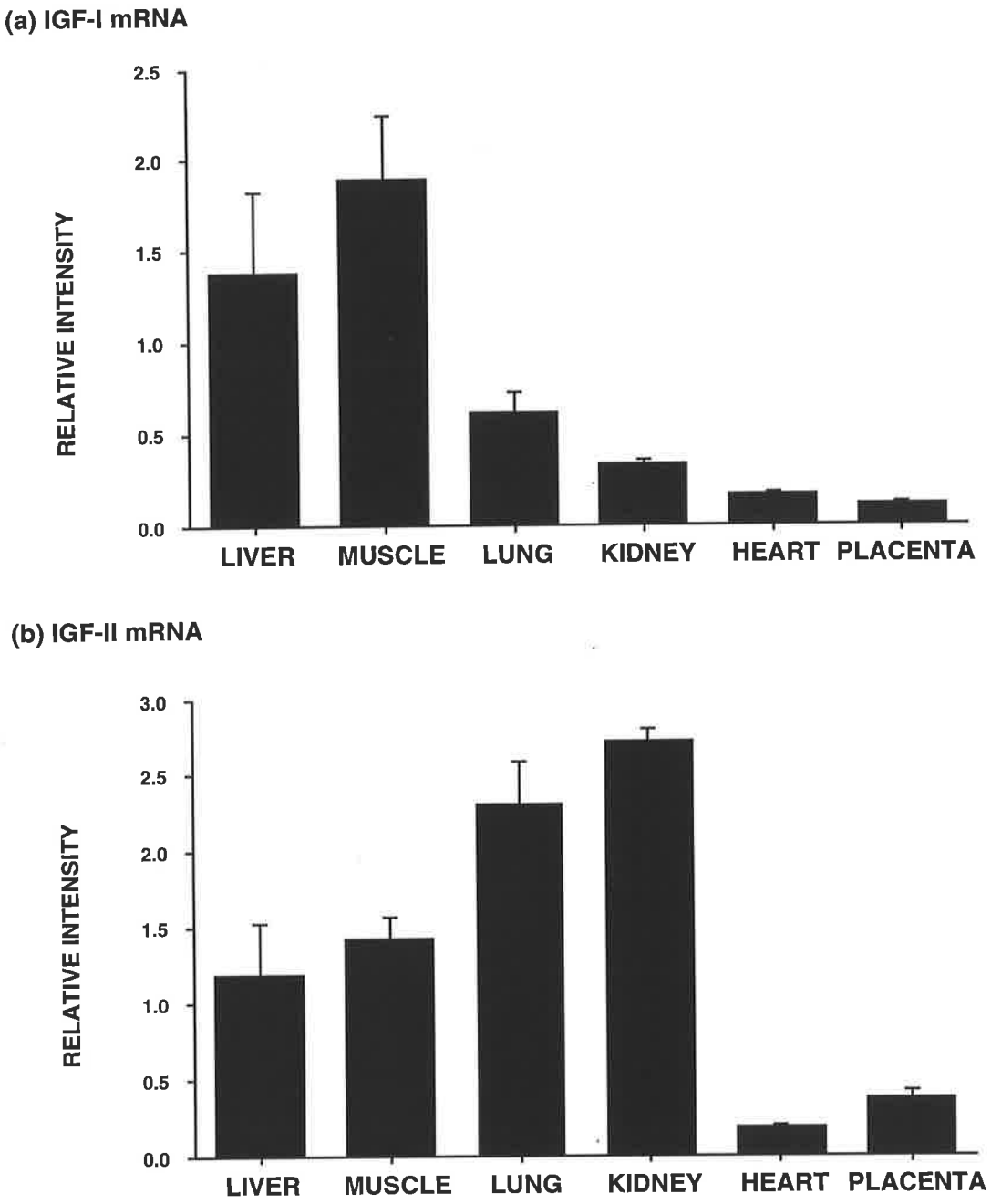
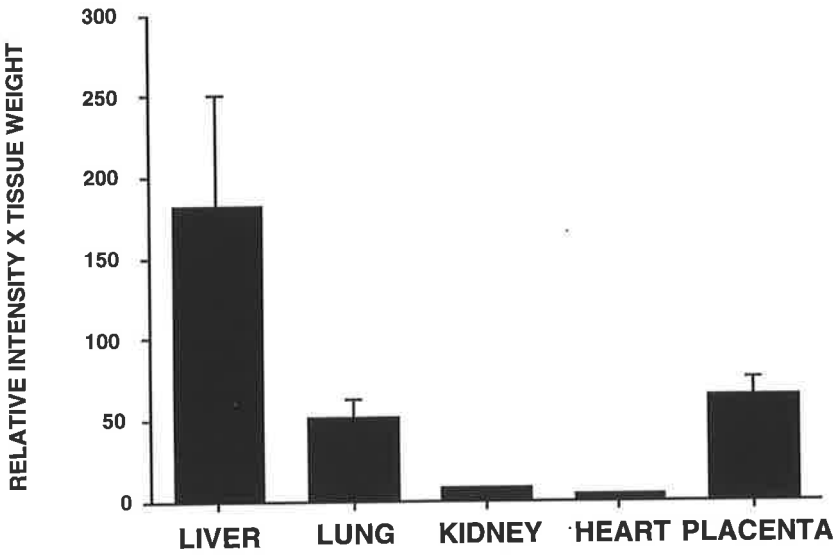


FIGURE 3.8 Relative abundance of IGF-I and -II mRNA in tissues from normal fetal sheep at 121 days gestation.

(a) Relative abundance of IGF-I mRNA measured in 50 µg of total RNA from fetal sheep tissues.
(b) Relative abundance of IGF-II mRNA measured in 25 µg of total RNA from fetal sheep tissues.

The abundance of IGF-I and -II mRNA is expressed relative to the amount of IGF-I or -II mRNA measured in replicates of a fetal liver RNA sample that was included in all assays. Results represent the mean ± SEM of measurements obtained from four fetal sheep in two ribonuclease protection assays. The abundance of IGF-II mRNA in fetal liver is approximately 20 times that of IGF-I mRNA.

(a) IGF-I mRNA



(b) IGF-II mRNA

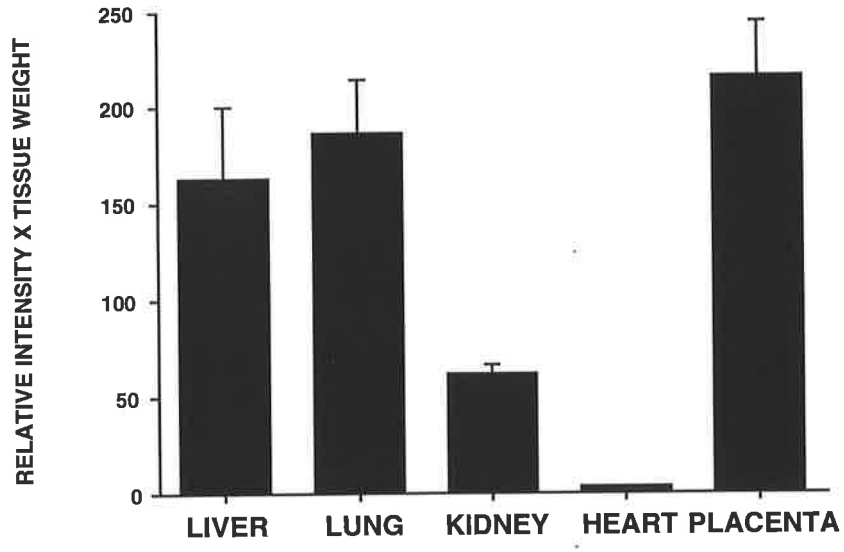


FIGURE 3.9 Estimated relative amounts of IGF-I and -II mRNA in tissues of normal fetal sheep at 121 days gestation.

(a) Relative abundance of IGF-I mRNA in 50 μg of total RNA from fetal sheep tissues multiplied by tissue weight.

(b) Relative abundance of IGF-II mRNA in 25 μg of total RNA from fetal sheep tissues multiplied by tissue weight.

The abundance of IGF-I and -II mRNA is expressed relative to the amount of IGF-I or -II mRNA measured in replicates of a fetal liver RNA sample that was included in all assays. Relative abundance was multiplied by tissue weight (g). These values are estimates only as the concentration of total RNA per gram of tissue has not been corrected for. Results represent the mean ± SEM of results obtained for four fetal sheep in two analyses.

Effect of restriction of placental size on IGF mRNA abundance in fetal tissues

Representative ribonuclease protection assays of (a) IGF-I mRNA in fetal skeletal muscle and (b) IGF-II mRNA in fetal kidney from normal and placentally restricted fetal sheep are shown in Figure 3.10. The relative abundance of IGF mRNAs in tissues from normal fetal sheep and the eight fetal sheep with restricted placental size are shown in Figure 3.11. IGF-I mRNA was reduced in skeletal muscle ($P < 0.002$), kidney ($P < 0.01$) and lung ($P < 0.05$) of fetuses with restricted placental growth (Figure 3.11a). No difference was detected in the abundance of IGF-II mRNA between groups (Figure 3.11b).

Kidney weight and liver weight correlated positively with their IGF-I mRNA abundance (kidney, r=0.79, P=0.001; liver, r=0.66, P=0.03). Similar associations were not detected between the weight of other tissues and their IGF-I mRNA abundance. Abundance of IGF-II mRNA was not related to tissue weight for any fetal tissue studied, although muscle IGF-II mRNA did tend to correlate positively with fetal weight (r= 0.53, P< 0.08).

Relationships were observed between the relative abundance of IGF-I mRNA in different tissues. For example, IGF-I mRNA in skeletal muscle correlated positively with IGF-I mRNA in liver, r=0.87, in kidney, r=0.78 and in lung, r=0.75 (all P <0.005) and weakly with IGF-I mRNA in placenta, (r=0.57, P = 0.052), but not with IGF-I mRNA in heart (r=-0.15, NS).

A positive association was observed between the relative abundance of IGF-II mRNA in fetal kidney and that of fetal lung (r= 0.72, P< 0.01), while IGF-II mRNA in heart was negatively related to IGF-II mRNA in lung (r= -0.64, P< 0.05) and in kidney (r= -0.61, P< 0.05). A negative association was also observed between the relative abundance of IGF-II mRNA in liver and IGF-II mRNA in placenta (r= -0.59, P< 0.05). No relationships were observed between the relative abundance of IGF-I and -II mRNA within any tissue.

For liver and placenta, the RNA content per gram of tissue was known for each animal (Dr. Fong Lok, Department of Obstetrics and Gynaecology, University of Adelaide, unpublished observations) (Table 3.4). Therefore, the total amount of IGF-I and -II mRNA was estimated

for liver and placenta by multiplying tissue weight (g) by the RNA content (mg/g) and by the relative abundance of IGF mRNA (relative to assay control) (Table 3.4). The amount of total RNA per gram of liver was increased in fetal sheep with restricted placental size, while placental RNA concentration was not different between groups. Liver weight and placental weight were reduced when placental growth was restricted (Table 3.1). The total hepatic IGF-I mRNA content in fetal sheep with restricted placental size was not significantly different from that of fetuses with normal placental size. In contrast, total placental IGF-I mRNA content was reduced ($P < 0.01$), when placental size was restricted. Hepatic IGF-II mRNA content in fetal sheep with restricted placental size was not altered, and the apparent reduction observed in total placental IGF-II mRNA content in fetal sheep with restricted placental size was not statistically significant (Table 3.4).

Total hepatic IGF-I mRNA correlated positively with placental IGF-I mRNA content (r= 0.74, P< 0.01, n= 10), but no association was observed between the total IGF-II mRNA content of liver with that of placenta (r= -0.53, n= 10, NS). In addition, the total amount of IGF-I mRNA in placenta was positively related to the total amount of IGF-II mRNA in placenta (r= 0.82, P< 0.002, n=11). No relationship between the total content of IGF-I and -II mRNA in liver was evident.

TABLE 3.4 IGF-I and -II mRNA content of liver and placenta from normal fetal sheep and those with restricted placental size, calculated according to tissue weight and tissue total RNA content.

	NORMAL (4)	PLACENTAL RESTRICTION (7)
Liver RNA concentration	3.51 ± 0.31 (mg/g)	4.83 ± 0.24 (mg/g)*
Placenta RNA concentration	2.48 ± 0.25 (mg/g)	2.87 ± 0.24 (mg/g)
Total liver RNA †	454 ± 87 (mg)	369 ± 34 (mg)
Total placental RNA †	1464 ± 162 (mg)	777 ± 102 (mg)*
Total liver IGF-I mRNA § (relative to assay control)	566 ± 128	289 ± 76
Total placental IGF-I mRNA § (relative to assay control)	151 ± 13	65 ± 20 *
Total liver IGF-II mRNA § (relative to assay control)	460 ± 79	447 ± 57
Total placental IGF-II mRNA § (relative to assay control)	527 ± 66	313 ± 74

Values are mean ± SEM (number of animals). *$P < 0.05$ compared to normal, one way analysis of variance and Bonferroni Dunn. All measurements were made at 121 ± 2 days gestation.

† Total liver RNA (liver RNA concentration (mg/g) x liver weight (g))
Total placental RNA (placenta RNA concentration (mg/g) x placental weight (g)).

§ IGF-I and -II mRNA are expressed relative to the amount of IGF-I or -II mRNA detected in an assay control liver RNA sample.
Total liver IGF-I or -II mRNA (relative to assay control)= Total liver RNA x IGF-I or -II mRNA relative abundance.
Total placental IGF-I or -II mRNA (relative to assay control)= Total placental RNA x IGF-I or -II mRNA relative abundance.

The abundance of IGF-II mRNA in fetal liver is approximately 20 times higher than that of IGF-I mRNA, while the abundance of IGF-II mRNA in placenta is approximately 78 times higher than that of IGF-I mRNA (see page 3-29).

Liver and placenta RNA concentrations were generously provided by Dr. Fong Lok.

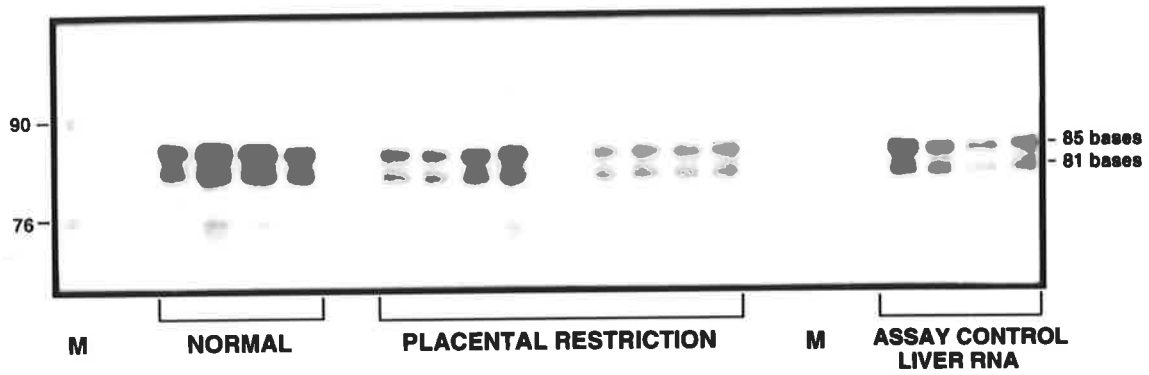
Figure 3.10 Autoradiographs of electrophoresis of ribonuclease protected products of solution hybridisation of IGF-I and -II mRNA in tissues from normal and growth retarded fetal sheep at 121 days gestation.

(a) Representative autoradiograph of electrophoresis of ribonuclease protected products of solution hybridisation of IGF-I mRNA in 50 μg total RNA extracted from quadriceps muscle of four normal fetal sheep and eight fetal sheep with restricted placental development at 121 days gestation.

