Hormonal Regulation of the Class B Scavenger Receptors CD36 and SR-BI, in the Rat Liver

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Chapter 1 Introduction

1.1 Project Scope and Context

CD36 is a molecule with many potential functions (see below). However, the one that has attracted interest recently is its role as a fatty acid translocase (FAT) that is involved in uptake of long chain fatty acids (LCFAs) by striated muscle, cardiac muscle and adipose tissue (Brinkmann et al., 2002, Koonen et al., 2005). The molecule is, therefore, often referred to as FAT or FAT/CD36. However, this should not be taken to imply that the sole hepatic function of CD36 is transport of fatty acids – an important qualification, because of all organs that express the molecule, its function is least understood in the liver. Indeed, hepatic expression of CD36 was discovered only recently in this laboratory, following a chance observation on female rats (Zhang et al., 2003). The male Sprague Dawley rat is a standard model for lipid metabolism and surveys of tissue expression of CD36 had failed to detect significant levels of transcripts in the liver (Abumrad et al., 1993). The starting point of this project was the observation that across a number of rat strains, there is an almost qualitative difference in expression of CD36 between females and males (Zhang et al., 2003). This observation has been confirmed in rats by others and there is also a gender difference in hepatic expression of CD36 in humans (Stahlberg et al., 2003) .

A number of developments in the field of lipid biology made this gender-bias in hepatic expression of CD36 potentially significant. Firstly, CD36 has been found to bind long chain fatty acids (LCFAs) with high affinity (Abumrad et al., 1993, Baillie et al., 1996) and as discussed above, it is one of a number of transporter molecules that facilitate the influx of FA into cells. The absence of CD36 from male rat liver led to an assumption that other transporters must be responsible for protein-mediated LCFA uptake by hepatocytes (e.g. fatty acid transport proteins, FATPs or fatty acid binding proteins FABPs). However, none of these proteins have accounted for the long-standing observation that FA uptake by the liver is more rapid in female rats than in males (Luxon et al., 1998, Sorrentino et al., 1992). Furthermore, female rats are less prone to insulin resistance than males following FA infusion (Hevener et al., 2002). Gender regulation of hepatic CD36 expression could contribute to these differences.

A second important development was the discovery of the Class B Scavenger Receptor family of surface membrane glycoproteins, of which CD36 is the founding member (Greaves et al., 1998, Krieger, 1997). Three members of this family, namely CD36, SR-BI and SR-BII, are capable of high affinity interactions with high density lipoproteins (HDL), native and modified low density lipoproteins (LDL) and anionic phospholipids. SR-BI, and to a lesser extent SR-BII and CD36, can mediate cellular uptake of cholesteryl ester (CE) from HDL by a process known as selective cholesterol ester (CE) uptake (Connelly et al., 2003, Xu et al., 1997, Webb et al., 1998, Connelly et al., 2001). The reported absence of CD36 from hepatocytes, which engage in selective CE uptake, has contributed to the view that the molecule is not involved in cholesterol transport in this tissue. However, when not only liver, but also adrenal cortical cells, Leydig cells and corpus luteal cells were found to express CD36 (Zhang et al., 2003), it appeared that this assumption might be incorrect and that further investigation was warranted. The large mass of the liver, together with the high levels of CD36 expressed by hepatocytes in females suggested that on the basis of mass action, the molecule might have a significant influence on reverse cholesterol transport. If this was the case, and a similar gender difference occurred also in humans, then CD36 in the liver might contribute to the more beneficial levels of HDL-cholesterol in the serum of pre-menopausal women and their relatively low risk of cardiovascular disease (CVD) (reviewed in Kalin and Zumoff, 1990). In contrast to CD36, the few studies on gender and expression of SR-B1 in the liver have noted an inverse relationship, with lower expression by hepatocytes in females and after estrogen treatment (Graf et al., 2001, Landschulz et al., 1996). Comparison of CD36 and SR-B1 was, therefore, included in this project.

The scope and objective of this project was, therefore, investigation of the effects of gender on expression of CD36 in rat liver.

1.2 The Scavenger Receptors

CD36 and SR-BI both belong to one class in a larger group of proteins known as scavenger receptors (SRs). The SRs are divided into eight classes, designated A to H. The various classes are not structurally related but they share a number of properties.

SRs have in common that they are multi-ligand receptors that bind a range of polyanionic molecules, particularly those created by chemical or enzymatic modification. Examples of modified ligands are oxidised lipoproteins or maleylated BSA (Gabrielsson et al., 1995, Greaves et al., 1998, Krieger, 1998). Intriguingly, despite the lack of sequence homology between the various classes of scavenger receptors, there is considerable overlap in ligand-binding activity. This redundancy is partial, however, as some receptors bind only specific ligands (or ligands with specific modifications). Close investigation of the regulation, distribution, three-dimensional structure and function of the SRs is likely to be highly rewarding, as they are implicated in important human diseases such as atherosclerosis, diabetes and Alzheimer's disease (Krieger and Stern, 2001). Of the eight classes, A and B are the best characterized and will be discussed further, whereas Classes C to H have been recognized more recently and new members are still being identified (reviewed in (Murphy et al., 2005)). The 24 SRs that have been identified to date are listed in Table 1.1.

Table 1.1 List of Scavenger Receptors[a]

SCAVENGER RECEPTOR CLASSES							
A	B	C	D	E	F	G	H
MEMBRANE TOPOLOGY[b]							
TYPE II	TYPE III	TYPE I	TYPE I	TYPE II	TYPE I	TYPE I	TYPE I
THE SCAVENGER RECEPTORS							
SR-AI/II/III	CD36 (FAT, GPIV, PAS IV)	dSR-C	CD68 (Microsialin)	LOX-1	SREC-1	SR-PSOX (CXCL16)	FEEL-1 (Stabilin-1)
MARCO	SR-BI/II (CLA-1)		Lamp1[c] (LGP-A)		SREC-2		FEEL-2 (Stabilin-2)
SRCL (CL-P1)	LIMP-II[c] (LGP85)		Lamp-2[c] (LGP-B)		CED-1		
	Croquemort		Lamp-3 (DC-Lamp)				
	Emp						
	Cm08h8						
	CD36/LIMP-II_SD						
	SnmP-1						
	SnmP-2						

[a] *Much of the data in this table has been compiled from an excellent review by J. Murphy et al 2005. Alternative nomenclature is shown in brackets.*

[b] *All Scavenger receptors are integral membrane proteins, with either single (types I and II) or multiple (type III) transmembrane regions. Type I proteins are orientated with an extracellular N-terminus whereas type II proteins have an extracellular C-terminus.*