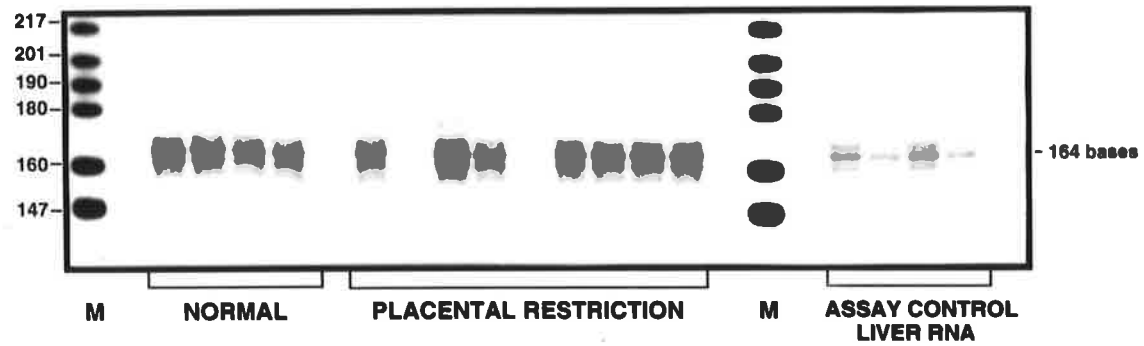
(b) Representative autoradiograph of electrophoresis of ribonuclease protected products of solution hybridisation of IGF-II mRNA in 25 μg total RNA extracted from kidney of four normal fetal sheep and seven fetal sheep with restricted placental development at 121 days gestation.

M= ^{32}P labelled DNA markers, Assay control liver RNA= 50 μg (IGF-I) and 25 μg (IGF-II) of total RNA extracted from fetal sheep liver. Autoradiographs were exposed for 4 days at -70°C (IGF-I) or 15 hours at room temperature (IGF-II). The same assays were shown in Figures 3.4 and 3.5.

(a) IGF-I mRNA



(b) IGF-II mRNA



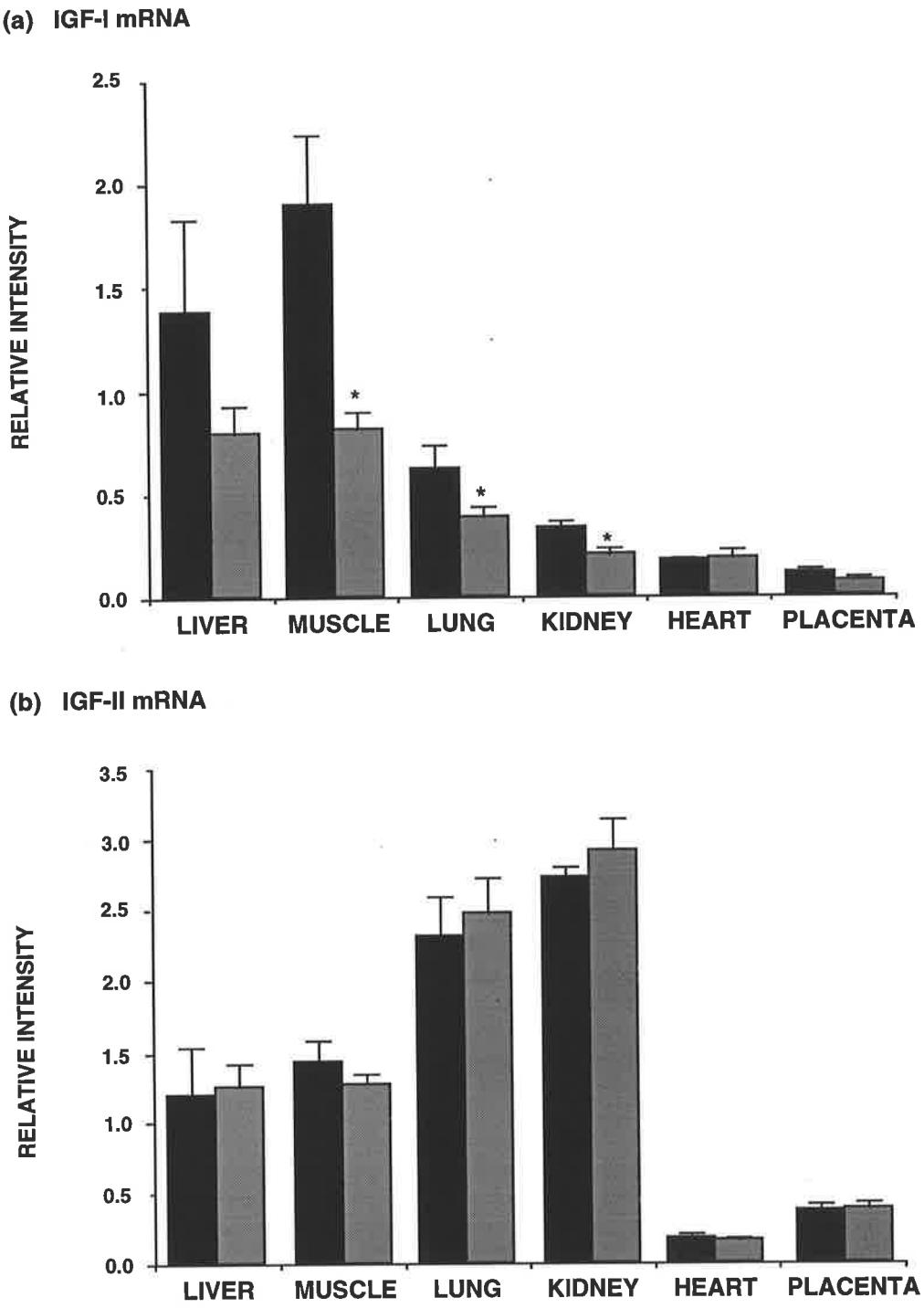


FIGURE 3.11 Relative abundance of IGF-I and -II mRNAs in tissues from fetal sheep with normal and restricted placental development at 121 days gestation.

(a) Relative abundance of IGF-I mRNA in fetal tissues from fetuses with normal (n=4, solid bars) and restricted (n=8, hatched bars) placental development. Results are expressed as the scanned intensity of fragments protected in an IGF-I ribonuclease protection assay relative to replicates of a control liver RNA. Values are mean ± SEM. * $P < 0.05$ compared to normal.

(b) Relative abundance of IGF-II mRNA in fetal sheep tissues. Results are expressed as the scanned intensity of fragments protected in an IGF-II ribonuclease protection assay relative to replicates of a within assay control liver RNA.

3.3.2 HEPATIC IGF-I AND -II GENE EXPRESSION

To further investigate the effect of restriction of placental and fetal growth on hepatic expression of IGF-I and -II in the fetus, RNA was extracted from the liver of an additional eight animals. Therefore, liver IGF-I and -II mRNA abundance was measured at 121 ± 2 days gestation in a total of eight normal fetal sheep and twelve fetuses from ewes with restricted placental growth.

Fetal growth, blood gases and metabolites

Placental weight ranged from 619 g to 156 g and correlated positively with fetal weight (r= 0.78, $P < 0.0001$, n=20). Fetal weight ranged from 1398 g to 2908 g. Placental weight correlated positively with the pO_2 of fetal arterial (r= 0.62, $P < 0.005$, n= 20) and umbilical venous (r= 0.49, $P < 0.05$, n= 17) blood. Placental weight was also positively associated with the concentration of glucose in fetal arterial plasma (r= 0.51, $P < 0.05$, n= 18). Similar positive associations were observed between fetal weight and fetal arterial blood pO_2 (r= 0.50, $P < 0.05$, n= 20) and arterial plasma glucose (r= 0.46, $P < 0.05$, n= 18).

Plasma IGF-I and IGF-II proteins

IGF-I concentrations in fetal blood ranged from 63 ng/ml to 176 ng/ml. Plasma IGF-I correlated positively with placental weight (r= 0.61, P< 0.006, n=19) and fetal weight (r= 0.63, P< 0.005, n=19) as expected from the previous study (Chapter 2). The concentration of IGF-I protein in fetal blood also correlated positively with fetal plasma glucose (r= 0.78, P< 0.0002, n= 17), with the pO_2 of fetal arterial blood (r= 0.56, P< 0.01, n=19) and with fetal arterial blood pH (r= 0.53, P< 0.02, n= 19). Plasma IGF-I was positively related to the weight of individual fetal tissues including, liver (r= 0.77, P< 0.0001, n=19), kidney (r= 0.71, P< 0.006, n=19), heart (r= 0.48, P< 0.04, n=19) and lung (r= 0.45, P< 0.05, n=19).

IGF-II protein in fetal blood ranged from 423 ng/ml to 931 ng/ml. Plasma IGF-II was not related to placental weight, fetal weight or the concentration of substrates in fetal blood.

Hepatic IGF-I and -II mRNA abundance: Relationship to fetal size

No associations were detected between hepatic IGF-I or -II mRNA and fetal or placental size. Placental restriction did not affect hepatic IGF-I mRNA or -II mRNA abundance (Table 3.5).

TABLE 3.5 Morphometry of fetal sheep from ewes with normal and restricted placental development.

	NORMAL (8)	PLACENTAL RESTRICTION (12)
Fetal weight (g)	2578 ± 126	2055 ± 72 *
Placental weight (g)	501 ± 35	285 ± 22 ***
Number of cotyledons formed	73 ± 2	27 ± 2 ***
Fetal weight/ placental weight	5.3 ± 0.4	7.6 ± 0.5 **
Liver weight (g)	113 ± 9	79 ± 4 **
Blood pO_2, arterial (torr)	20.5 ± 1.3	16.7 ± 0.9 *
Blood pH	7.34 ± 0.002	7.33 ± 0.006
Plasma IGF-I (ng/ml)	157 ± 7	121 ± 10 *
Plasma IGF-II (ng/ml)	663 ± 45	697 ± 45
Plasma glucose (mmol/l)	1.79 ± 0.1	1.39 ± 0.1 *
Liver IGF-I mRNA §	1.33 ± 0.19	1.12 ± 0.15
Liver IGF-II mRNA §	1.23 ± 0.17	1.22 ± 0.11

Values are mean ± SEM, *** $P< 0.0001$, ** $P< 0.005$, * $P<0.05$, compared to normal. One way analysis of variance (ANOVA) and Bonferroni Dunn. All measurements were made at 121 ± 2 days gestation.

§ The abundance of IGF-I or -II mRNA is expressed relative to the amount of IGF-I or -II mRNA detected in a normal fetal liver assay control sample. The abundance of IGF-II mRNA in normal fetal sheep liver is approximately 20 times higher than that of IGF-I mRNA (see page 3-28).

3.3.3 *Relationship between IGF mRNA abundance in fetal tissues and IGF protein levels in fetal blood*

The relative abundance of IGF-I mRNA in fetal liver, (r= 0.81, P< 0.0001, n= 19), quadriceps muscle (r= 0.74, P< 0.01, n=12) and kidney (r= 0.74, P< 0.01, n=11) correlated positively with the concentration of IGF-I protein in fetal blood (Figure 3.12). No associations were observed between IGF-I mRNA in other fetal tissues and IGF-I protein in fetal blood (lung, r= 0.5, n=11; placenta, r= 0.3, n=12; heart, r= -0.49, n=12).

No relationships were observed between IGF-II protein in blood and IGF-II mRNA in fetal tissues or placenta (Figure 3.12d).

The total content of IGF-I mRNA in fetal liver, estimated as described in Section 3.3.1 (Table 3.4), also correlated positively with the concentration of IGF-I protein in fetal blood (r= 0.73, P < 0.01, n=11). A weak association was observed between the total IGF-I mRNA content of placenta (Table 3.4) and plasma IGF-I (r= 0.55, P < 0.08, n=12). The total IGF-II mRNA content of liver or placenta was not related to the concentration of IGF-II protein in fetal blood.

3.3.4 *Relationship between IGF mRNA abundance in fetal tissues and fetal substrates.*

The relative abundance of IGF-I mRNA in fetal liver correlated with fetal arterial blood pO_2 (r= 0.52, P < 0.02, n= 19) (Figure 3.13), but was not related to the concentration of glucose in arterial blood plasma (Figure 3.14). IGF-I mRNA abundance in fetal liver also correlated positively with the pH of arterial blood (r=0.51, P < 0.03, n=19) (Figure 3.13).

IGF-I mRNA abundance in fetal muscle and lung correlated positively with fetal plasma glucose (muscle, r= 0.65, P< 0.02, n=12; lung, r= 0.71, P< 0.01, n=11) (Figure 3.15) and fetal blood pO_2 (muscle, r= 0.74, P< 0.01, n=12; lung, r= 0.73, P< 0.01, n=11) (Figure 3.16). IGF-I mRNA abundance in fetal muscle (r= 0.71, P< 0.01) and kidney (r= 0.72, P< 0.01) was positively associated with fetal blood pH.

No associations were observed between the abundance of IGF-II mRNA in fetal tissues and the concentration of glucose or oxygen in or the pH of fetal blood.

3.3.5 *Relationship between IGF mRNA abundance in fetal tissues and insulin concentrations in fetal blood.*

Sufficient plasma was available from sixteen of the twenty fetuses for analysis of insulin concentrations. Insulin concentrations in fetal arterial plasma ranged from 1204 pg/ml to 162 pg/ml. Insulin concentrations were not significantly altered by restriction of placental size (normal, 722 ± 132 ρg/ml (n=6), placental restriction, 564 ± 119 ρg/ml (n=10), mean ± SEM). Plasma insulin was not associated with fetal weight, placental weight, fetal arterial plasma glucose or blood pH. However, plasma insulin was positively related to fetal arterial blood pO_2 (r= 0.72, P< 0.04, n= 16). Insulin concentrations in arterial plasma were related to plasma IGF-I (r= 0.54, P< 0.03) and to the abundance of IGF-I mRNA in fetal liver (r= 0.57, P< 0.02) (Figure 3.14). No associations were observed between plasma insulin and IGF-I expression in other fetal tissues, or IGF-II gene expression in any fetal tissue.

3.3.6 *Analysis of IGF binding proteins in fetal plasma*

Western ligand blot analysis (Figure 3.17) revealed up to seven size forms of IGFBPs in fetal sheep plasma with apparent molecular weights of 25, 29-30, 34, 43, 49 and >200 kDa, as previously reported (Osborn *et al.*, 1992; McLellan *et al.*, 1992). The 43 and 49 kDa forms are IGFBP-3 and the 34 kDa species is IGFBP-2 (McLellan *et al.*, 1992; Lord *et al.*, 1994). The 25 kDa form most likely represents oIGFBP-4 (Lord *et al.*, 1994; Carr *et al.*, 1994). IGFBP-1 antiserum cross reacts with a 31 kDa IGFBP in lamb plasma (Lord *et al.*, 1994), while IGFBP-4 antiserum also cross reacts with a 29 kDa protein (Lord *et al.*, 1994; Carr *et al.*, 1994). Therefore the 29-30 kDa species, which appeared as an unresolved doublet in this study, most likely represents the N-glycosylated form of IGFBP-4 as well as IGFBP-1. The >200 kDa protein corresponds to the circulating type 2 IGF / mannose 6-phosphate receptor. IGFBP-3 and the type 2 receptor appear to be major forms of IGFBP in fetal sheep blood at this gestation, while the putative IGFBPs 1, 2 and 4 appear to be minor forms, according to

their intensity on Western ligand blot. The amount of all binding proteins appeared variable in fetuses with normal and restricted placental development, however, no attempt was made to quantitate these IGFBPs by scanning densitometry.

Concentrations of IGFBP-3 were analysed by radioimmunoassay in fetal sheep plasma. IGFBP-3 levels ranged from 1.27 to 2.73 μg/ml. Plasma IGFBP-3 levels were not significantly different in fetuses with restricted placental size (normal, 2.17 ± 0.18 μg/ml, (n=6); placental restriction, 1.72 ± 0.14 μg/ml, (n=10), mean ± SEM). However, plasma IGFBP-3 correlated positively with fetal size (r= 0.63, P< 0.01, n= 18) and plasma IGF-I (r= 0.63, P< 0.01, n= 18). IGFBP-3 concentrations were not related to plasma IGF-II or to the total plasma concentration or molar concentration of IGF-I and -II.