[c] *These receptors are also localised to the lysosomal/endosomal membrane.*

Scavenger receptors have been described in many mammalian species, as well as a number of invertebrate species. Classes B and F consist of structurally related mammalian and non-mammalian proteins, while the single representative of class C (dSR-C protein from *Drosophila melanogaster*) currently has no mammalian orthologue (Pearson et al., 1995). Although identified initially on either macrophages or endothelial cells, it is now clear that expression of many SRs is much more widespread. Multiple SRs have been detected on muscle cells (smooth, skeletal and cardiac), platelets, monocytes, dendritic cells and adipocytes. In view of (or perhaps because of) the large number of ligands that have been identified evidence relating to the function(s) of many SRs is inconclusive. It is of interest that nearly all of the mammalian SRs have affinity for modified (eg oxidised or acetylated) low density lipoproteins (LDL). Some SRs mediate endocytosis of lipoprotein particles and have been implicated in removal of modified LDL from the bloodstream (Dhaliwal and Steinbrecher, 1999). They may, therefore, have anti-atherogenic potential. On the other hand, excessive uptake of oxLDL by macrophages appears to be an early event in the pathogenesis of atherosclerosis, leading to the view that some SRs have pro-atherogenic effects (Dhaliwal and Steinbrecher, 1999, Kunjathoor et al., 2002). A number of SRs, both mammalian (CD36) and invertebrate (Croquemort, Emp, dSR-C, Cn08h8 and CED-1) are also involved in the recognition of apoptotic cells by macrophages and endothelial cells, although the mechanism for this is not well understood.

The discussion that follows will be limited to the class B scavenger receptors and principally to CD36 and SR-B1.

1.2.1 Class B Scavenger Receptors

The class B super-family is the largest of the SR families, with nine known members (listed in Table 1.1). These include three mammalian receptors, two *D. melanogaster* proteins and one each from silk moth species (*A. polyphemus, M.sexta*), *C. elegans* and *S. domuncula* (sea-sponge). It has been speculated that this family arose from a single ancestral gene that underwent a number of gene duplication events (Calvo et al., 1995). Very recently, a candidate for the ancestral gene was identified in the metazoan species S. domuncula and designated LIMP-II/CD36_SD (Muller et al., 2004). The genes encoding the three class B receptors found in humans (CD36, LIMP-II and SR-BI/II)

are now dispersed through the genome and have considerable differences in amino acid sequences, suggesting an ancient divergence.

1.2.1.1 Class B Scavenger Receptor Structure

Although class B SRs members share only approximately 30% amino acid sequence identity (Krieger, 2001), they do share many structural features. They are the only SRs that are predicted to pass through the membrane twice (Table 1.1), with hydrophobic transmembrane domains at both N- and C- termini and a large extracellular loop (Fig. 1.1). The cytoplasmic tails are short, and there is an uncleaved signal peptide at the N-terminus. This basic model has been proposed for CD36 (Oquendo et al., 1989), SR-BI/II (Acton et al., 1994), SNMP-1 (Rogers et al., 1997) and Emp (Hart and Wilcox, 1993). This model is well supported with evidence from *in vitro* expression of CD36 c-terminal truncation mutants (Gruarin et al., 2000, Tao et al., 1996). LIMP-II is proposed to have a similar structure but in this case the molecule is a component of lysosomal membranes rather than the plasma membrane and the middle portion of the protein forms a loop within the lysosome lumen (Vega et al., 1991).

The notable structural features of class B scavenger receptors include two palmitoylation sites at each end of the protein (Tao et al., 1996), six cysteine residues that form three disulfide bridges in the extracellular portion of the protein (Rasmussen et al., 1998), and extensive N-glycosylation in the central region. The mammalian class B SRs each contain approximately ten potential N-glycosylation sites (Asn-X-Ser/Thr) which are highly conserved between species and moderately conserved between different members of the class (Vega et al., 1991, Vinals et al., 2003). Glycosylation accounts for the difference between the observed molecular masses (80-90 kDa) and the sizes predicted from amino acid sequence (50-60 kDa) for CD36, LIMP-II and SR-BI (Acton et al., 1994, Oquendo et al., 1989, Vega et al., 1991). All of these structural features are shown in the diagramatic representation of CD36 protein (Fig. 1.1) that has been adapted from Collot-Teixeira et al. (2007).

1.2.2.2 Class B Scavenger Receptor Ligands

The ligands that have been identified for the mammalian class B SRs are shown in Table 1.2.

Figure 1.1: Diagramatic representation of CD36 protein.

(adapted from Collot-Teixeira, S. et al. 2007)

(A) The structure of CD36 with transmembrane domain (yellow/orange box), hydrophobic region (light blue) and proline-rich region (dark blue). The ten predicted N-linked glycosylated sites and ten cysteines are shown. (B) Representation of the CD36 receptor on cellular membrane. Shown are binding sites for thrombospondin-1/-2 (TSP-1 and TSP-2) and *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1) respectively. The immuno-dominant 155–183 amino acid region is the binding site for OxLDL, AGE, growth hormone-releasing peptide heraxelin (GHRP), and apoptotic neutrophils (see Table 1.2 for references). The cysteine residues desginated + in A form disulphide bonds in a 1-3, 4-5, 2-6 conformation in the proline rich region and palmitoylation sites at the N and C termini.

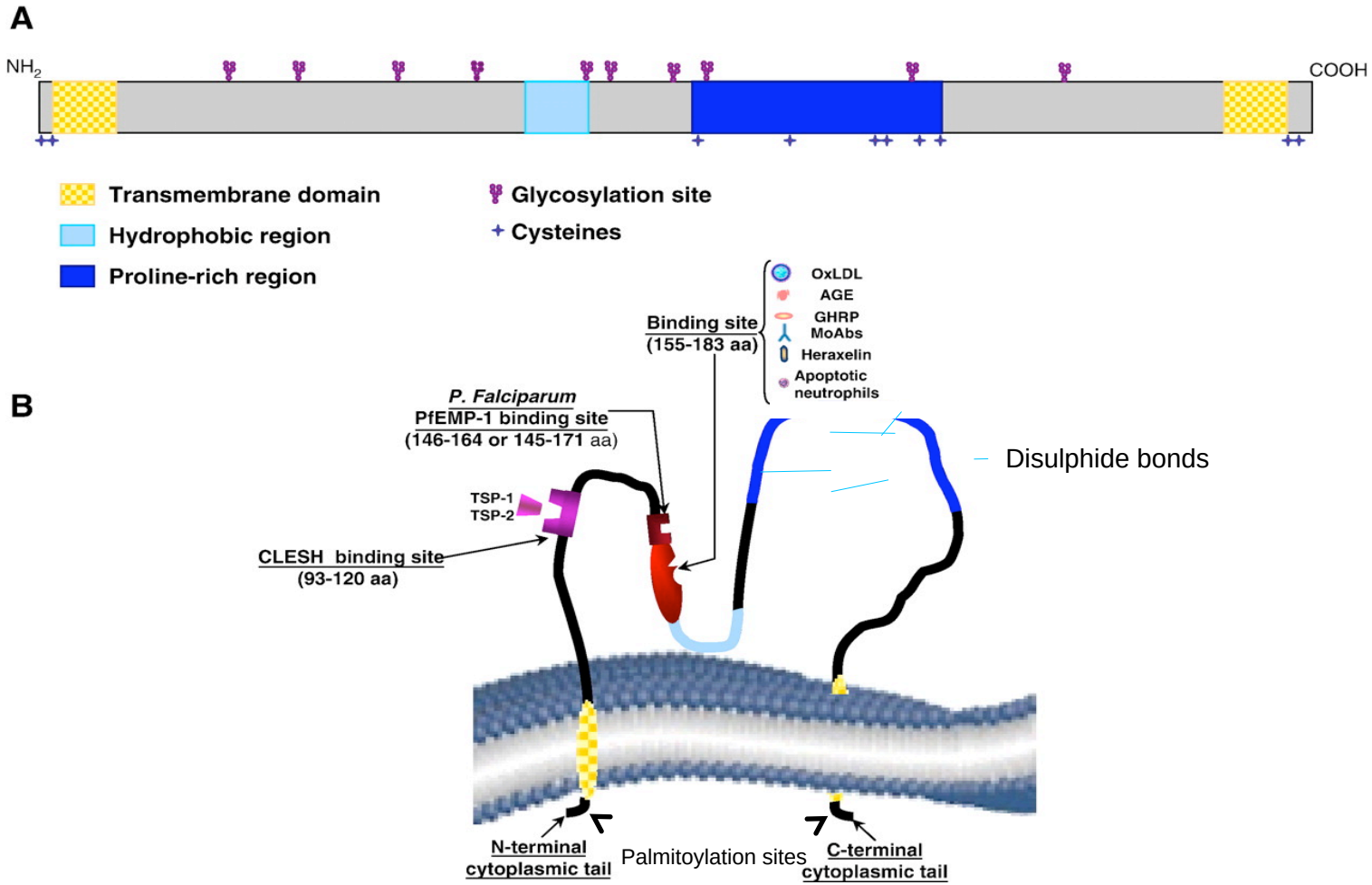

A
NH2
COOH
Transmembrane domain
Hydrophobic region
Proline-rich region
Glycosylation site
Cysteines
B
Binding site
(155-183 aa)
OxLDL
AGE
GHRP
MoAbs
Heraxelin
Apoptotic
neutrophils
P. Falciparum
PfEMP-1 binding site
(146-164 or 145-171 aa)
TSP-1
TSP-2
CLESH binding site
(93-120 aa)
Disulphide bonds
N-terminal
cytoplasmic tail
Palmitoylation sites
C-terminal
cytoplasmic tail

Table 1.2 Proposed Ligands for Mammalian Class B SRs

RECEPTOR	LIGAND	REFERENCE
CD36	oxLDL	(Endemann et al., 1993)
	Plasmodium falciparum- infected erythrocytes	(Oquendo et al., 1989)
	Anionic phospholipids	(Rigotti et al., 1995)
	Fibrillar beta amyloid	(Moore et al., 2002)
	T3	(van der Putten et al., 2003)
	AGE	(Ohgami et al., 2001a)
	Hexarelin	(Bodart et al., 2002)
	HDL	(Calvo et al., 1998)
	acLDL	(Acton et al., 1994)
	Long chain fatty acids	(Abumrad et al., 1993)
	LDL	(Calvo et al., 1998)
	VLDL	(Calvo et al., 1998)
	Bacterial diacylglycerides	(Hoebe et al., 2005)
	Apoptotic cells	(Savill et al., 1992)
	thrombospondin	(Asch et al., 1993)
	Collagen (I & IV)	(Tandon et al., 1989), (Asch et al., 1993)
	HOCL-LDL	(Marsche et al., 2003)
LIMP-II	thrombospondin	(Crombie and Silverstein, 1998)
SR-BI	HDL	(Acton et al., 1996)
	LDL	(Acton et al., 1994)
	VLDL	(Calvo et al., 1997)
	oxLDL	(Acton et al., 1994)
	acLDL	(Acton et al., 1994)
	Anionic phospholipids	(Rigotti et al., 1995)
	Apoptotic cells	(Fukasawa et al., 1996)
	Hepatitis C E2 protein	(Scarselli et al., 2002)
	AGE	(Ohgami et al., 2001b)
	Maleylated serum albumin	(Acton et al., 1994)
	HOCl-LDL	(Marsche et al., 2002)
	Chylomicron remnants	(Out et al., 2004)

Abbreviations: oxLDL, oxidized LDL; T3, triiodothyronine; AGE, advanced glycation end-products; AcLDL, acetylated LDL; HOCl-LDL, hypochlorite-modified LDL

CD36 and SR-BI are unique amongst the scavenger receptors in their affinities for native as well as modified lipoproteins. CD36 has, in addition, affinity for long chain fatty acids (LCFA), T3, types I and IV collagen and the growth hormone secretagogues hexarelin and ghrelin (See Table 1.2 for references).

1.2.2.3 Functions of Mammalian Class B Scavenger Receptors

It appears that in addition to those that they share with other members (eg the uptake of apoptotic cells by phagocytic cells), the mammalian class B SRs have evolved some quite specific functions. One that has been studied extensively is the non-endocytic ("selective") uptake of cholesteryl ester (CE) from HDL by SR-BI (Acton et al., 1996). The importance of SR-BI in "reverse transport" of cholesterol from peripheral tissues to the liver was demonstrated firstly by over-expressing SR-BI in the liver, using adenoviral transduction (de Villiers et al., 2001) and secondly by observing the lipid phenotype of SR-BI knock out mice (Rigotti et al., 1997). However, both SR-BII and CD36 are also capable of mediating CE transfer, although this is much less efficient (Section 1.1) and it remains unclear whether it has physiological significance. CD36 knock out mice display defects in fatty acid metabolism and also have abnormalities in lipoproteins, indicating that the molecule may have a significant role in cholesterol as well as fatty acid metabolism (Febbraio et al., 1999). This will be discussed in more detail in sections 1.4.1.4 and 1.4.2.6.