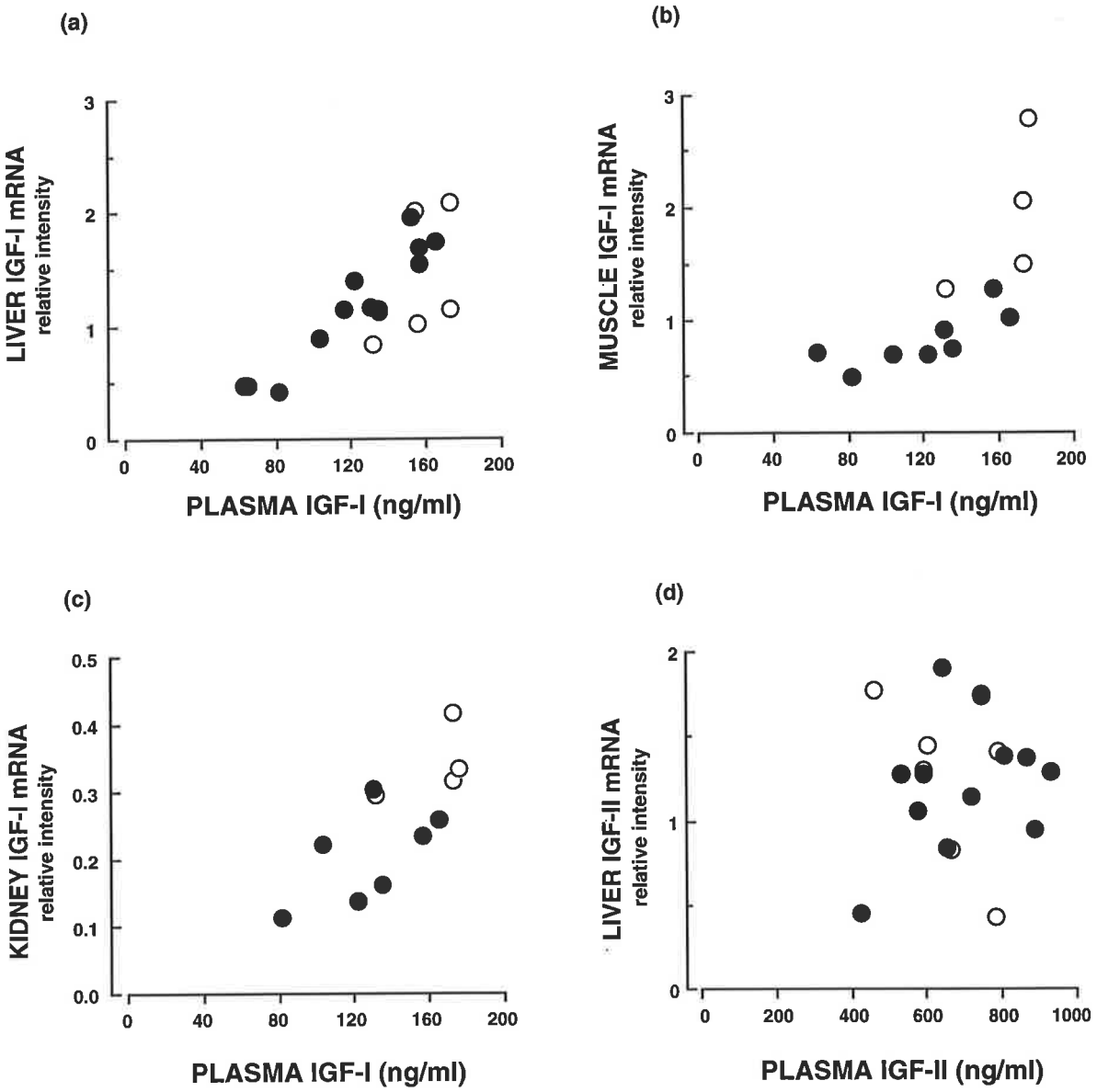


FIGURE 3.12 Relationships between IGF proteins in blood and relative IGF mRNA abundance in fetal tissues at 121 days gestation.

Correlations of the relative abundance of

(a) IGF-I mRNA in liver (r= 0.81, $P < 0.0001$, n= 19),

(b) IGF-I mRNA in skeletal muscle (r= 0.74, $P < 0.01$, n= 12),

(c) IGF-I mRNA in kidney (r= 0.74, P < 0.01, n=11),

with concentrations of IGF-I in plasma from fetuses of (○) normal and (●) placentally restricted ewes.

(d) Correlation of the relative abundance of IGF-II mRNA in fetal liver with the concentration of IGF-II in fetal plasma (NS, n=19).

IGF mRNA abundance is expressed relative to a within assay control liver RNA sample. All results represent the mean of two analyses, corrected to the within assay control.

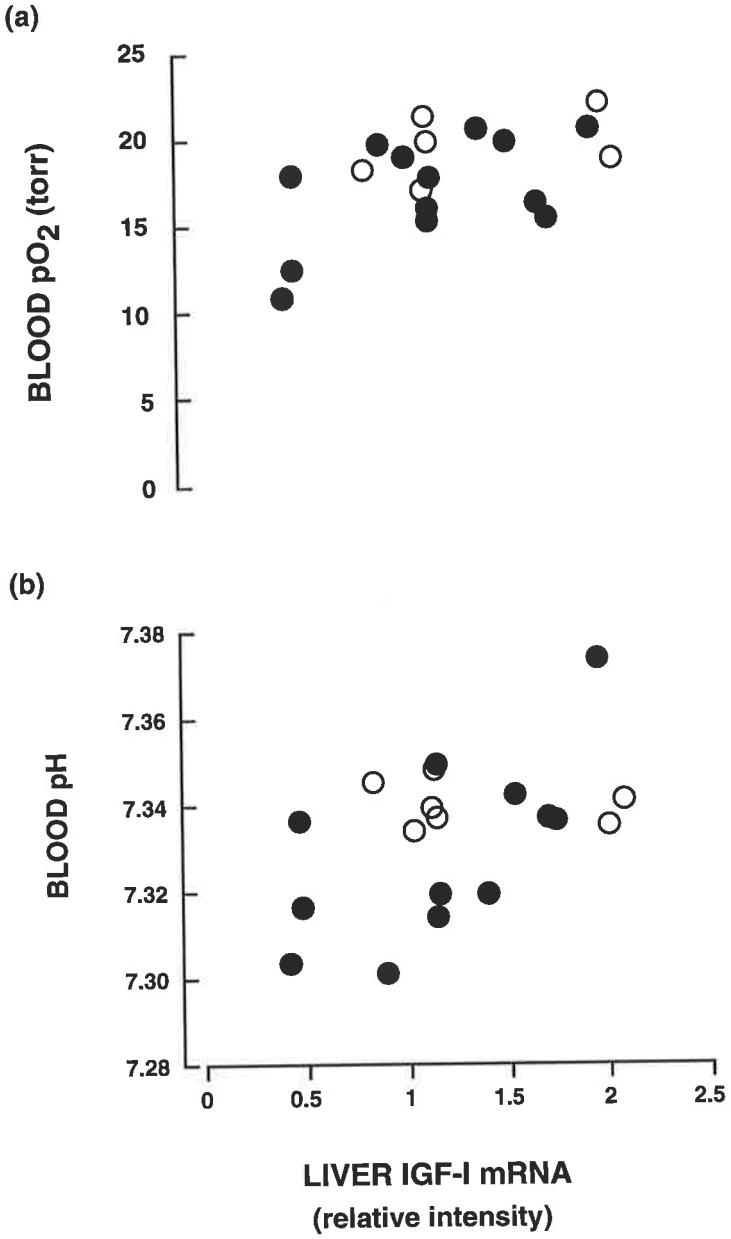


FIGURE 3.13 Relationship between the abundance of IGF-I mRNA in total RNA from fetal liver and fetal arterial blood pO_2 and pH at 121 days gestation.

Correlations of

(a) pO_2 of fetal arterial blood (r= 0.52, P< 0.02, n= 19) and,

(b) pH of fetal arterial blood (r= 0.51, P< 0.03, n= 19)

with the relative abundance of IGF-I mRNA in liver of fetal sheep with normal (O) and restricted (●) placental development.

IGF-I mRNA is expressed relative to the amount of IGF-I mRNA measured in a fetal liver RNA sample that was included in all assays.

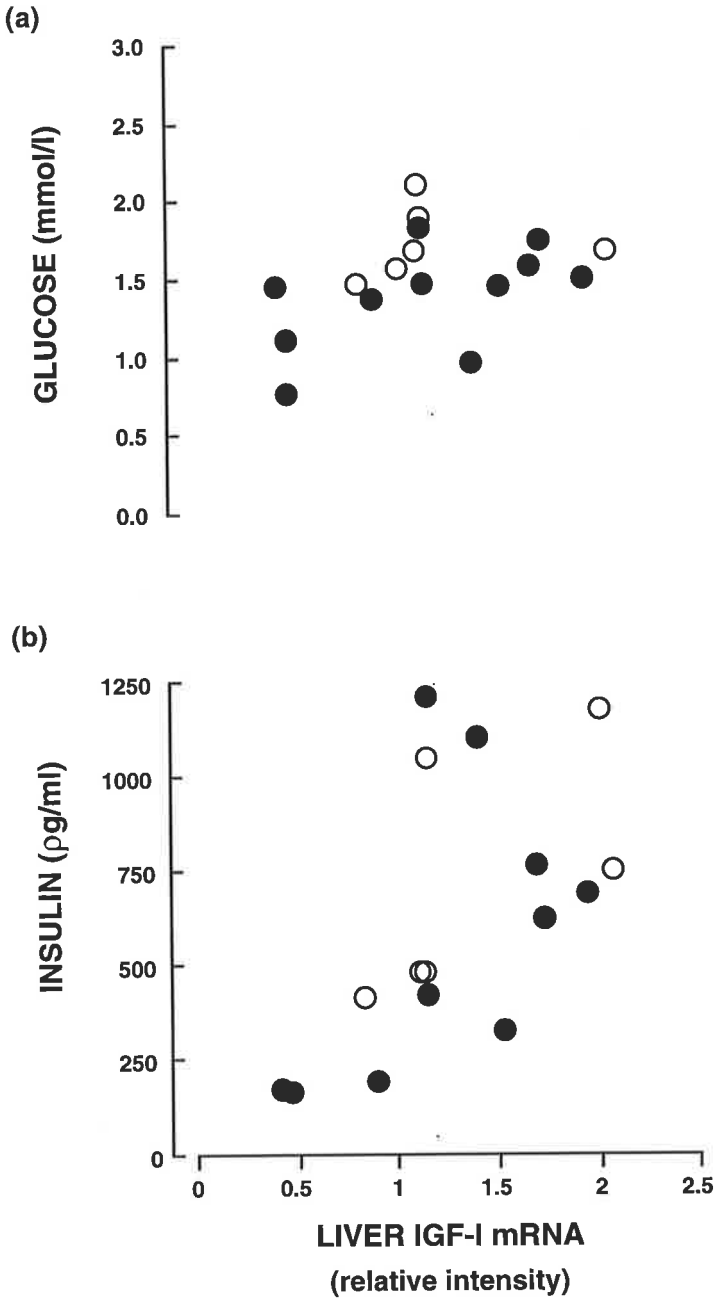


FIGURE 3.14 Relationship between the abundance of IGF-I mRNA in total RNA from fetal liver and fetal arterial plasma glucose and insulin at 121 days gestation.

Correlations of

(a) fetal plasma glucose concentration (NS) and,

(b) fetal plasma insulin concentration (r= 0.57, P< 0.02, n= 19)

with the relative abundance of IGF-I mRNA in liver of fetal sheep with normal (O) and restricted (●) placental development.

IGF-I mRNA is expressed relative to the amount of IGF-I mRNA measured in a fetal liver RNA sample that was included in all assays.

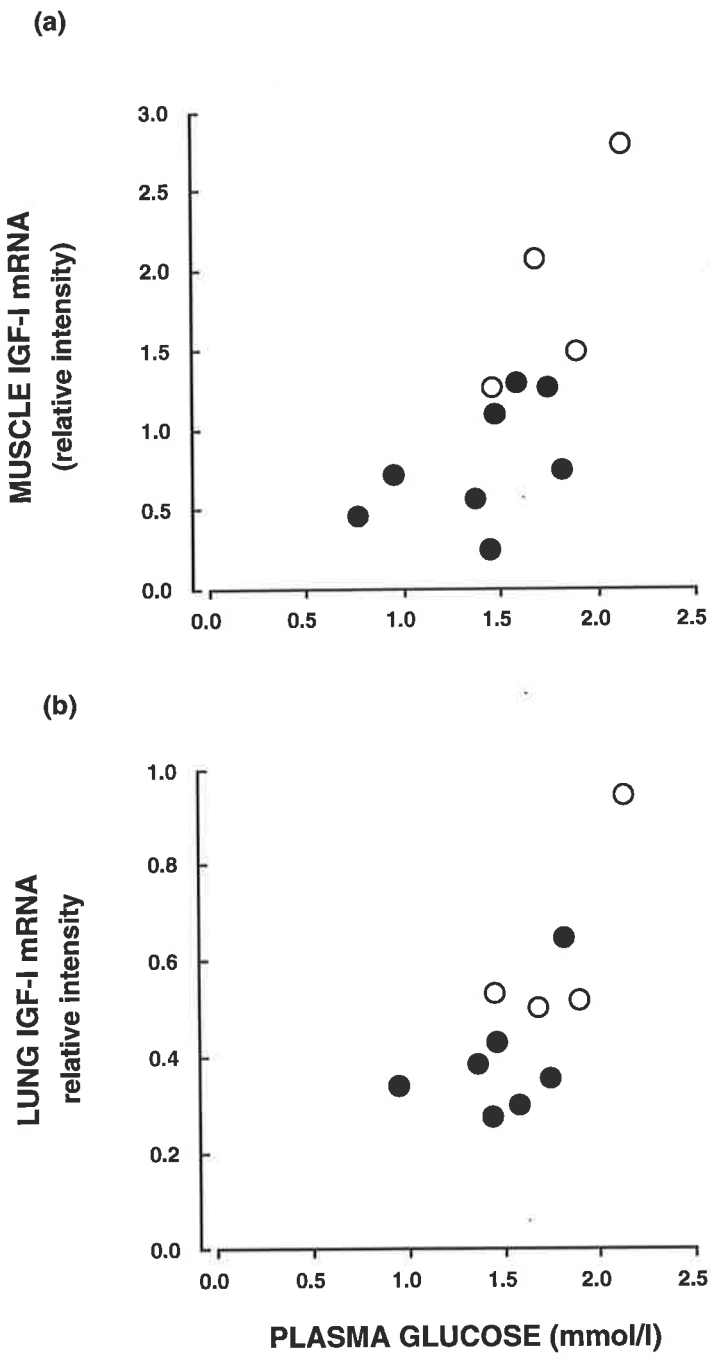


FIGURE 3.15 Relationship between fetal arterial plasma glucose and the relative abundance of IGF-I mRNA in fetal skeletal muscle and lung at 121 days gestation.

Correlations of

(a) Relative abundance of IGF-I mRNA in fetal muscle (r= 0.65, P< 0.02, n= 12) and,

(b) Relative abundance of IGF-I mRNA in fetal lung (r=0.71, P< 0.01, n=11)

with fetal arterial plasma glucose with in fetal sheep with normal (O , n=4) or restricted (●, n=8) placental development.

IGF-I mRNA is expressed relative to the amount of IGF-I mRNA measured in a fetal liver RNA sample that was included in all assays.