LIMP-II, with thrombospondin-1 (TSP-1) as its only known ligand, has been relatively neglected. However, a role in lysosome biosynthesis and maintenance has been proposed recently (Kuronita et al., 2002).

1.3 Structure and Distribution of CD36

1.3.1 Discovery of CD36

CD36 has been isolated and partially characterized by independent groups working in very different fields. As early as 1973, a proteolysis-resistant membrane protein was isolated from bovine milk fat globule membranes (Kobylka and Carraway, 1973). It was designated periodic acid/Schiff-positive protein IV or PAS IV (Greenwalt and Mather, 1985, Mather et al., 1980), an early indication of its high level of glycosylation. Independent studies on human platelets identified a glycoprotein IV or IIIb (Okumura and Jamieson, 1976), while monoclonal antibody (mAb) OKM5 defined the OKM5 antigen on human monocytes (Knowles et al., 1984). OKM5-ag was designated CD36

following the First Workshop on Human Leucocyte Differentiation Antigens (Bernard and Boumsell, 1984). In 1985, OKM5-ag was recognized as the receptor for P.falciparum-infected erythrocytes on endothelium (Barnwell et al., 1989, Barnwell et al., 1985). With the successful isolation and N-terminal amino acid sequencing of human OKM5-ag and bovine PAS IV from milk globule membranes it became evident that CD36 and GPIV were orthologues (Greenwalt et al., 1990). Finally, the cloning of an 88 kDa rat adipocyte membrane protein called fatty acid translocase (FAT) revealed that this molecule was also an orthologue of human CD36 (Abumrad et al., 1993, Harmon and Abumrad, 1993).

Cd36 cDNA has been cloned from humans (Tandon et al., 1989), rats (Abumrad et al., 1993), mice (Endemann et al., 1993) and bovines (Berglund et al., 1996). However, the sequences of an additional six orthologues have been published in Genbank, providing information on CD36 from: chimpanzee (XM519573), rhesus monkey (AY600441), chicken (AW355734), rainbow trout (AY606034), rabbit (AF412572) and golden hamster (U42430).

1.3.2 Distribution of CD36 in the Rat

1.3.2.1 General

In the initial studies on rat *Cd36*, transcripts were detected by Northern analysis in heart muscle, intestine, spleen, skeletal muscle, adipose tissue and testis. Notably, *Cd36* message was not detected in either kidney or liver (Abumrad et al., 1993, Van Nieuwenhoven et al., 1995). This pattern of *Cd36* expression was widely accepted until quite recently, when a monoclonal antibody against rat CD36 (mAb UA009) was produced in this laboratory (Zhang et al., 2003). A complete tissue survey revealed that CD36 had a distribution that was similar to that in other species, with some notable exceptions. CD36 was detected widely on vascular endothelium, except within the brain, normal endometrium and large arteries. It was also detected on monocytes, macrophages, erythrocytes, adipocytes (from both brown and white fat), cardiomyocytes, and red (but not white) skeletal muscle. However, it was not detected on either megakaryocytes or platelets. CD36 was also expressed by a number of epithelia, including the small intestine, lactating mammary gland, kidney, lung, oviduct, sebaceous gland and lingual circumvallate papillae of the tongue all expressed CD36.

Finally, there was strong expression of CD36 in the splenic red pulp and marginal zone and by the parenchymal cells of the liver.

The most novel findings were that steroidogenic cells in the ovary (corpus luteal granulose cells), testes (Leydig cells) and adrenal cortex (zona fasiculata and zona reticularis all expressed CD36. Most surprising was the observation that hepatocytes were stained strongly, in a centrilobular distribution. Expression of CD36 was confirmed on isolated hepatocytes and *Cd36* transcripts were detected readily in liver RNA using RT-PCR (Zhang et al., 2003). This difference from the results of earlier work (Abumrad et al., 1993, Van Nieuwenhoven et al., 1995) was found by R. Fitzsimmons to be due to a gender difference in expression of CD36 in the liver, rather than to either strain difference or difference in sensitivity of techniques used to detect *Cd36* transcripts. A recent study on rat liver using RT-PCR has confirmed that *Cd36* mRNA is present in not only endothelial cells and Kupffer cells, but also in hepatocytes (Malerod et al., 2002a).

1.3.2.2 The Gastrointestinal Tract

Expression of CD36 by epithelial cells of the gastrointestinal tract has been observed in a number of species, although there may be some species differences in distribution along the gut. In rats, there are high levels of *Cd36* mRNA in the duodenum and jejunum, with lower levels in the ileum and levels that were below detection in the stomach and colon (Poirier et al., 1996). These findings are supported by immuno-histochemical studies using mAb UA009 (Zhang et al., 2003). Others have detected low levels of *Cd36* mRNA in rat stomach and colon mucosae (Chen et al., 2001). In humans, CD36 protein was observed only in the duodenum and jejunum (Lobo et al., 2001). In mice, expression of CD36 appears to be high in the ileum and jejunum, with only low expression in the duodenum (van Bennekum et al., 2005). Although these differences appear subtle, they may represent different specialization of segments of the gut in the various species for particular aspects of lipid absorption.

1.3.2.3 Cardiac and Skeletal Muscle

CD36 has been reported in cardiac and skeletal muscle in all species studied (mouse, rat and human). In rats, CD36 is expressed on the sarcolemma of red skeletal muscle

myocytes (Bonen et al., 1998, Zhang et al., 2003) and by cardiac myocytes from both neonatal and adult rats (Abumrad et al., 1993, Van Nieuwenhoven et al., 1995). In human skeletal muscle, CD36 is expressed at high levels on the sarcolemma of type I myocytes, while expression on type II fibres is low (Keizer et al., 2004, Vistisen et al., 2004). The pattern of expression in human heart is less well known, but a study on a small number of pathological biopsy samples reported the presence of CD36 protein on both endothelial cells and cardiomyocytes (Pohl et al., 2000). Information about CD36 expression in mouse cardiac and skeletal muscle is less conclusive. Some reports have not detected the protein on myocytes in either cardiac (Greenwalt et al., 1995, Zibara et al., 2002) or skeletal muscle (Greenwalt et al., 1995). However, Irie et al (2003) detected CD36 on mouse cardiomyocytes using immuno-fluoresence. Ibrahimi et al (1999) found *Cd36* mRNA and protein in skeletal muscle homogenates, but did not distinguish between expression on vascular endothelial cell and myocytes. Interestingly, it was found that *Cd36* mRNA is expressed at higher levels in skeletal muscle from women than in men (Kiens et al., 2004), suggesting that gender may be one reason for the differing reports, as proved to be the case in the liver.

1.3.2.4 Steroidogenic Tissues

Cd36 transcripts were reported in rat testis (Abumrad et al., 1993) and CD36 was detected on Leydig cells by mAb UA009 (Zhang et al., 2003). However, the rat may be unique in this regard. Although both protein and mRNA was detected in extracts of mouse testis, immuno-histochemical localization showed that CD36 was expressed by capillary endothelial cells, spermatids and spermatozoids but not by either Leydig or Sertoli cells (Zibara et al., 2002). In human testes, CD36 was detected by Western blot analysis in all samples that were tested but none was detected by immuno-histochemistry in sections from testis tissue obtained from young normal individuals. Interestingly, when normal specimens from aging men (65 – 80 years) and pathological specimens from various ages were examined by immuno-histochemistry, CD36 was observed on Sertoli cells (Arenas et al., 2004). Further work is necessary to reconcile these findings.

The presence of CD36 in the ovary has only been recognized relatively recently. Petrik et al. (2002) reported cyclical expression of *Cd36* mRNA and CD36 by granulosa cells during the early antral phase and on the corpus luteum post-ovulation but there was no

expression by thecal cells. These findings were confirmed in rats (Zhang et al., 2003) and in cows (Greenaway et al., 2005). A single study of human ovarian tissue reported the presence of *Cd36* mRNA in inner thecal cells, but expression by granulosa cells was not discussed (Yao et al., 2004).

Our laboratory reported that CD36 is expressed by parenchymal cells of the zona fasiculata and zona reticularis in the rat adrenal cortex (Zhang et al., 2003). There have been no reports for other species, although expression of SR-BI is well established in this tissue.

1.3.2.4 The Liver

Uncertainty relating to expression of CD36 in the liver in rats was clarified by finding that the level of expression exhibits an almost qualitative gender dimorphism (Zhang et al., 2003). Evidence for hepatic expression of the molecule in humans and mice is no less conflicting. Early work, using mAb OKM5, suggested that CD36 in human liver was expressed by the sinusoidal endothelial cells (Fukuda et al., 1986, Nagura et al., 1986). Curiously, a second mAb (8C9) did not detect any FAT/CD36 on endothelial cells, either in the liver or in spleen and lymph nodes (Kudo et al., 1992). Volpes et al (1990) described CD36-positive hepatocytes in inflamed liver from subjects with hepatitis B virus, but control liver samples were not included in the study. Finally, a detailed ultrastructural investigation using a third mAb (MO30) described expression of CD36 by both sinusoidal endothelial cells and hepatocytes in normal human liver, as well as by the human hepatoma cell line HepG2-A16 (Maeno et al., 1994). More recently, CD36 has been detected on cultured human hepatic stellate cells (Schneiderhan et al., 2001) and *Cd36* transcripts have been detected in human liver RNA samples (Stahlberg et al., 2003). The latter study found that as in rats, there is a gender dimorphism in levels of CD36 expression in humans, with higher levels in women than in men.

CD36 was not detected on sinusoidal endothelial cells in liver from C57BL/6 mice but there was no comment on liver parenchymal cells (Greenwalt et al., 1995). Other studies either detected low levels of *Cd36* transcripts by Northern blot on mouse liver RNA (Memon et al., 2000, Yu et al., 2001, Motojima et al., 1998), or failed to detect transcripts (Motojima et al., 1998). In contrast, *Cd36* mRNA was detected in

parenchymal cells, endothelial cells and Kupffer cells purified from mouse liver (Malerod et al., 2002a). Because these studies were conducted in three mouse strains, inter-strain differences in hepatic CD36 expression such as occur in rats (Zhang et al., 2003) might account for the observed differences.

To summarize, the weight of evidence suggests that CD36 is expressed by hepatocytes in rat and human liver, but the evidence in mice is inconclusive.

1.3.3 The Structure of the Gene Encoding CD36

The structural gene (*Cd36*) that encodes CD36 is well characterized in humans, rats and mice. *Cd36* is located in the q11.2 band of chromosome 7 in humans. There are 15 exons, extending across approximately 32 kb of DNA. Exons 1 and 2 are not translated but they contain a proximal promoter region (discussed further in section 1.5.1.1). The translational start codon is positioned 290 bp downstream of the transcriptional start site in exon 3. Exon 3 contains the entire sequence encoding the N-terminal intracellular region and transmembrane domain, while exon 14 contains the C-terminal intracellular and transmembrane portions. The sequence encoding the major extracellular portion of the protein is distributed between exons 4 to 13, which range from 47 to 188 nucleotides in length. It has been hypothesized that the segments of CD36 encoded by individual exons may correspond to discrete functional units within the extracellular loop (Armesilla and Vega, 1994). Two alternative exons have been identified in the 5' un-translated region, designated exons 2a and 2b (Taylor et al., 1993). There is also an internal splice donor site within exon 14 that can join to the start of exon 15, thus generating a shorter *Cd36* transcript (Armesilla and Vega, 1994, Noguchi et al., 1993). The significance and relative frequency of the resulting alternative transcripts is not known. While none affect the coding region, they may influence regulation, transcript stability and/or translational efficiency.