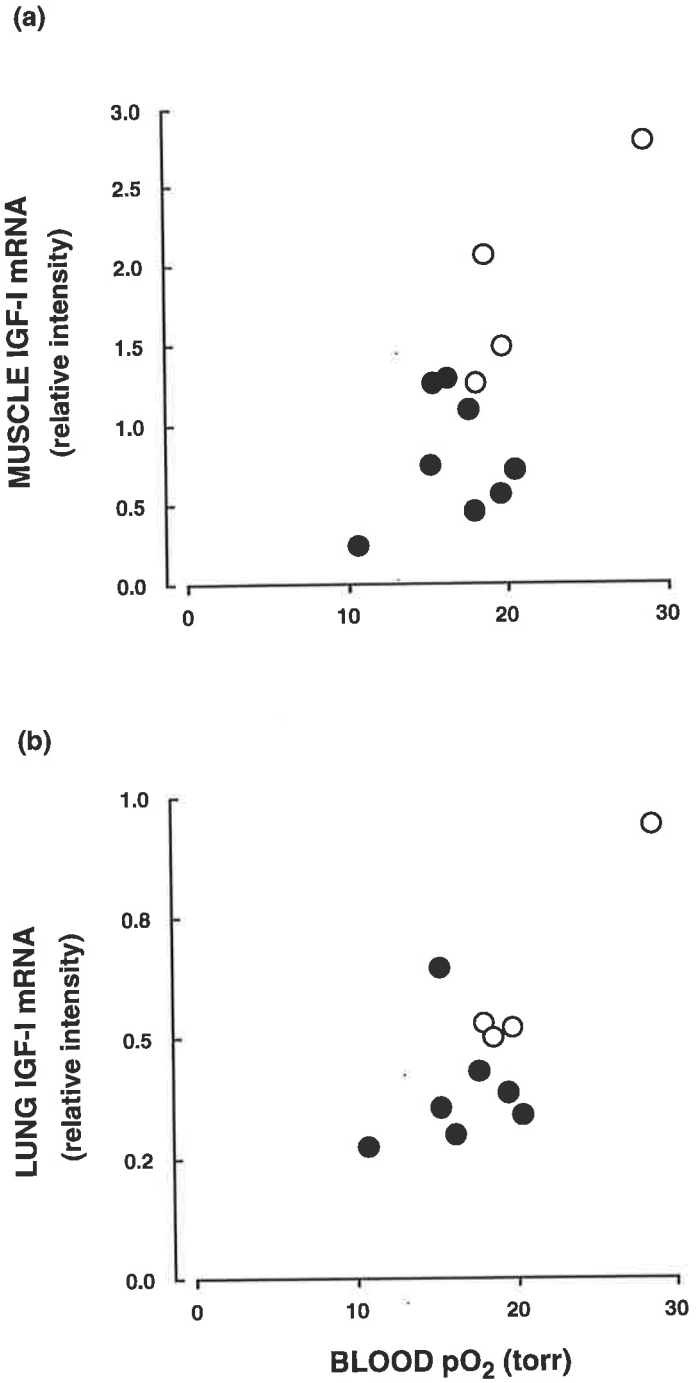


FIGURE 3.16 Relationship between fetal arterial blood pO_2 and the relative abundance of IGF-I mRNA in fetal skeletal muscle and lung at 121 days gestation.

Correlations of

(a) Relative abundance of IGF-I mRNA in fetal muscle (r= 0.74, P< 0.01, n= 12) and,

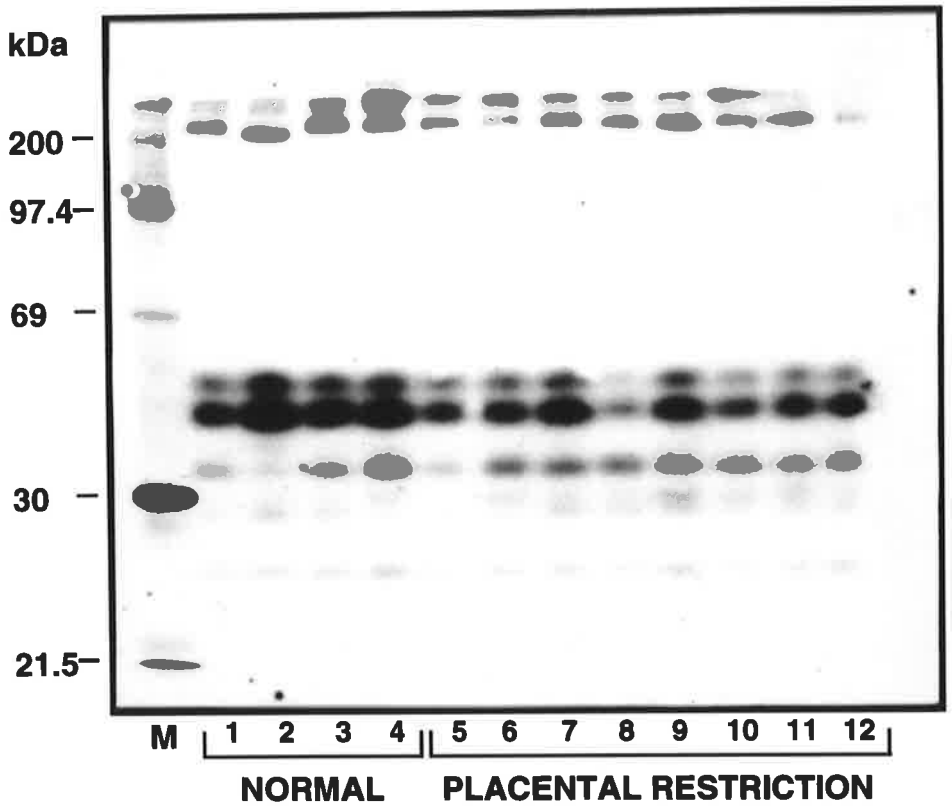
(b) Relative abundance of IGF-I mRNA in fetal lung (r=0.73, P< 0.01, n=11)

with arterial blood pO_2 in fetal sheep with normal (O , n=4) or restricted (●, n=8) placental development.

IGF-I mRNA is expressed relative to the amount of IGF-I mRNA measured in a fetal liver RNA sample that was included in all assays.

FIGURE 3.17 Western ligand blot analysis of IGF binding proteins in fetal sheep plasma with normal and restricted placental development at 121 days gestation.

Western ligand blot analysis of IGF binding proteins in plasma from fetal sheep with normal (lanes 1-4) and restricted (lanes 5-12) placental development. Each lane contains 2.5 µl of fetal sheep plasma. The sizes (kDa) of ^{14}C labelled molecular weight markers (M) is shown on the left. The nitrocellulose was probed with [^{125}I]-labelled IGF-II and exposed to X-ray film for 7 days at -70°C with intensifying screens.


kDa
200
97.4
69
30
21.5
M
1 2 3 4
5 6 7 8 9 10 11 12
NORMAL
PLACENTAL RESTRICTION

3.4 DISCUSSION

Major tissue sites of expression of IGF-I and -II in fetal sheep at 121 days gestation were identified in this study and abundance of IGF-I and -II in these tissues was quantitated. Other reports have identified high levels of expression of IGF-I in skeletal muscle at 84 days of gestation in fetal sheep, when compared to liver, kidney, lung, spleen and smooth muscle (Dickson *et al.*, 1991). However, by 134 days of pregnancy Dickson *et al.* (1991) found that fetal liver was the predominant site of IGF-I expression. Yang *et al.* (1991) also suggest liver and lung as major sites of IGF biosynthesis in late gestation fetal sheep, however, skeletal muscle was omitted from their study.

Of the fetal sheep tissues examined in the current study, the relative abundance of IGF-I mRNA was highest in liver and skeletal muscle at 121 days of gestation. Absolute amounts of IGF-I mRNA in tissues were estimated by considering tissue mass (Figure 3.9). However, skeletal muscle weights were not collected in the current study. Skeletal muscle represents approximately 25% of weight in normal sheep at birth (Butterfield, 1988). Therefore, although the amount of total RNA per gram of muscle tissue is around half that of liver in fetal sheep at this gestation (Dr. F. Lok, Dept. Obstetrics & Gynaecology, University of Adelaide, personal communication), skeletal muscle is potentially a major site of IGF-I production in the sheep fetus, in addition to liver.

IGF-II mRNA abundance was higher than that of IGF-I in all fetal sheep tissues studied, apart from brain, consistent with previous reports (Yang *et al.*, 1991; McLellan *et al.*, 1992). In fetal cerebellum, IGF-I mRNA was present in low levels, but no IGF-II mRNA was detected. Delhanty & Han (1993) also report that the IGF-II gene is expressed only in choroid plexus in the fetal sheep brain.

IGF-II mRNA abundance was highest in fetal lung and kidney, followed by skeletal muscle and liver, consistent with previous reports (O'Mahoney *et al.*, 1991; Delhanty & Han, 1993; Li *et al.*, 1993). IGF-II mRNA has also been detected in the fetal sheep adrenal in high concentrations (Delhanty & Han, 1993). Absolute abundance of IGF-II mRNA was also

estimated by correction for tissue mass (Figure 3.9). Placenta and lung were implicated as quantitatively major sites of IGF-II production. In addition, if skeletal muscle weight is estimated as 25% of fetal body weight as discussed, muscle is also potentially a major site of IGF-II synthesis.

Fetal weight correlated positively with placental size at 121 days gestation in the current study, as observed at 130 days gestation in the preceding study (Chapter 2). Since fetal and placental weights were obtained at 121 days gestation, in contrast to 130 days in the previous study (Chapter 2), direct comparison of the extent of growth restriction cannot be made. However, in the previous study (Chapter 2), blood pO_2 was reduced by around 18% and glucose levels by 27% when all growth-retarded fetuses are combined at 120 days gestation. Therefore, overall the extent of substrate restriction observed at 120 days gestation in Chapter 2 is similar to the 19% decrease in blood pO_2 and 22% decrease in plasma glucose observed at 121 days gestation in this study (Chapter 3). However, the absolute levels of glucose in normal and restricted fetuses in the current study are higher than those described in Chapter 2, even after correcting for haematocrit. This suggests that maternal condition or body energy stores, and hence fetal nutrition, may have been better during the season in which the current study was performed.

Consistent with the higher basal glucose levels in the fetus in the current study (Chapter 3), plasma levels of IGF-I are also higher on average than in the preceding study (Chapter 2) (157 ± 7 ng/ml v 140 ± 9 ng/ml in normal fetal sheep). However, as observed in the previous study (Chapter 2), the concentration of IGF-I in fetal blood, but not that of IGF-II, was lower at 121 days gestation, in fetal sheep with restricted placental size. The results of the current study indicate that the relative abundance of IGF-I mRNA in skeletal muscle, kidney and lung is reduced in fetal sheep whose placental growth is limited. Restriction of placental and fetal growth did not change IGF-I mRNA abundance in placenta, heart or liver. IGF-I mRNA in liver, muscle and kidney correlates positively with the concentration of IGF-I protein in fetal blood. Thus, low circulating IGF-I is accompanied by reduced production of IGF-I in skeletal muscle, lung and kidney. In contrast, IGF-II expression in fetal tissues and IGF-II

protein in fetal blood were not altered by restriction of placental size, the latter being consistent with the earlier study (Chapter 2).

The positive associations of IGF-I mRNA in liver, muscle and kidney with the concentration of IGF-I protein in blood, supports the view that IGF-I in fetal blood may originate from a number of tissue sites. No distinction could be made between the potential contribution of each of these tissues to plasma IGF-I using partial correlation and multiple regression statistical analysis. However, larger organs such as liver and muscle are implicated as major sites of IGF-I production, whereas kidney is a smaller tissue with a lower IGF-I mRNA abundance compared to liver and muscle, suggesting that any contribution of IGF-I produced in the kidney to circulating IGF-I would be minor. Postnatally, liver is the major site of IGF-I expression in the sheep and appears to be the major source of circulating IGF-I (Pell *et al.*, 1993). Both the relative abundance of hepatic IGF-I mRNA and the estimated total content of IGF-I mRNA in fetal liver correlate positively with IGF-I protein in fetal blood. Given the decrease in liver weight associated with restriction of placental size in the current study, and the positive association of hepatic IGF-I mRNA with plasma IGF-I protein, it is possible that the decrease in circulating IGF-I could be accounted for by altered hepatic expression alone. Nevertheless, previous reports indicate that weights of individual skeletal muscles are significantly reduced in growth-retarded fetal sheep (Alexander, 1974) suggesting that total production of IGF-I by skeletal muscle may also be substantially reduced following placental restriction of fetal growth.

As in the preceding study (Chapter 2), plasma IGF-I correlates positively with fetal weight, consistent with the view that circulating IGF-I may be involved in the endocrine regulation of fetal growth (Chapter 2). However, the decrease in IGF-I gene expression observed in several fetal tissues (Chapter 3), when fetal growth is restricted, also suggests that altered local expression and actions of IGF-I may contribute to retarded fetal growth. IGF-I mRNA abundance in fetal kidney and liver correlated positively with the weights of these organs. However, as observed previously (Chapter 2), the weight of these tissues was also positively associated with the concentration of IGF-I in fetal blood. Since, infusion of IGF-I into the

late gestation fetus promotes the growth of these organs (Lok *et al.*, 1994), endocrine actions of IGF-I on the growth of these tissues cannot be excluded.

In addition to the positive association of plasma IGF-I with arterial blood pO_2 and with circulating glucose concentrations in the fetus (Chapters 2 & 3), a positive association of plasma IGF-I with plasma insulin and with blood pH was observed in the current study (Chapter 3). Therefore, restriction of substrate supply may regulate fetal IGF-I production through more than one mechanism. Insulin, but not glucose, correlated positively with hepatic IGF-I mRNA, suggesting that regulation of hepatic IGF-I production by glucose availability may be mediated by insulin. A role for insulin in the regulation of fetal IGF-I production is supported by the observation that pancreatectomy of fetal sheep reduces plasma insulin, elevates blood glucose and lowers plasma IGF-I (Gluckman *et al.*, 1987). Short term restriction of uterine blood flow lowers fetal blood pO_2 and reduces IGF-I mRNA abundance in fetal liver, but does not alter plasma glucose or insulin (McLellan *et al.*, 1992), indicating that oxygen availability can regulate hepatic IGF-I gene expression independently of insulin in fetal sheep. However, partial correlation analysis indicated that the relationships of oxygen and insulin with hepatic IGF-I expression were not independent in the present study. Furthermore, in contrast to previous studies (Robinson *et al.*, 1980), plasma insulin concentrations were not directly related to fetal plasma glucose in this study. It is possible that other factors, such as catecholamines, which are increased in hypoxaemic and hypoglycaemic fetal sheep (Robinson *et al.*, 1985), are inhibiting insulin secretion and altering the relationship between glycaemic state and circulating levels of insulin in the restricted fetus.

Fetal blood pH also correlated positively with fetal hepatic IGF-I mRNA. However, partial correlation analysis showed that the relationship of pH to IGF-I mRNA in fetal liver was not independent of the influence of oxygen. Blood pH is determined by the balance between fetal oxygen supply and nutrient availability, and this relationship may be secondary to the association of hepatic IGF-I mRNA with blood pO_2. No relationship was observed between fetal blood pH and circulating IGF-I in the fetal sheep in Chapter 2. The fall in blood pH in the current study (Chapter 3) may be a consequence of the interaction between their hypoxaemia and higher prevailing glucose levels compared to the earlier study (Chapter 2).

Associations were also observed between IGF-I mRNA in extrahepatic tissues and substrate concentrations in fetal blood, but these were different from those seen with liver. IGF-I mRNA abundance in skeletal muscle and lung was related to oxygen and glucose availability and partial correlation analysis indicated that the effects of glucose and oxygen on muscle and lung IGF-I mRNA were independent. In further contrast to liver, IGF-I mRNA abundance in these tissues was not related to plasma insulin concentration. Therefore, altered glucose availability may regulate IGF-I expression in these tissues, by a different mechanism than in liver. A positive association between fetal arterial blood pH and IGF-I mRNA in muscle and kidney was also observed, which was independent of the effects of oxygen and glucose. This suggests that pH, or determinants of pH such as lactate, may regulate IGF-I production in kidney.