There is a high level of nucleotide sequence identity between human *Cd36* and the rat, (Abumrad et al., 1993) and mouse (Endemann et al., 1993) orthologues, and the exon/intron boundaries are predicted to be conserved between the human and rat genes (Aitman et al., 1999). The recent observation that a sub-strain (SHR/NCr1 strain) of spontaneous hypertensive rats (SHR) carries a defective copy of *Cd36* has triggered

intensive research effort to understand the structure of the gene (Aitman et al., 1999, Gotoda et al., 1999, Pravenec et al., 1999). Quantitative trait locus mapping in this strain has linked the defective *Cd36* gene to other phenotypic features of SHR rats that resemble the metabolic syndrome in humans, suggesting that CD36 may play a key role in the pathogenesis of type II diabetes. The defective copy of *Cd36* in the SHR/NCr1 strain appears to have resulted from a spontaneous homologous recombination event between the known *Cd36* gene (mapped to chromosome 4) and a silent *Cd36* pseudogene (Aitman et al., 1999). Further analysis revealed that in the parent WKY strain and BN strain rats (and presumably most other strains), there are three genomic copies of *Cd36*. These consist of the structural gene encoding *Cd36*, plus two normally silent pseudogenes, designated *Cd36-ps1* and *Cd36-ps2*. Consequently, WKY, BN (and 27 other laboratory rat strains) express a single 2.8kb *Cd36* mRNA transcript (Aitman et al., 1999, Collison et al., 2000). SHR/N rats express a chimeric transcript that contains sequence from the 5'end up to the end of exon 4 of the *Cd36* gene, fused to sequence from exon 5 of *Cd36-ps2* onwards. Although *Cd36-ps2* has high similarity with *Cd36*, an 8 bp deletion at the end of exon 14 destroys the splice acceptor site, thus preventing removal of intron 14 and resulting in 3.8kb and 5.4kb transcripts that are translationally mute (Glazier et al., 2002). Duplication of *Cd36* appears to be a unique characteristic of rats.

1.3.4 Amino Acid Sequence, Structure and Post-Translational Modification of CD36

CD36 is a 472 amino acid polypeptide with over 80% similarity of amino acid sequence between humans, bovines, mice and rats. The predicted hairpin structure described in section 1.2.2.1 and illustrated in Figure 1.1 is preserved in all of these species. As mentioned earlier, class B scavenger receptors exhibit a range of post-translational modifications. In the case of CD36, it is not known whether these indicate precursor forms of the protein, or various states of functional competency that may be cell-type specific.

The amino acids that are important for the tertiary structure of CD36 and for post-translational modification are well conserved between species. All ten cysteine residues and eight out of ten potential glycosylation sites are maintained between human and rat

(Abumrad et al., 1993). Of the cysteine residues, the middle six (in human, residues 243, 272, 311, 313, 322 and 333) are clustered in a small region in the C-terminal half of the extra-cellular loop. These residues form three intra-chain disulfide bridges in a 1-3, 4-5, 2-6 conformation (Rasmussen et al., 1998). Inhibition of disulfide bond formation has been shown to block the progression from nascent CD36 to mature protein and results in degradation within the ER (Gruarin et al., 1997).

The remaining four cysteine residues are at the ends of the molecule and are intracellular, located close to the cytoplasmic face of the cell membrane (residues 3, 7 464 and 466). They are sites that can be modified reversibly by palmitoylation (Tao et al., 1996). Although the function of palmitoylation is poorly understood, CD36 may be like many other integral membrane proteins that contain a palmitoylation recognition motif (ACP/RSKT) and undergo extensive endosomal cycling (Gubitosi-Klug et al., 2005).

Finally, bovine, rat and human CD36 exhibit extensive N-glycosylation. There are ten potential N-glycosylation sites in human CD36 (Tandon et al., 1989) and of these, nine have identical positions in the bovine orthologue and eight are conserved in the rat molecule (Abumrad et al., 1993, Greenwalt et al., 1990). The observation that CD36 from platelets and milk fat globule membranes have slightly different molecular weights prior to de-glycosylation suggests that the nature of carbohydrate decoration may be cell-type specific (Greenwalt et al., 1990). Glycosylation and protein function appear to be linked in SR-BI (Vinals et al., 2003) but unfortunately there has been no comparable work on CD36.

1.4 Functions of CD36

As shown in Table 1.2, CD36 has binds a large number ligands, although its physiological relevance as a receptor for all of these compounds is still uncertain. This thesis focuses principally on CD36 expression by hepatocytes, where its function is likely to have a metabolic context and for this reason, only interactions that have clear relevance to lipid metabolism will be discussed in this section. These are the putative functions of CD36 in FA transport, the relevance of its interactions with native lipoproteins, and its role in clearance of modified lipoproteins.

1.4.1 CD36 AND FATTY ACID TRANSPORT

Uptake of long-chain fatty acids (LCFA) is the best-characterized function of CD36. However, CD36 is certainly not the only protein that facilitates FA uptake and a transporter may not be obligatory for FA entry into all cell types. This section examines the evidence that CD36 significantly enhances cellular uptake of fatty acids.

1.4.1.1 Overview of FA metabolism

Fatty acids (FA) perform diverse physiological functions, ranging from formation of structural lipids to generation of energy. They are derived from dietary triglycerides, or by lipogenesis from single carbon sources. They are the basis of storage and provision of energy, and they are the preferred energy source for some tissues that have high energy requirements (such as cardiac and red skeletal muscles). FA are stored as triacylglycerol (TG) in adipose tissue when energy is plentiful and released during fasting, exercise or stress. In the circulation, they are transported bound to albumin and delivered to various tissues. At a number of points in the TG biosynthetic pathway, FA derivatives can be sequestered for other roles, such as formation of cholesterol, steroid hormones, structural lipids (such as phospholipids and glycolipids), or participate in regulation of a variety of cell functions as intra-cellular and inter-cellular messengers (Potter et al., 1989).

1.4.1.2 Mechanisms of Cellular FA Uptake

Most FAs in circulation are bound to albumin, with only about 0.1% in the unbound (free fatty acid, FFA) form. The role of albumin in the process is to improve the water solubility of FAs and to provide a circulating reservoir. FAs dissociate from albumin prior to being taken up by cells and local lowering of the concentration of FFA leads to further dissociation and restoration of the bound/unbound ratio. Two mechanisms have been proposed for the entry of LCFAs into cells. One mechanism involves passive diffusion, where FAs essentially perform a ‘flip-flop’ maneuver to cross the membrane lipid bilayer. However, to do this they must first negotiate the hydrophilic outer leaflet of the plasma membrane (Pownall, 2001, Schaffer, 2002). An alternative proposal

suggests that large scale FA uptake involves either active transport or facilitated diffusion, mediated by membrane protein transporter molecules. Both mechanisms appear to operate *in vivo*, although there is ongoing debate about which mechanisms is most important in particular physiological settings (Hamilton et al., 2002, Hamilton et al., 2001). Many studies have indicated both saturable and non-saturable components of FA uptake in various cell types such as hepatocytes, myocytes and adipocytes (Abumrad et al., 1984, Sorrentino et al., 1989, Sorrentino et al., 1996, Stump et al., 2001, Stump et al., 1992) There is a consensus that passive diffusion is unlikely to be the sole mechanism by which FAs move into cells.