In contrast to IGF-I mRNA, IGF-II mRNA was not altered in tissues of fetal sheep made growth-retarded by restriction of placental size. This suggests that IGF-II gene expression is less sensitive to restriction of oxygen and nutrient supply *in utero* than IGF-I gene expression. The lack of association of both fetal growth and nutrient supply with IGF-II gene expression, agrees with previous reports of unchanged IGF-II mRNA in fetal rat liver following maternal starvation (Davenport *et al.*, 1990; Straus *et al.*, 1991b). In contrast, liver IGF-II mRNA is increased in fetal rats following uterine artery ligation in late pregnancy (Price *et al.*, 1992a). Hepatic IGF-II mRNA is increased at 135 days gestation in fetal sheep by maternal underfeeding from early pregnancy and by insulin-induced maternal hypoglycaemia from mid-pregnancy (Townsend *et al.*, 1992a,b), while fetal kidney IGF-II mRNA is reduced following infusion of insulin into the pregnant ewe (Townsend *et al.*, 1992a). Maternal underfeeding throughout pregnancy reduced fetal weight by 15% and fetal blood glucose by 24%, a similar degree of restriction to that achieved in the current study. However, restriction of placental size is also associated with a reduction in fetal oxygenation in sheep. Thus, as discussed previously (Chapter 2), whether oxygen alone, nutrients alone or both in combination are restricted may be important in determining the effect on IGF-II gene expression.

Furthermore, the gestational age at which nutrients become limiting may be important in relationship to developmental control of IGF-II gene expression. Short-term starvation of the pregnant ewe at 140 days gestation reduces IGF-II mRNA in fetal liver (Li *et al.*, 1993). This is consistent with the decrease in IGF-II protein levels in fetal blood which occurs very late in gestation in fetuses with restricted placental size and limited substrate supply (Chapter 2). The fetal endocrine response to restricted nutrient supply may be a determinant of the effect of restriction on IGF-II gene expression. As discussed previously, fetal blood cortisol has been shown to negatively regulate hepatic IGF-II expression (Li *et al.*, 1993). At 140 days gestation maternal fasting is associated with an increase in cortisol in fetal blood, and the decrease in hepatic IGF-II expression is related to the elevated cortisol concentrations (Li *et al.*, 1993). However, when nutrient supply is limited earlier in gestation, or with less severe maternal undernutrition, fetal blood cortisol concentrations are not increased or increase to a much lesser extent than that observed in very late gestation (Bassett & Maddill, 1974; Fowden & Silver, 1985) and IGF-II expression may not be influenced by cortisol under these conditions. In contrast, chronic fetal hypoxaemia can elevate blood cortisol concentrations from around 120 days of gestation in sheep (Jacobs, 1987). As discussed in Chapter 2, the cortisol surge occurs prematurely in growth-retarded fetal sheep with small placentas and may account for the decrease in fetal plasma IGF-II in severely growth retarded fetal sheep at 127 days gestation (Robinson *et al.*, 1980). However, at 120 days gestation cortisol levels may not be elevated to an extent that will result in a significant alteration in IGF-II protein (Chapters 2 & 3) or IGF-II mRNA.

No relationships were observed between IGF-II mRNA in fetal tissues and IGF-II protein in fetal blood. IGF-II protein in blood may be produced by a number of tissues in the sheep fetus, including some not examined in this study (Delhanty & Han, 1993). Production of IGF-II may also be regulated at a post transcriptional level (Haselbacher *et al.*, 1987; De Moor *et al.*, 1994). A lack of association between IGF-II protein and mRNA abundance has been reported in cultured carcinomas (Haselbacher *et al.*, 1987). In human fetal liver, a proportion of the largest 6.0 kb IGF-II mRNA is not associated with polysomes and appears not to be translated (De Moor *et al.*, 1994). The 6.0 kb IGF-II mRNA form is abundant in

fetal sheep tissues (Delhanty & Han, 1993). Thus analysis of tissue levels of IGF-II protein synthesis may be required to assess IGF-II production by individual fetal tissues.

Among the factors known to modulate both the circulating concentration of IGFs and the interactions of IGFs with IGF receptors, and thus their biological actions, are the IGF binding proteins (IGFBPs) (1.2.4). IGFBP-3 is the major species of IGFBP in fetal sheep blood, as previously reported (Delhanty & Han 1993) and is also the predominant IGFBP in the blood of late gestation human fetuses (Crystal & Giudice 1991, Fant *et al.*, 1993). As observed in the fetal sheep in this study, IGFBP-3 concentrations in human fetal blood correlate positively with birthweight and fetal plasma IGF-I in the human fetus (Fant *et al.* 1993). In contrast to postnatal life, where IGFBP-3 concentrations are related to the total amount of IGF-I and -II proteins in the circulation (Baxter & Martin 1986; Baxter 1988; Owens *et al.*, 1991), no associations were observed between IGFBP-3 and plasma IGF-II in the sheep fetus in the present study. However, other IGF binding proteins, including the circulating type 2 receptor, may carry significant proportions of the IGF-II protein *in utero* (Gallaher *et al.*, 1992).

Variable levels of the smaller molecular weight IGFBPs were observed in the blood of fetuses with normal and restricted placental development by western ligand blot analysis. Blood levels and gene expression of IGFBP-1 are increased in growth retarded fetal rats and humans (Crystal & Giudice, 1991; Straus *et al.*, 1991b; Price *et al.*, 1992a,b), suggesting that IGFBP-1 may act to inhibit the actions of the IGFs when fetal growth is limited. IGFBP-1 increases rapidly when fetal sheep are made hypoglycaemic (Osborn *et al.*, 1992), hypoxaemic (McLellan *et al.*, 1992) or are infused with catecholamines (Hooper *et al.*, 1994), suggesting that substrate supply and/or associated endocrine changes regulate IGFBP-1 production *in utero*. Increased levels of IGFBP-2 have been detected in plasma of fetal sheep at 120 days gestation, following restriction of placental size (Carr, 1995) and IGFBP-2 is elevated in chronically growth-retarded human fetuses (Crystal & Giudice, 1991). Further studies are required to assess the effect of placental restriction of substrate supply, in sheep, on production and circulating levels of these IGFBPs. These will require more specific assays than western ligand blotting.

3.5 SUMMARY

• In summary, IGF-I and -II genes are expressed in a number of fetal sheep tissues in late gestation. Liver and skeletal muscle contain the highest relative abundance of IGF-I mRNA. IGF-II gene expression is highest in kidney and lung, followed by liver and skeletal muscle. On a weight basis skeletal muscle and liver are indicated as major sites of IGF-I synthesis, while skeletal muscle, placenta and lung are suggested as major sites of IGF-II production.

• IGF-I gene expression in several major fetal organs and IGF-I protein levels in fetal blood are reduced following restriction of placental size in sheep. IGF-I mRNA abundance in liver, muscle and kidney correlated positively with the concentration of IGF-I protein in fetal blood. Since liver and muscle are major sites of IGF-I production in the sheep fetus, these tissues are suggested as likely major sources of circulating IGF-I. In contrast, IGF-II protein in blood and IGF-II mRNA in fetal tissues were not altered by restriction of placental size.

• IGF-I mRNA in fetal liver correlated positively with fetal arterial blood pO_2, suggesting oxygen supply as a potential regulator of hepatic IGF-I expression in the sheep fetus. Plasma insulin, but not plasma glucose, was also related to hepatic IGF-I mRNA abundance. Therefore insulin may mediate the effects of glucose availability on hepatic IGF-I gene expression. No relationship was observed between IGF-II mRNA in any tissue and concentrations of substrates in fetal blood.

• The results of this study suggest that altered production of IGF-I, but not IGF-II, at a number of tissue sites in the fetus may contribute to retarded fetal growth when the placental supply of substrates is limited, through altered local actions and/or as a consequence of reduced circulating levels and thus through endocrine mechanisms.

CHAPTER 4

THE EFFECT OF INTRAVENOUS INFUSION OF IGF-I ON EXPRESSION OF IGF-I AND IGF-II GENES IN LIVER AND SKELETAL MUSCLE OF FETAL SHEEP.

4.1 INTRODUCTION

The highest levels of expression of IGF-I in tissues of the fetal sheep are normally found in skeletal muscle and liver (Chapter 3). The abundance of IGF-I mRNA in skeletal muscle and liver also correlates positively with the concentration of IGF-I protein in fetal blood. Thus liver and kidney are suggested as major sites of IGF-I production and potential sources of circulating IGF-I in the ovine fetus.

Postnatally, the liver is the major source of circulating IGF-I in adult rats (Schwander *et al.*, 1983; Murphy *et al.*, 1987a). Administration of IGF-I to malnourished or growth hormone deficient rats (Schalch *et al.*, 1989; Butler *et al.*, 1994; Gosteli-Peter *et al.*, 1994) reduces hepatic IGF-I mRNA. This suggests that, in conditions where the growth hormone axis is not operative, IGF-I can regulate hepatic IGF-I production. Before birth, the role of growth hormone in the regulation of IGF-I production has been inferred to be minor in sheep (Mesiano *et al.*, 1989) and circulating growth hormone concentrations in the fetus are high indicating prenatal life as a state of growth hormone insensitivity. Thus, it is possible that production of endocrine IGF-I in the fetus may be regulated by negative feedback mechanisms, as occurs postnatally.

In addition, IGF-I is suggested as a potential regulator of IGF-II gene expression. IGF-I treatment reduces IGF-II mRNA abundance of differentiating rat and human myoblasts *in vitro* (Magri *et al.*, 1994).

The ***specific aim*** of this study was to determine whether IGF-I regulates IGF-I and -II gene expression *in vivo* before birth.

IGF-I and -II mRNAs were therefore measured in liver and muscle from fetal sheep following ten days intravenous infusion of IGF-I.

4.2 MATERIALS AND METHODS

4.2.1 SURGICAL PROCEDURES

Twelve pregnant Merino ewes of known mating dates carrying singleton fetuses were studied. Vascular catheters were inserted as described in Section 2.2.1 with the modifications described in Section 3.2.1. Catheter patency was maintained as described in Section 3.2.1.

4.2.2 EXPERIMENTAL DESIGN

Recombinant human IGF-I (GroPep Pty. Ltd.) was dissolved at a concentration of 0.42 mg/ml in 0.9% sterile saline containing 10 g/l bovine serum albumin (Sigma Chemical Co., RIA grade). At 120 days gestation infusion of IGF-I (n=6) or saline (n=6) into the fetal tarsal vein was commenced at 0900 h. Continuous infusion at a rate of 0.195 ml /h or 81 µg/h was maintained for ten days. Based on fetal weights measured at 130 days gestation this is equivalent to an average rate of 26 ± 4 µg /h /kg body weight (mean ± SD). Blood samples (2.5 ml) were collected from the fetal femoral artery on days 8, 9 and 10 of treatment. On day 9, blood samples were collected at 0830, 0845 and 0900 h. On days 8 and 10 a single sample was collected at 0900 h. Samples were treated as described in Section 2.2.2 and plasma was recovered and stored at -20°C.

At 130 days gestation, the ewe and fetus were sacrificed by maternal intravenous overdose of sodium phenobarbitone (Lethobarb, Arnolds of Reading). Fetal weight, placental weight and fetal organ weights were recorded and samples of tissues were collected for RNA analysis (3.2.2).

4.2.3 BLOOD GASES AND METABOLITES

Blood gases were measured as described (2.2.3). The blood gas results reported in this chapter are the mean results of samples collected on days 8, 9 and 10 of treatment.

Glucose concentrations in arterial plasma were measured as described (3.2.3). All samples collected from a single animal were analysed in one assay. The intra- and inter- assay co-efficients of variation, assessed by repeat analysis of a diagnostic quality control sample (Precinorm, Roche Diagnostics Systems) were 4% and 4.7% respectively. The results presented are the mean value of measurements made in samples collected on days 8, 9 and 10 of treatment.

4.2.4 PLASMA IGF-I AND IGF-II PROTEINS

IGF-I and -II proteins were measured in plasma samples collected from the fetal femoral artery on day 10 of IGF-I infusion. IGF-I and -II were measured by radioimmunoassay, in acid gel chromatographed plasma exactly as described in Section 3.2.4. All samples were analysed in triplicate within a single assay. The minimum detectable concentrations were 5 ng/ml plasma for IGF-I and 12 ng/ml plasma for IGF-II. The within assay variation, assessed by analysis of a standard reference plasma sample in quadruplicate, was 4% for both assays.

4.2.5 MEASUREMENT OF IGF-I AND IGF-II mRNA IN FETAL TISSUES

Total RNA extraction

Total RNA was isolated from 1 g portions of fetal liver and quadriceps muscle as described in Section 3.2.5. Total RNA extracted from normal fetal sheep liver at 130 days gestation was pooled as an assay control RNA.

Solution hybridisation / ribonuclease protection assay

Ribonuclease protection assays of IGF-I and IGF-II mRNA in fetal tissues were performed as described in Section 3.2.5. Fifty µg total RNA was analysed in IGF-I mRNA assays and 25 µg total RNA was analysed in IGF-II assays. Control liver RNA was analysed in quadruplicate. The specific activity of the antisense IGF-I and -II RNAs ranged from 4×10^8 to 1×10^9 cpm/µg. Dried gels were exposed to X-ray film with intensifying screens for 15 h to 24 h (IGF-II assays) and 10 days to 20 days for IGF-I assays. Autoradiographs were

analysed by scanning densitometry as described (3.2.5), and the intensity of protected fragments in each experimental sample was expressed relative to the mean intensity of the assay control RNA replicates. The within assay coefficient of variation for analysis of control RNA ranged from 13% - 20% (n=6) for IGF-I and -II mRNA measurements.

4.2.6 INSULIN RADIOIMMUNOASSAYS

Concentrations of insulin were measured in fetal arterial plasma collected on day 10 of treatment. Samples were assayed as described (3.2.6). The within assay co-efficient of variation was 4.5%. The minimum detectable concentration of insulin was 6 ρg/ml.

4.2.7 STATISTICS

All results are expressed as mean ± SEM with the number of animals in parentheses. Treatment effects were analysed using one way analysis (ANOVA) and the means compared by Bonferroni / Dunn tests using the SuperANOVA (Abacus Concepts, Inc., Berkeley, CA, USA) program. Relationships between variables were tested using simple correlation analysis (Statview SE + Graphics, Abacus Concepts, Inc., Berkeley, CA, USA and SAS/STAT software, Cary, NC, USA)

4.3 RESULTS

4.3.1 *Fetal growth*

IGF-I infusion reduced fetal body weight by 12% compared to saline infusion ($P< 0.05$; Table 4.1). Liver weight and quadriceps muscle weights were not altered by IGF-I infusion whether compared in absolute terms (Table 4.1) or relative to fetal body weight (data not shown).

TABLE 4.1 Body weight and tissue weights of fetal sheep at 130 days gestation following infusion of saline or IGF-I from 120 to 130 days gestation.

	SALINE INFUSED	IGF-I INFUSED
Fetal weight (g)	3607 ± 81 (6)	3179 ± 168 (6) *
Liver weight (g)	133 ± 6 (6)	127 ± 7 (6)
Quadriceps muscle weight (g)	24.9 ± 0.9 (6)	23.2 ± 1.7 (6)

Values are mean ± SEM, (number of animals). * $P < 0.05$ compared to saline infused, One way analysis of variance (ANOVA). Recombinant human IGF-I or saline was infused into the fetal tarsal vein at a rate of 26 ± 4 μg /kg/h from 120 to 130 days gestation. All measurements were made at 130 days gestation.

4.3.2 *Concentrations of IGF proteins in fetal blood*

Intravenous infusion of IGF-I to fetal sheep from 120 to 130 days gestation increased the concentration of IGF-I in fetal plasma approximately three fold ($P < 0.0002$, Table 4.2). The amount of IGF-II protein in fetal blood was not significantly altered by IGF-I infusion (Table 4.2).

TABLE 4.2 Concentrations of IGF proteins in arterial plasma from fetal sheep following infusion of saline or IGF-I from 120 to 130 days gestation.

	SALINE INFUSED	IGF-I INFUSED
Plasma IGF-I (ng/ml)	123 ± 21 (6)	377 ± 41 (5) ***
Plasma IGF-II (ng/ml)	928 ± 60 (6)	845 ± 43 (5)

Values are mean ± SEM, (number of animals). *** $P < 0.0005$ compared to saline infused, One way analysis of variance (ANOVA). Recombinant human IGF-I or saline was infused into the fetal tarsal vein at a rate of 26 ± 4 µg /kg/ h from 120 to 130 days gestation. All measurements were made at 130 days gestation.

4.3.3 *IGF-I and -II mRNA abundance in fetal tissues*

Representative ribonuclease protection / solution hybridisation assays of IGF-I and -II mRNA in liver and muscle from IGF-I infused and saline infused fetal sheep are shown in Figures 4.1 and 4.2. The scanned intensity of fragments protected in the solution hybridisation assays, expressed relative to the intensity of a within assay control liver RNA sample, are shown in Figure 4.3, as mean values for IGF-I infused and saline infused groups. The abundance of IGF-I mRNA in fetal liver was reduced by approximately 50% by ten days intravenous infusion of IGF-I (Figure 4.3a, $P < 0.0002$). In contrast, the concentration of IGF-I mRNA in fetal quadriceps muscle was not different in IGF-I infused fetal sheep, when compared to saline infused controls (Figure 4.3a).

IGF-II mRNA abundance was also reduced in fetal liver following infusion of IGF-I (Figure 4.3b, $P < 0.01$). IGF-I infusion did not alter the abundance of IGF-II mRNA in fetal skeletal muscle (Figure 4.3b).

No significant associations were evident between IGF-I or -II mRNA and the weights of fetal liver or quadriceps muscle.

4.3.4 *Effect of IGF-I infusion on insulin concentrations.*

Concentrations of insulin, measured in fetal arterial blood plasma on day ten of infusion, were reduced by IGF-I infusion ($P< 0.05$; Table 4.3). No significant associations were detected between plasma insulin and IGF-I or -II mRNA abundance in fetal liver (IGF-I mRNA, $r= 0.51$, NS; IGF-II mRNA, $r= 0.39$, NS) or muscle.

4.3.5 *Effect of IGF-I infusion on fetal blood pO_2, pH and glucose concentrations.*

Fetal arterial plasma glucose concentrations and blood pO_2 and pH, measured on days 8, 9 and 10 of treatment, were not altered by infusion of IGF-I (Table 4.3). Fetal plasma glucose correlated negatively with IGF-I mRNA abundance in fetal liver ($r= -0.71$, $P< 0.01$) and positively with IGF-I mRNA abundance in fetal skeletal muscle ($r= 0.67$, $P< 0.02$) (Figure 4.4). No associations were observed between IGF-II mRNA abundance and plasma glucose levels in either tissue. The pH and pO_2 of fetal blood were not related to IGF-I or -II mRNA abundance in liver or muscle.

TABLE 4.3 Concentrations of insulin and metabolites in the blood of fetal sheep following infusion of saline or IGF-I from 120 to 130 days gestation.

	SALINE INFUSED	IGF-I INFUSED
Plasma insulin (pg/ml)	441 ± 53 (6)	201 ± 80 (6) *
Plasma glucose (mmol/l)	1.39 ± 0.10 (6)	1.69 ± 0.13 (6)
Blood pO_2 (torr)	18.9 ± 1.0 (6)	17.7 ± 1.3 (6)
Blood pH	7.340 ± 0.006 (6)	7.336 ± 0.005 (6)

Values are mean ± SEM, (number of animals). * $P < 0.05$ compared to saline infused, One way analysis of variance (ANOVA). Recombinant human IGF-I or saline was infused into the fetal tarsal vein at a rate of 26 ± 4 µg /kg/ h from 120 to 130 days gestation. Insulin concentrations were measured in fetal arterial blood plasma collected on day 10 of IGF-I infusion (130 days gestation). Glucose, pO_2 and pH values are a mean value for measurements made on days 8, 9 and 10 of infusion (128 to 130 days gestation).

FIGURE 4.1 Autoradiograph of electrophoresis of ribonuclease protected products of solution hybridisation of IGF-I and -II mRNA in liver from fetal sheep following infusion of saline or IGF-I from 120 to 130 days gestation.

Autoradiograph of electrophoresis of ribonuclease protected products of solution hybridisation of (a) IGF-I mRNA in 50 μg total RNA and (b) IGF-II mRNA in 25 μg total RNA extracted from liver of fetal sheep following intravenous infusion of saline (saline infused) or IGF-I (IGF-I infused) (26 μg/kg/hr) from 120 to 130 days of gestation. Autoradiographs were exposed for 10 days (IGF-I) or 18 hours (IGF-II) at -70^{o}C with intensifying screens. M= ^{32}P labelled DNA molecular weight markers.

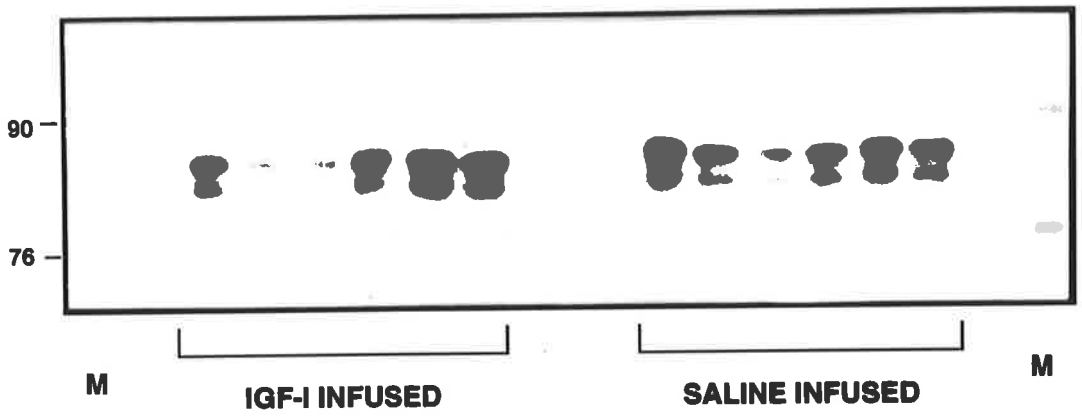
(a) IGF-I mRNA

(b) IGF-II mRNA

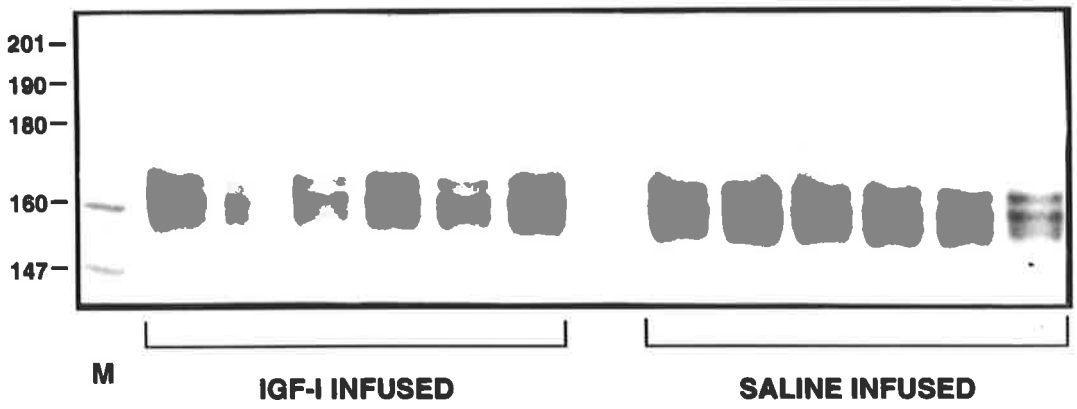
FIGURE 4.2 Autoradiograph of electrophoresis of ribonuclease protected products of solution hybridisation of IGF-I and -II mRNA in skeletal muscle from fetal sheep following infusion of saline or IGF-I from 120 to 130 days gestation

Autoradiograph of electrophoresis of ribonuclease protected products of solution hybridisation of (a) IGF-I mRNA in 50 μg total RNA and (b) IGF-II mRNA in 25 μg total RNA extracted from quadriceps muscle of fetal sheep following intravenous infusion of saline (saline infused).or IGF-I (IGF-I infused) (26 μg/kg/hr) from 120 to 130 days of gestation. Autoradiographs were exposed for 20 days (IGF-I) or 15 hours (IGF-II) at -70°C with intensifying screens. M= ^{32}P labelled DNA molecular weight markers.

(a) IGF-I mRNA



(b) IGF-II mRNA



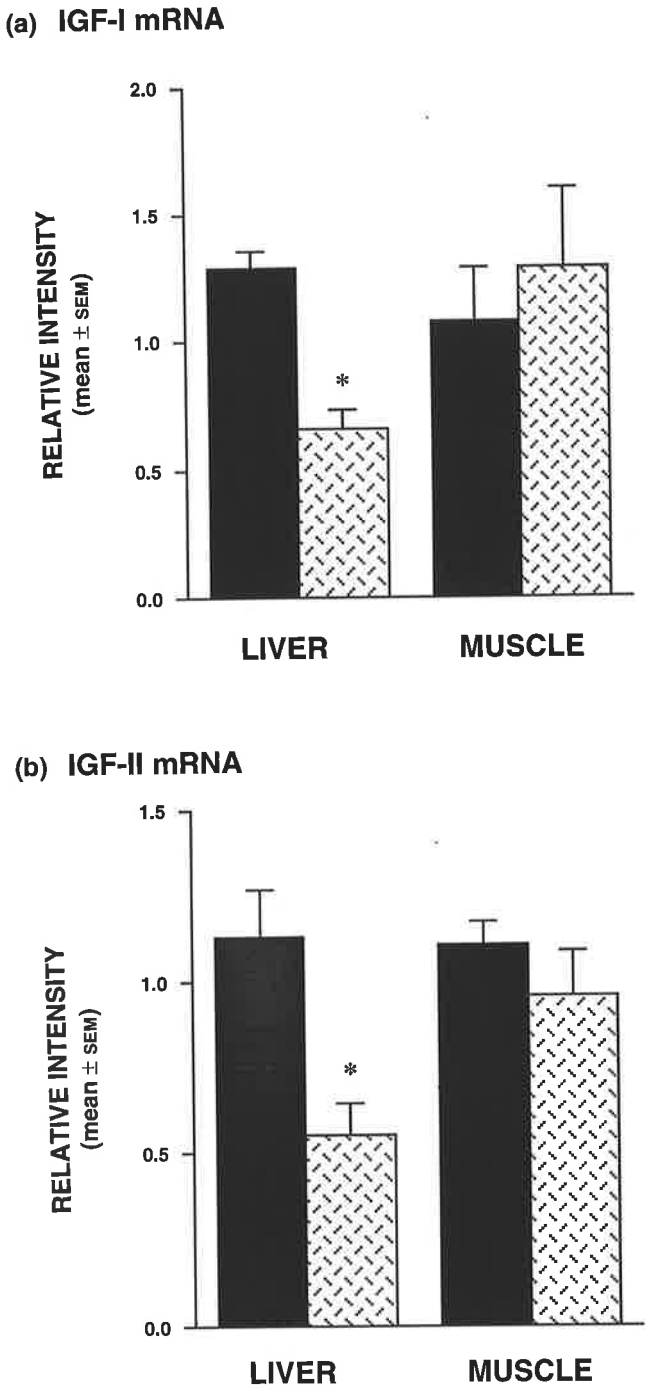


FIGURE 4.3 Effect of IGF-I infusion on IGF-I and -II gene expression in fetal liver and muscle.

(a) IGF-I mRNA abundance in 50 μg of total RNA from liver and muscle of saline infused (closed bars, n=6) and IGF-I infused (hatched bars, n=6) fetal sheep.
(b) IGF-II mRNA abundance in 25 μg of total RNA in the same fetal sheep as (a).

* $P < 0.05$ compared to saline infused. The abundance of IGF-I and -II mRNA is expressed relative to the amount of IGF-I or -II mRNA measured in a fetal liver RNA sample that was included in all assays. Recombinant human IGF-I was infused at a rate of 26 ± 4 μg/kg/h from 120 to 130 days gestation. All measurements were made at 130 days gestation.

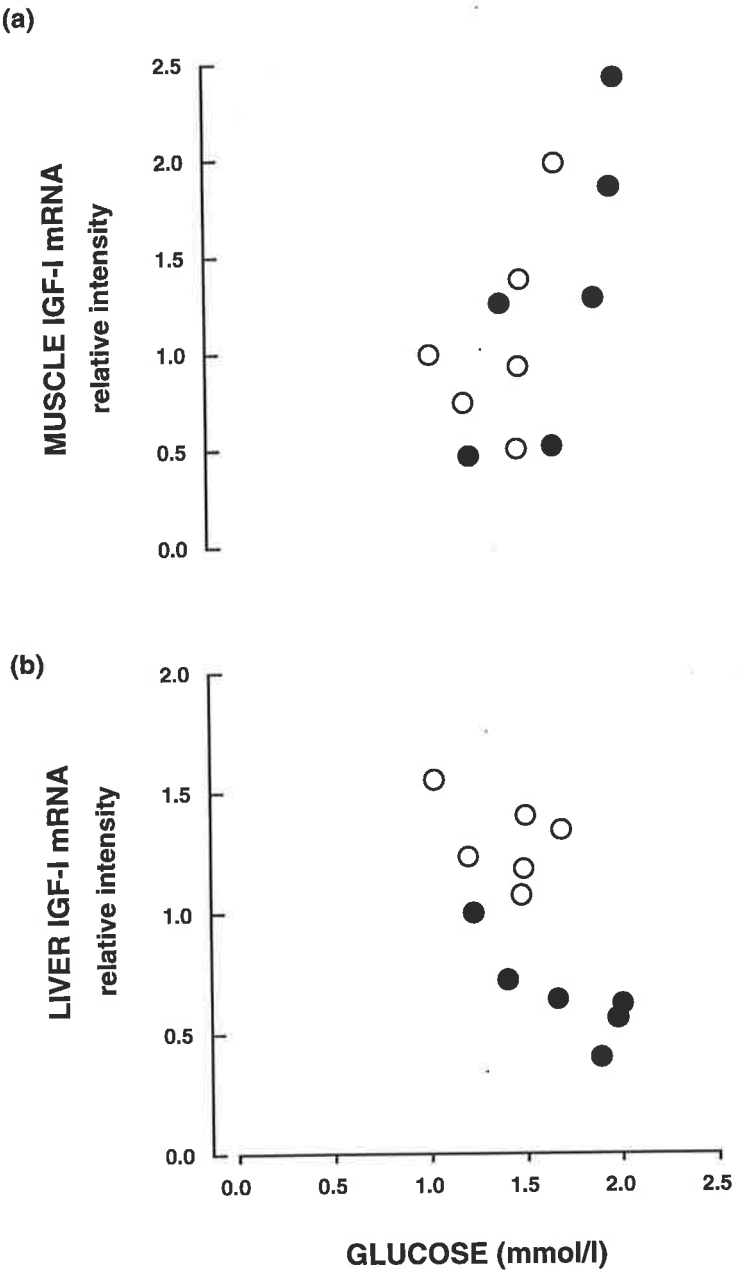


FIGURE 4.4 Relationship of IGF-I mRNA abundance in fetal muscle and liver to fetal plasma glucose concentrations following IGF-I or saline infusion into fetal sheep.

Correlations of

(a) IGF-I mRNA relative abundance in skeletal muscle with fetal arterial plasma glucose concentrations (r=0.67, P< 0.02, n= 12), in fetal sheep infused with saline (O , r=0.53, NS, n=6) or IGF-I (●, r=0.76, P< 0.08, n=6,).

(b) IGF-I mRNA relative abundance in liver with fetal arterial plasma glucose concentrations (r=-0.71, P< 0.01, n=12, in fetal sheep infused with saline (O , r=-0.43, NS, n=6) or IGF-I (●, r=0.83, P< 0.04, n=6,).

Recombinant human IGF-I was infused at a rate of 26 µg/kg/h from 120 to 130 days gestation. Glucose values are the mean of measurements made on days 8, 9 and 10 of infusion. IGF-I mRNA was measured at 130 days gestation. The abundance of IGF-I mRNA is expressed relative to the amount of IGF-I mRNA in a control fetal liver RNA sample.