1.4.1.3 Early Evidence for Facilitated Transport of FA

Initial investigations focused on measuring FA uptake and efflux kinetics, and then moved towards identifying potential protein transporter molecules. Stremmel *et al* (1985) reported saturable binding of [^{14}C]-oleate-albumin complexes to rat liver plasma membrane preparations that was heat- and trypsin-sensitive. In addition, oleate-agarose affinity chromatography identified a single 40kDa protein that bound oleate with high affinity. These findings suggested the existence of a cell surface FA receptor and when monoclonal/polyclonal antibodies were raised against the purified protein, it was found on the surface of hepatocytes, cardiomyocytes and jejunal mucosal cells. Finally, the polyclonal antibodies inhibited initial influx of [^{3}H]-oleate into short-term cultures of rat hepatocytes (Stremmel and Theilmann, 1986). This protein was named Fatty Acid Binding Protein (FABPpm).

Work on FA uptake in adipocytes also showed kinetics and specificity consistent with involvement of a membrane protein transporter. At physiologically relevant ratios of FA to BSA (eg 0.2:1.0), uptake of oleate was shown to be saturable and to occur with high affinity, while non-saturable diffusion occurred only at un-physiologically high concentrations of unbound FA. High-affinity uptake was trypsin-resistant, restricted to FA with a minimum hydrocarbon chain length of 9, required the carboxyl group to be free and it was inhibited by 4, 4'diisothiocyanostilbene-2, 2'disulfonate (DIDS). [^{3}H]DIDS displaced FA from albumin, bound irreversibly to adipocyte membranes and affinity labelled an 88 kDa membrane protein (Abumrad et al., 1984).

Although the physiological necessity for a facilitated mechanism of FA uptake is still debated (Guo et al., 2006, Meshulam et al., 2006), attention has turned to elucidating the specific roles played by a number of candidate transporters. In the following section, three of these will be discussed. These structurally unrelated proteins are fatty acid translocase (designated here as CD36), fatty acid binding protein (plasma membrane) (FABPpm), and fatty acid transport protein (FATP).

1.4.1.4 CD36 as a Fatty Acid Translocase

Isolation and characterization of the 88 kDa protein that was labeled by [^{3}H]DIDS on adipocytes (Section 1.4.1.3) revealed that it was the rat orthologue of human CD36 (Abumrad et al., 1993). The molecule was purified by virtue of its affinity for the fatty acid derivative sulfo-N-succinimidyl oleate (SSO) (Harmon and Abumrad, 1993). SSO had been shown to inhibit LCFA uptake irreversibly in a number of cell types and the inhibition was due to its specific covalent binding to CD36 (Coort et al., 2002, Harmon et al., 1991). Investigation of FA binding to CD36 revealed similar curves for all LCFA with chains of 16 to 20 carbons, irrespective of the degree of saturation. CD36 also competed with albumin for binding FA. The region of the extracellular domain comprising amino acids 127-279, which has similarity with FA binding protein M-FABP, may contain a potential binding site for FA (Baillie et al., 1996). However, this has not been investigated directly. The predicted topology of CD36 (Fig. 1.1) is different to classical membrane transporters, which often traverse the membrane four or more times, thereby creating a channel for passage of the substrate. Thus, the mechanism of FA transport by CD36 remains unclear.

Nevertheless, expression of CD36 correlates closely with the metabolic demands for FA in a number of cell types, the most important being adipocytes, cardiac myocytes and red skeletal muscle fibres. In pre-adipocytes, maturation is accompanied by increased expression of CD36 and a commensurate increase in FA uptake (Abumrad et al., 1993). The up-regulation of CD36 in the heart in young rats parallels increases in FA utilization that occur during this period of development (Abumrad et al., 1993, Van Nieuwenhoven et al., 1995). Indeed, in various types of muscle CD36 expression correlates positively with the rate of FA uptake (Luiken et al., 1999). Furthermore, experiments using transfection show that expression of CD36 in a fibroblast cell line leads to increased FA uptake and that this increase is saturable (Ibrahimi et al., 1996).

More recently, it has been shown that the amount of CD36 in the sarcolemma is regulated during chronic muscle contraction (Bonen et al., 1999). Furthermore, acute muscle stimulation is also followed by a rapid increase in the amount of sarcolemmal CD36 and this appears to come from pre-formed intracellular pools. This increase in surface CD36 is accompanied by increased FA uptake into giant vesicles (Bonen et al., 2000). Induced translocation of CD36 to the plasma membrane, and increased FA uptake, was also observed in cardiac myocytes in response to both insulin and the adenosine analogue AICAR (Chabowski et al., 2005, Luiken et al., 2002b). Finally, in rats with streptozotocin-induced diabetes, the rate of FA uptake increased in both the heart and skeletal muscle as the disease progressed and this was accompanied by increases in both total and membrane-associated CD36 levels. However this direct relationship between FA uptake and plasma membrane CD36 did not hold in adipose tissue or the liver. In these tissues, enhanced rates of FA transport appeared to correlate with changes in levels of FABPpm (Luiken et al., 2002a). This study highlights two important points with respect to the role played by CD36 in FA transport. Firstly, modulation of CD36 expression can occur in response to a pathological metabolic state. Secondly, it shows that various tissues may use different mechanisms to regulate uptake of FA, and the presence of CD36 does not mean necessarily that it is the only FA transporter in that tissue. The latter is exemplified in the liver, where the rate of FA uptake decreases in severe insulin deficiency, while the quantity of plasma membrane CD36 actually increases. Whether this is because another transporter is more important in the diabetic liver, or because downstream events neutralize the activity of CD36, remains to be explored.

An additional interesting facet to the potential role of CD36 as a fatty acid transporter is the correlation that has been observed between exercise-induced increases in mitochondrial CD36 protein expression with increased *ex vivo* palmitate oxidation rates in human skeletal muscle (Holloway et al., 2006). This effect could be blocked with SSO and was hypothesized to be a consequence of increased CD36-mediated transport of FA into the mitochondria. Furthermore, studies using *Cd36* knockout mice also showed impaired induction of mitochondrial fatty acid oxidation, following muscle contraction (Holloway et al., 2009). However, addition of SSO did not differentially effect fatty acid uptake at the mitochondrial membrane between wildtype and *Cd36* knockout mice. This suggested that the primary inhibitory effect of SSO was localised

to the plasma, not mitochondrial membranes. Independent studies have not confirmed a definitive role for CD36 at the mitochondrial membrane and King et al (2007) reported no differences in mitochondrial fatty acid transport between *Cd36* knockout and wildtype mice.