4.4 DISCUSSION

Intravenous infusion of IGF-I into fetal sheep for ten days in late gestation inhibited expression of the genes for IGF-I and -II in fetal liver, but not fetal muscle. These results indicate that IGF-I can directly or indirectly regulate hepatic IGF-I and -II production *in utero*. Postnatal administration of IGF-I to growth-hormone deficient dwarf rats (Butler *et al.*, 1994), malnourished rats (Schalch *et al.*, 1989) or hypophysectomised rats (Gosteli-Peter *et al.*, 1994) reduces hepatic IGF-I mRNA. Type 1 IGF receptors are not detected in the liver of adult (Lowe *et al.*, 1989) and dwarf rats (Butler *et al.*, 1994), suggesting that the effects of exogenous IGF-I on hepatic IGF-I gene expression in postnatal rats are indirect. However, type 1 IGF receptors are present in liver of fetal rats and pigs (Werner *et al.*, 1989; Lee *et al.*, 1993). Although liver weight was not altered in the present study, IGF-I infusion to fetal sheep has been shown to stimulate hepatic growth in a study of a larger group of animals (Lok *et al.*, 1994). Therefore, direct actions of IGF-I on IGF gene expression in fetal sheep liver are possible. IGF-I treatment of cultured rat dermal fibroblasts does not alter IGF-I mRNA abundance, also indicating that IGF-I does not directly regulate IGF-I gene expression in that cell type (Lowe *et al.*, 1990). In contrast, addition of IGF-I to differentiating rat and human myoblasts *in vitro* suppresses IGF-II mRNA (Magri *et al.*, 1994). These effects of IGF-I appear to be mediated via the type 1 IGF receptor, suggesting that IGF-I can act directly to inhibit IGF-II gene expression in these cells.

Acute infusion of IGF-I into fetal sheep alters a number of metabolic and hormonal factors, known to be potential regulators of IGF-I and/ or -II gene expression in the fetus (De Ze gher *et al.*, 1988; Harding *et al.*, 1994). Thus, IGF-I could act through indirect mechanisms to regulate hepatic IGF-I and -II production in the current study. Glucose concentrations in the fetus showed a tendency to increase, but this did not reach statistical significance. This contrasts with the hypoglycaemic effect of IGF-I infusion after birth (Jacob *et al.*, 1989; Douglas *et al.*, 1991). IGF-I infusion into fetal sheep for two hours, at double the dose used in the current study, also has no effect on blood glucose levels, despite a decrease in plasma insulin (De Zegher *et al.*, 1988). Insulin concentrations in fetal plasma were also reduced by IGF-I infusion in the current study. As previously discussed, insulin is implicated as a

potential regulator of IGF-I and -II production *in utero*, since pancreatectomised fetal sheep have reduced plasma levels of insulin and IGF-I (Gluckman *et al.*, 1987). Also, addition of insulin to fetal sheep hepatocytes *in vitro* increases IGF-II mRNA (Townsend *et al.*, 1991). Therefore, the reduction in hepatic IGF-I and -II mRNA abundance in response to IGF-I administration may have resulted indirectly via reduced plasma insulin concentrations in this study.

In contrast to the decrease in hepatic IGF gene expression in IGF-I infused fetal sheep, neither IGF-I or -II mRNA abundance was altered in fetal muscle by IGF-I infusion. Type 1 IGF receptors are present in skeletal muscle of fetal rats (Werner *et al.*, 1989) and pigs (Lee *et al.*, 1993), indicating that IGF-I can bind to this fetal tissue. However, ten days infusion of IGF-I does not stimulate muscle growth in fetal sheep in the present study, or in a larger study in which hepatic growth was stimulated (Lok *et al.*, 1994). Postnatally, IGF-I infusion does not alter IGF-I mRNA levels in skeletal muscle of dwarf rats, but reduces hepatic levels (Butler *et al.*, 1994), suggesting that tissue specific factors may regulate the effects of IGF-I on IGF gene expression, both pre-and postnatally. A positive association was observed between plasma glucose concentrations and skeletal muscle IGF-I mRNA in the present study. This association is the reverse of that observed between plasma glucose and liver IGF-I mRNA, suggesting tissue specific regulation of IGF-I gene expression. The lack of effect of IGF-I on IGF-II gene expression in muscle contrasts with the suppression of IGF-II mRNA by exogenous IGF-I in differentiating myoblasts *in vitro* (Magri *et al.*, 1994). However, at 130 days gestation, skeletal muscle differentiation will be well advanced in the sheep fetus, with skeletal muscle consisting predominantly of differentiated muscle fibres (Ashmore *et al.*, 1972).

In the preceding study (Chapter 3), positive associations were observed between the amount of IGF-I in the blood of fetal sheep and the abundance of IGF-I mRNA in fetal liver and muscle (3.3.3), implicating these tissues as possible sources of circulating IGF-I in the sheep fetus. Reduced endogenous production of IGF-I in fetal liver, when circulating IGF-I is increased using exogenous peptide, suggests that negative feedback inhibition of hepatic IGF-

I synthesis may occur *in utero*. This supports the view that liver may be a major source of endocrine IGF-I in fetal sheep

Whether such a negative feedback regulation of hepatic IGF production occurs *in vivo* is not certain. Exogenous administration of IGF-I to fetal sheep is unlikely to reflect the physiological conditions under which fetal blood levels of IGF-I are elevated by an increase in endogenous production . Concentrations of IGF-I *in utero* are closely related to substrate supply and insulin. Therefore under normal physiological conditions, plasma IGF-I is increased when fetal blood glucose and insulin are high (Oliver *et al.*, 1993). Conversely, as observed in the preceding study, low blood glucose and insulin concentrations are associated with reduced plasma levels of IGF-I. In the present study, elevated concentrations of IGF-I in fetal blood were accompanied by low plasma insulin and no significant change in blood glucose levels. However, multiple tissues in the sheep fetus produce IGF-I (Chapter 3). Therefore, it is possible that negative feedback regulation may provide a mechanism, whereby hepatic IGF-I production could be reduced, when IGF-I expression by extrahepatic tissues is increased.

Despite a reduction in hepatic IGF-II mRNA, plasma IGF-II concentrations were not significantly affected by IGF-I. In the preceding study (Chapter 3), skeletal muscle and lung were identified as potential major sites of IGF-II production in the sheep fetus, based on IGF-II mRNA abundance and relative size of these tissues. Muscle IGF-II mRNA was not altered by IGF-I infusion. Therefore the results of this study are consistent with the suggestion that a number of tissues, including skeletal muscle, may be sources of circulating IGF-II protein in the sheep fetus. Alternatively, if the liver is a major source of IGF-II in fetal blood, increased production of IGF-II by other fetal tissues or altered clearance of IGF-II from blood would be required to maintain fetal plasma concentrations of IGF-II, in the face of reduced hepatic IGF-II production in the current study. In contrast to these results, administration of IGF-I to adult humans reduces plasma IGF-II (Guler *et al.*, 1989). Thus, IGF-I may also inhibit the synthesis of IGF-II postnatally. However, the decrease in plasma IGF-II in adult subjects when IGF-I is increased, may be associated with an increase in the clearance of IGF-II from blood since IGF-I and -II compete for the same binding proteins, including IGFBP-3.

4.5 SUMMARY

• This study has shown that expression of the IGF-I and -II genes in fetal liver, but not skeletal muscle is inhibited by chronic intravenous IGF-I infusion in late gestation. Thus IGF gene expression in liver and muscle are differentially regulated in the fetal sheep.

• These results suggest that negative feedback mechanisms may regulate IGF-I and -II production in fetal liver and are consistent with the suggestion that the liver may be a major source of circulating IGF-I in the fetus. In contrast, liver does not appear to be the major source of circulating IGF-II in the sheep fetus.

CHAPTER 5

GENERAL DISCUSSION

5.1 *Insulin-like growth factors in the fetal sheep*

As has been reported for the fetus of several mammalian species (1.3.2), the current studies show that IGF-I and -II are produced by many tissues in the late gestation sheep fetus. They further confirm that circulating concentrations of IGF-II in fetal blood and expression of the IGF-II gene in the majority of fetal sheep tissues are higher than those of IGF-I. Of the tissues examined here, liver and muscle contained the highest relative and estimated absolute abundance of IGF-I mRNA in the sheep fetus at 0.8 gestation. This contrasts to postnatal life, where liver is the major site of IGF-I expression in the sheep, and the relative abundance of IGF-I mRNA in liver is approximately twenty times that of skeletal muscle (Pell *et al.*, 1993). Ontogenic studies in fetal sheep suggest that this is due to a reduction in expression of IGF-I in skeletal muscle, and an increase in hepatic abundance of IGF-I mRNA with advancing gestation (Dickson *et al.*, 1991; Yang *et al.*, 1991). The high relative abundance of IGF-II mRNA in kidney, lung, skeletal muscle and liver of fetal sheep has also been observed by others (O'Mahoney *et al.*, 1991; Delhanty & Han, 1993). IGF-II expression in fetal sheep tissues decreases in late gestation and in the early perinatal period (Delhanty & Han, 1993).

5.2 *Circulating levels and tissue production of IGF-I and -II in the growth-retarded fetus.*

The pattern of expression of the IGFs *in utero* suggests that the dominant mechanisms of action of the IGFs may be different in the fetus to those in postnatal life. In particular, it has been suggested that autocrine/paracrine actions of the IGFs may be more important than endocrine actions before birth (1.3.6). However, endocrine actions of IGF-I in the fetus are suggested by a number of studies (1.3.6). In addition, class 2 IGF-I mRNA transcripts, which are believed to be produce IGF-I preproproteins targeted to secretion, (1.2.1.2) are present in several fetal sheep tissues. In further support of endocrine actions, the concentration of IGF-I protein in fetal plasma correlated positively with fetal weight in the current study (Chapter 2 & 3). However, expression of the IGF-I gene was concomitantly reduced in several tissues in the growth retarded fetus. Thus, altered expression of IGF-I in fetal sheep tissues could contribute to retarded fetal growth either through altered local actions or as a consequence of decreased circulating levels, and thus via endocrine mechanisms.

As discussed in Chapter 3, distinction between the potential influence of circulating IGF-I and locally produced IGF-I on growth of fetal tissues in this study was difficult. The weight of fetal kidney and liver correlated positively with both circulating IGF-I and with IGF-I expression within the tissue. Growth of the kidney and liver is stimulated by infusion of IGF-I into fetal sheep (Lok *et al.*, 1994), indicating that IGF-I can promote the growth of these tissues. However, a more accurate assessment of the relationship between tissue growth and circulating or locally produced IGF-I may be obtained by analysis of the rates of synthesis of DNA or protein by fetal tissues, rather than tissue weight alone. In addition, analysis of class 1 or -2 IGF-I mRNAs in fetal tissues and examination of the relationship of expression of these transcripts to tissue growth may provide further information as to the mode of action of IGF-I in particular fetal tissues.

Restriction of placental size disproportionately reduces the growth of individual skeletal muscles (Alexander, 1974). Muscle hypoplasia is observed in fetal mice with null mutations of the IGF-I gene (Liu *et al.*, 1993). The decrease in IGF-I mRNA abundance in muscle of growth retarded fetal sheep suggests that local actions of IGF-I may be important in determining muscle growth *in utero*. In support of this view, muscle growth is not enhanced by infusion of IGF-I into fetal sheep (Lok *et al.*, 1994). Circulating concentrations of IGF-I, rather than local expression of IGF-I, are related to muscle growth in young growing lambs when nutritional and growth hormone status is altered (Pell *et al.*, 1993). In contrast to postnatal life, high levels of expression of IGF-I in fetal muscle (5.1) may influence muscle growth, at least in part, through local actions.

IGF-I mRNA was not altered in the heart of growth-retarded fetal sheep. Hypoxaemia in the sheep fetus is associated with peripheral vasoconstriction (Thornburg & Morton, 1994). In the growth-retarded fetus, transient periods of decreased heart rate and increased blood pressure occur in association with periods of acute hypoxia (Robinson *et al.*, 1985; Robinson *et al.*, 1994). Increased levels of IGF-I mRNA are detected in adult rat heart in association with hypertension (1.2.3.1). It is possible that the relative hypertension and transient hypertensive episodes in growth retarded fetal sheep provide a stimulatory influence on IGF-I mRNA expression in fetal heart. Whether maintenance of IGF-I gene expression affects the growth or

development of fetal heart is uncertain. Certainly, as a proportion of body weight, heart size was not altered by restriction of placental size.

At 0.8 gestation, circulating levels of IGF-II and tissue expression of the gene for IGF-II were not altered in fetal sheep with restricted placental size, suggesting that IGF-II has not been involved in the significant growth retardation which has occurred in these fetal sheep by this stage of gestation. The effect of restriction of placental size on the supply of substrates to the fetus and on fetal growth becomes significant in the third trimester (Robinson *et al.*, 1994). However, IGF-II gene expression in the majority of tissues of the fetal sheep is highest at around 60 to 70 days of gestation (Delhanty & Han, 1993), suggesting that the major influence of IGF-II on fetal growth and development may occur early in gestation.

Other studies have reported unchanged or elevated concentrations of IGF-II in fetal blood and IGF-II mRNA in fetal liver in growth-retarded fetal sheep and rats (Jones *et al.*, 1988; Townsend *et al.*, 1992, 1.3.3, 1.3.7). The significance of normal or elevated levels of IGF-II protein and mRNA in growth-retarded fetuses is uncertain. The amount of IGF-II which is available to fetal tissues will be modulated by both the concentrations of binding proteins in fetal blood and the abundance of type 1 and 2 IGF receptors in fetal tissues, both of which may be altered when the supply of substrates to the fetus is altered (Gallaher *et al.*, 1994; Carr, 1995; 1.3.4). It is also possible that the normal levels of IGF-II play a role in maintaining minimally essential growth or development of the fetus, when IGF-I is low. Jones *et al.* (1988) suggest that IGF-II may have a metabolic role, such as enhancing the maintenance of glycogen stores in fetal liver, when the supply of substrates to the sheep fetus is reduced. In addition, a role for IGF-II in stimulating the differentiation of particular cell types in a number of fetal tissues, including skeletal muscle and the adrenal gland, is indicated (1.3.6). Normal or increased expression of IGF-II in the growth-retarded fetus may prevent premature or even normal differentiation of important fetal tissues in the early stages of the third trimester. Immaturity of a number of key organs and systems is characteristic of the growth retarded fetal guinea pig and sheep (Harding *et al.*, 1985; Lafeber *et al.*, 1985; Avila *et al.*, 1989).

In contrast, in later gestation (0.9 gestation), IGF-II concentrations are reduced in the blood of fetal sheep with restricted placental size (Chapter 2). Whether this is associated with a similar decrease in IGF-II mRNA in fetal tissues is uncertain. As previously discussed, this later reduction in circulating IGF-II in the fetus may be mediated by the premature increase in blood cortisol which occurs in the small fetus (Robinson *et al.*, 1980). In tissues, such as the adrenal, the decrease in IGF-II mRNA may be associated with cellular differentiation (Lu *et al.*, 1994). Blood concentrations of IGF-II also correlate with fetal size at this stage of gestation, suggesting that nearer term circulating IGF-II may be involved in regulating fetal growth. In support of this, restriction of maternal nutrient supply in late gestation fetal sheep reduces hepatic IGF-II mRNA (Li *et al.*, 1993). Furthermore, fetal growth in mice lacking an allelle of the IGF-II gene becomes significantly retarded in late gestation (Baker *et al.*, 1993). Therefore, the role of IGF-II in fetal growth and development may differ throughout gestation.

The relative abundance of IGF-I or -II mRNA was not altered in the growth-restricted placenta. IGF-I mRNA is reduced in the rat placenta following maternal starvation (Bernstein *et al.*, 1991) while placental IGF-II mRNA decreases following uterine artery ligation in this species (Price *et al.*, 1992a). Limiting the number of placentomes in the sheep placenta, by removal of potential implantation sites, results in compensatory growth of the existing placentomes. Individual placentomes are larger and have an increased abundance of fetal trophectoderm and increasesd surface area of trophectoderm per gram of placenta (Chidzanja, 1994). Semi-quantitative studies further suggest that natural restriction of implantation, as occurs in twins and triplets, increases vascularity of the fetal villi in the sheep placenta in late gestation (Stegeman, 1974). Preliminary reports suggest that in the sheep placenta, as in other species, IGF-II gene expression is abundant in fetal stromal cells, fibroblasts and endothelium (Bassett & Challis, 1992). The maintenance of IGF-II gene expression may contribute to or reflect increased angiogenesis and vascularity in these compensatory placentomes. Whether the maintenance of IGF-I or -II gene expression in the small placenta is associated with a role for the IGFs in this and other aspects of compensatory growth of the placenta should be further investigated.

5.3 *Sources of circulating IGFs in the fetus*

Potential sources of circulating IGFs in the fetus were also identified by the studies in this thesis. Liver and skeletal muscle were indicated as the most likely sources of IGF-I protein in fetal blood. Liver is implicated as the major source of circulating IGF-I in the postnatal sheep (Pell *et al.*, 1993) and the normal ontogenic increase in plasma IGF-I, in the sheep, is paralleled by an increase in hepatic IGF-I mRNA (Dickson *et al.*, 1991; Carr *et al.*, 1994). Whether other tissues, such as muscle, release IGF-I into fetal blood is not certain. Infusion of IGF-I into the fetal sheep inhibited IGF-I production in fetal liver, but not skeletal muscle, suggesting that a negative feedback mechanism may regulate hepatic, but not muscle, IGF-I expression. This would also be consistent with the liver as a major source of IGF-I in fetal blood. As discussed in Chapter 4, this decrease may be mediated indirectly through suppression of insulin levels in the blood of IGF-I infused fetuses. Furthermore, it is uncertain whether negative feedback regulation of hepatic IGF-I expression is likely to occur under normal physiological conditions in the fetus.

In the sheep, expression of class 2 IGF-I mRNA transcripts has been detected in fetal liver in late gestation (Dr. Stewart Gilmour, Department of Cellular Physiology, Babraham Cambridge, UK, personal communication). The preproprotein translated from the class 2 IGF-I mRNA is proposed to target the mature IGF-I protein to the circulation (1.2.1.2., Gilmour, 1994). Using polymerase chain reaction analysis expression of class 2 IGF-I mRNAs can also be detected in other in fetal sheep tissues, including muscle (Ohlsen *et al.*, 1993). However whether these transcripts are expressed in physiologically significant amounts in extrahepatic tissues of the sheep fetus remains to be determined. Analysis of class 1 and 2 IGF mRNAs in fetal liver and extrahepatic tissues in normal and growth-retarded fetal sheep may further identify the likely sources of circulating IGF-I in the fetus and whether these vary with pertubation.

No single tissue could be suggested as the major source of IGF-II protein in fetal blood. It is possible that a number of fetal tissues release IGF-II into the fetal circulation. IGF-I infusion suppressed hepatic IGF-II mRNA, but did not alter plasma IGF-II, suggesting that liver is not

the major source of circulating IGF-II in the fetus. As discussed in Chapter 3, the production of IGF-II protein may be regulated at a post-transcriptional level (De Moor *et al.*, 1994). Furthermore, the type 2 IGF receptor, which mediates the degradation of the IGF-II protein by receptor mediated internalisation, is present in high quantities in fetal sheep tissues (Owens *et al.*, 1980). Therefore, the rate of turnover as well as synthesis of IGF-II within a tissue may determine the amount of IGF-II protein produced. In the mid-gestation human fetus, Han *et al.* (1988) noted that the ratio of IGF-II mRNA to IGF-I mRNA in fetal tissues, (650:1 in fetal liver), was much higher than the ratio of IGF-II to IGF-I protein in fetal blood (2:1 to 4:1), This suggests that not all IGF-II mRNA was translated or released into blood, or that IGF-II is cleared more rapidly from fetal blood than IGF-I. A similar comparison can be made in fetal sheep, where the estimated ratio of IGF-II to IGF-I mRNA in fetal tissues ranged from 200: in kidney to 17: in muscle, while the amount of IGF-II protein was only 4 to 6 times higher than that of IGF-I in fetal blood. Measurement of the IGF-II protein content of fetal tissues is required to investigate this further.

No associations were observed between the relative or estimated absolute abundance of IGF-I or -II mRNA within the placenta (Chapter 3). The low levels of IGF-I mRNA observed in fetal sheep placenta contrast with the mid-gestation human fetus, where the placenta was the major site of IGF-I expression (Han *et al.*, 1988). However, the placenta is the largest and also the most growth-retarded tissue in this study and was quantitatively a major site of IGF-I and -II gene expression. Therefore, the placenta cannot be excluded as a potential source of circulating IGF proteins in the fetus.

5.4 *Regulation of IGF-I and -II gene expression in the sheep fetus*

The studies described in this thesis also suggest possible chronic regulators of IGF-I gene expression in the sheep fetus. However, the relationships upon which these suggestions are based are associative only, as restriction of placental supply of substrates to the fetus alters the concentration of a number of metabolites and hormones in fetal blood, including many that were not measured in these studies.

Supply of oxygen and nutrients to the fetus is indicated as a regulator of circulating IGF-I and hepatic IGF-I gene expression, consistent with other studies in the rodent and sheep fetus (1.3.3). The positive association observed between plasma insulin and hepatic IGF-I mRNA suggests that the influence of altered substrate supply on IGF-I production in fetal liver may be regulated indirectly, via insulin. Consistent with possible regulation of hepatic IGF-I expression by insulin, plasma insulin concentrations and hepatic IGF-I mRNA were reduced following infusion of IGF-I into the sheep fetus. Others have shown that pancreatectomy of fetal sheep reduces plasma IGF-I and insulin and increases plasma glucose (Gluckman *et al.*, 1987). Insulin stimulates IGF-I gene transcription in adult rat hepatocytes, indicating that insulin may regulate IGF-I expression at the transcriptional level, as has been described for other genes (O'Brien & Granner, 1991). However, the specific mechanisms through which insulin regulates IGF-I gene expression have not yet been determined.

The positive association of blood pO_2 with hepatic IGF-I mRNA in normal and growth-retarded fetal sheep was not independent of the influence of insulin. However short term restriction of uterine blood flow in sheep reduces fetal blood pO_2 and hepatic IGF-I mRNA, but does not alter plasma glucose or insulin (McLellan *et al.*, 1992). The mechanism through which reduced blood pO_2 could regulate IGF-I expression in fetal tissues in that report is uncertain. Hypoxaemia in fetal sheep is associated with a number of endocrine changes, including elevated blood cortisol concentrations (Challis *et al.*, 1989; Hooper *et al.*, 1990). Cortisol inhibits IGF-I gene expression in fetal rat osteoblasts (McCarthy *et al.*, 1990), however, the effect of cortisol on IGF-I expression in fetal sheep liver is not known.

The mechanisms through which restricted substrate supply regulates IGF-I gene expression appeared to be tissue specific. In contrast to fetal liver, the association of fetal arterial blood pO_2 with IGF-I mRNA abundance in muscle and lung was independent of the influence of glucose and insulin. Hypoxaemia alters blood flow distribution in the sheep fetus (Thornburg & Morton, 1994) and the degree of oxygenation and the extent of its reduction with placental restriction as experienced by individual fetal tissues may vary. IGF-I mRNA in muscle and lung was positively related to fetal plasma glucose, independent of the influence of insulin, suggesting that glucose may regulate IGF-I production in these tissues. Consistent with this,

muscle IGF-I mRNA was not altered in IGF-I infused fetal sheep and correlated positively with plasma glucose, but not plasma insulin (Chapter 4). Nutrient supply is known to regulate IGF-I gene expression in extrahepatic tissues of postnatal rats (1.2.3.1). However, the specific mechanism through which nutrient availability regulates IGF-I production is not certain. In postnatal animals this appears to involve, at least in part, an associated growth hormone resistance (1.2.3.1). However, a preliminary report indicates that addition of glucose to cultured rat glioma and pituitary cells increases IGF-I mRNA abundance, suggesting that glucose can directly regulate IGF-I production in some cell types (Yang *et al.*, 1994).

Metabolic acidosis is associated with a decrease in liver IGF-I mRNA in adult rats, although this association is partially mediated through effects of nutrient intake (Challa *et al.*, 1994). In the fetus, the relationship observed between hepatic IGF-I mRNA and fetal blood pH may be secondary to the positive association of hepatic IGF-I mRNA and blood pO_2. In hypoxaemic fetal sheep cellular pH is related to hepatic IGFBP-1 gene expression, independent of fetal oxygenation, suggesting that cellular pH or factors associated with metabolic acidosis, such as blood lactate, may regulate gene expression in fetal tissues (Asano *et al.*, 1993). In support of this suggestion, a relationship between blood pH and IGF-I mRNA, independent of blood pO_2 was observed in fetal kidney.

At 120 days gestation the supply of oxygen and nutrients to the fetal sheep does not appear to regulate IGF-II protein levels or IGF-II gene expression in fetal tissues. As discussed in Chapter 3, others have observed increases in IGF-II expression in fetal liver when the supply of nutrients was restricted from early in gestation (Townsend *et al.*, 1992a,b). Although the degree of fetal hypoglycaemia associated with chronic maternal undernutrition was similar to that observed in the current study, the early onset and chronic nature of the nutrient restriction in the study of Townsend *et al.* (1992a,b) may influence IGF-II gene expression differently. Similarly, as previously discussed (2.4, 3.4), the specific limiting nutrients may be important, with both oxygen and nutrients being restricted in the current study. Treatment of fetal sheep hepatocytes with insulin increases IGF-II mRNA abundance (Townsend *et al.*, 1991), suggesting that insulin positively regulates IGF-II expression. Consistent with this, the fall in plasma insulin levels in IGF-I infused fetal sheep was suggested as a possible mediator of the

concomitant decrease in hepatic IGF-II mRNA (Chapter 4). However, under physiological conditions *in vivo* this relationship is not evident. In growth-retarded fetuses, plasma insulin is reduced while IGF-II protein or IGF-II mRNA are increased or unchanged (Chapter 3, Unterman *et al.*, 1993; Townsend *et al.*, 1992b) and pancreatectomised fetal sheep have elevated concentrations of IGF-II and glucose in fetal plasma and reduced concentrations of insulin (Gluckman *et al.*, 1987).

One factor known to regulate IGF-II expression in the sheep fetus in late gestation is cortisol (Li *et al.*, 1993; Lu *et al.*, 1994). Cortisol, which inhibits IGF-II gene expression in fetal sheep, is increased in the blood of growth-retarded fetal sheep in late gestation, in association with increasing fetal hypoxaemia and hypoglycaemia. Therefore, cortisol is a potential regulator of the decrease in IGF-II protein concentrations in severely growth retarded fetal sheep nearer term (Chapter 2). Whether IGF-II gene expression is also altered in some or all tissues of the growth-retarded fetus very late in gestation, and relates to circulating IGF-II, remains to be determined.

5.5 *Future directions*

The studies in this thesis suggest that restriction of placental supply of substrates reduces IGF-I gene expression in several fetal tissues and IGF-I protein in fetal blood. However, the relationships observed between the concentrations of substrates or related factors in fetal blood and IGF-I gene expression in fetal tissues are associative in nature. Studies directly manipulating factors identified as possible regulators of the IGF-I gene *in utero* are required to determine which, if any, of these associations are causative in nature. These types of experiments have been performed to examine the factors regulating expression of the genes for IGFBPs in fetal sheep tissues (Osborn *et al.*, 1992; McLellan *et al.*, 1992; Hooper *et al.*, 1994) and to a lesser extent that of IGF-II (Li *et al.*, 1993; Lu *et al.*, 1994) and largely in the short term time domain. Due to the lesser abundance of IGF-I mRNA, study of its regulation has been much more limited (McLellan *et al.*, 1992). Manipulation of insulin and glucose concentrations independently, using pertubations such as chemical or surgical pancreatectomy, may confirm the suggested regulation by insulin of IGF-I gene expression in fetal liver, but

not extrahepatic tissues. In addition, the current studies did not examine the molecular mechanisms through which IGF-I gene expression was altered. Both transcriptional and post-transcriptional regulation of IGF-I gene expression occurs with nutritional restriction in adult animals (1.2.3.1).

The relationship of IGF-I gene expression to tissue growth was examined in the current study using tissue weight as a measure of growth. Analysis of DNA and protein synthesis rates in and histological analysis of cellular composition and cell proliferation in fetal tissues may provide further information as to the relationship of locally produced IGF-I to tissue growth. Similarly the current study suggests that the role of IGF-II in tissue growth may be more important very late, and possibly earlier, in gestation. Analysis of tissue growth and development and tissue expression of IGF-II mRNA at these gestational stages during normal and restricted growth *in utero* is required to assess this further.

Preliminary analyses of the effect of restriction of placental substrate supply on circulating concentrations of the IGF binding protein were performed in these studies. Others have analysed circulating IGFBPs and expression of IGFBPs 2 and 4 in the growth-retarded fetal sheep (Carr, 1995), however, IGFBP-1 was not measured. Concomitant analysis of blood levels and tissue expression of the IGFBPs and the IGFs in normal and growth-retarded fetal sheep, is required to determine the mechanisms through which the IGFBPs may be modulating the actions of the IGFs when placental supply of substrates is limited.

In addition, studies now indicate that in contrast to the rat fetus, both class 1 and class 2 IGF-I mRNAs are expressed in fetal sheep liver and possibly other tissues. Studies to determine which fetal tissues express class 1 or 2 IGF-I mRNA transcripts in the sheep fetus and to investigate the relationship of expression of these alternative transcripts to blood levels of IGF-I and to local tissue growth in normal and growth-retarded fetal sheep may provide further understanding as to the relative importance of autocrine or endocrine actions in regulating growth of individual tissues in the fetus.

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APPENDIX

SOURCES OF REAGENTS

Abacus Concepts, Inc., Berkeley, CA, USA

Abbott , Sydney, Australia

Ajax Chemicals, Auburn, NSW, Australia

Alltech Associates Inc., Deerfield, IL, USA

Amersham Australia Pty. Ltd., North Ryde, NSW, Australia

Arnolds of Reading, Peakhurst, NSW, Australia

Australian Radioisotopes, Lucas Heights Research Laboratories, NSW, Australia

Beckman Instruments, Gladesville, NSW, Australia

Boehringer Mannheim, Castle Hill, NSW, Australia

Bresatec Pty Ltd. Adelaide, SA, Australia

Clontech, Palto Alto, CA, USA

Commonwealth Serum Laboratories, Melbourne, Victoria, Australia

Dako Corporation, Carpinteria, California

Dow Corning Medical Products, Midlands, Michigan, USA

Du-Pont, Wilmington, MA, USA.

Dural Plastics, Dural, NSW, Australia

Eppendorf, Hamburg, Germany

Fluka Chemika-Biochemika, Buchs, Switzerland

GroPep Pty Ltd, Adelaide, SA, Australia

Hoeffer Scientific Instruments, San Francisco, CA, USA

ICI Instruments, Sydney, NSW, Australia

IDS, Bolden Business Park, Bolden, UK

Janke & Kunkel, Staufen, Germany

Johnson & Johnson, North Ryde, NSW, Australia

Lilly Research Laboratories, Indianapolis, IN, USA

Molecular Dynamics, Sunnyvale, CA, USA

Nalgene Company, Rochester, NY, USA

Packard Instrument Co., Meriden, CT, USA

Pharmacia/LKB Wallac, Turku, Finland

Radiometer, Copenhagen, Denmark

Roche Diagnostics Systems Inc., New Jersey, USA

SAS/STAT software, Cary, NC, USA

Schleicher and Scheull, Dassel, West Germany

Shimadzu Corporation, Kyoto, Japan

Sigma Chemical Co., Castle Hill, NSW, Australia

Silenus, Hawthorn, Victoria, Australia

Stratagene Ltd, Cambridge, England

UltraLum, Carson, CA, USA

Wako Pure Chemical Industries Ltd., Japan

Waters /Millipore, Lane Cove, NSW, Australia

Whatman International Ltd, Maidstone, England