1.4.1.5 Fatty Acid Binding Protein (FABPpm)

As noted above, FABPpm was the first candidate transporter to be identified (Stremmel et al., 1985). This 40 kDa protein was isolated from rat hepatocytes (Section 1.4.1.4) and was designated FABPpm in order to distinguish it from a smaller, unrelated cytosolic fatty acid binding protein (FABPc). FABPpm is identical to the mitochondrial isoform of the enzyme aspartate aminotransferase (mAspAT). Thus FABP/mAspAT has been found in three cellular locations - on the inner mitochondrial membrane, as a Type I protein in the plasma membrane and also as a membrane protein in intracellular pools. It can be translocated rapidly from the low-density microsomes to the plasma membrane (Cechetto et al., 2002, Chabowski et al., 2005). Although it is thirty years since FABPpm was first recognized, there is still no clear understanding of how this protein facilitates FA uptake and the evidence that it is involved remains largely circumstantial. There remain a number of unresolved issues with respect to FABPpm and the process of FA transport. Firstly although FA binding can be inhibited by anti-FABPpm antibody, the precise binding site has not been identified. Secondly, it is uncertain how a Type I membrane protein is able to act as a trans-membrane transporter, although it has been suggested that FABPpm may act in concert with CD36 (Luiken et al., 1999). A number of lines of evidence are consistent with this hypothesis. CD36 and FABPpm are both expressed constitutively in rat skeletal muscle cells and cardiomyocytes. The specific blocking agents (SSO for CD36 or anti-FABPpm antibody) each inhibit palmitate uptake partially. However, addition of both agents simultaneously did not result in further inhibition. This suggested that the two agents block the same process, but at different points (Luiken et al., 1999). In addition, while over-expression of FABPpm in myocytes resulted in an increased rate of palmitate uptake, the amount of increase was modest in comparison with the large increase in cell-surface FABPpm expression that was achieved (Clarke et al., 2004).

The most compelling evidence for co-operative action between CD36 and FABPpm comes from an elegant study in cardiac myocytes, where insulin and AICAR were used to induce translocation of both proteins from intracellular pools to the cell surface.

While both agents induced translocation of CD36, only AICAR induced translocation of FABPpm. Addition of insulin alone (increased surface expression of CD36) led to a modest increase in palmitate uptake, whereas greater uptake followed addition of AICAR (increased surface expression of both CD36 and FABPpm). Significantly, the inhibition of palmitate uptake by SSO was comparable whether the cells were treated with AICAR or insulin. Therefore, increased surface expression of FABPpm only confers increased FA uptake in the presence of functional CD36 (Chabowski et al., 2005). The nature of the putative interaction between CD36 and FABP is still speculative and it is discussed briefly in section 1.4.1.7.

1.4.1.6 Fatty Acid Transport Protein (FATP)

Fatty acid transport protein (FATP) is a 63 kDa protein that was identified in adipocytes. This protein has high affinity for oleic acid and was found to confer increase in FA uptake when expressed in fibroblasts by transfection (Schaffer and Lodish, 1994). It was recognized subsequently that FATP contains a conserved sequence of 311 amino acids that is also present in five other proteins (now designated FATP1 to 6) and that these proteins are present many species (Hirsch et al., 1998). In mice, the various FATP family members are expressed in a tissue specific manner. FATP1 is the only member that has been identified in adipocytes and skeletal muscle cells. FATPs 2-5 are all expressed abundantly in the liver, while FATP1 and FATP6 are both found at high levels in cardiac muscle. It appears that FATP5 is expressed only in the liver (Pohl et al., 2004b). Although FATP5 knockout mice display impaired fatty acid uptake in the liver (reviewed in (Gimeno, 2007), there is still controversy about whether the FATP proteins behave as true FA transporters. They may possess acyl-CoA synthetase activity (Coe et al., 1999, Herrmann et al., 2001) and facilitate LCFA uptake by maintaining a trans-membrane diffusion gradient. They may also channel incoming FA into particular intra-cellular compartments or metabolic pathways. These proteins will not be discussed further.

1.4.1.7 A Cohesive Model for Facilitated Fatty Acid Transport

To summarise, the available evidence suggests strongly that a number of proteins may facilitate the cellular uptake of LCFA and that they may have importance in situations where demand exceeds the rate of uptake available via passive diffusion. Although all

three candidate FA receptors can improve the rate of saturable FA uptake, it is by no means conclusive that all act as transporters at the cell surface. The mechanisms responsible for FA translocation across the plasma membrane are not fully understood and it is likely that they may vary from one cell type to another. Depending on the receptors and enzymes that are available in a given cell, the following model is conceivable. Acting either alone or in concert with FABPpm, CD36 may scavenge LCFA from albumin at the cell surface and then transport it across the cell membrane. It is interesting that whilst a higher rate of oleate upatke has been observed in female rat liver compared to male liver, the expression of FABPpm was identical between the sexes (Sorrentino et al., 1992). CD36 could potentially confer a sex-dependent component to this process. The absorbed FA might then be "passed' to cytoplasmic fatty acid binding protein (FABPc) and following activation by acyl CoA synthetase (FATP) be passed on again to acyl CoA binding protein. These intracellular steps would confer uni-directionality to the flux of FA (Glatz et al., 2003, Schaffer, 2002). Alternatively, following translocation across the membrane, the FA might be passed from CD36 to FATP, whose primary role may be its acyl CoA synthetase activity and/or chaperone function to direct FA towards particular cytosolic compartments (Garcia-Martinez et al., 2005, Pohl et al., 2004a). Irrespective of the specific steps, in cells that express CD36, the molecule is likely to play a central role in protein-mediated FA uptake.

1.4.2 Interactions between CD36 and Lipoproteins

CD36 was recognized first as a receptor for ox-LDL (Endemann et al., 1993), but it was later discovered that the molecule bound a range of native and modified lipoproteins (see Table 1.2 for a full list). Unraveling the molecular mechanisms and the physiological significance of the various receptor-ligand interactions that have been observed *in vitro* is still an extremely active field of research. The high affinity interactions of CD36 with HDL and modified LDL are of particular interest because of possible links between cholesterol metabolism and various disease states, such as atherosclerosis, type 2 diabetes, obesity and Alzheimer's disease.

1.4.2.1 Background: The Role of Lipoproteins in the Body

Lipoproteins are macromolecules whose primary function is to facilitate the transportation of hydrophobic lipids such as cholesterol and triglyceride (TG) throughout the body (Arias and Boyer, 2001). Provision of an adequate supply of cholesterol to the peripheral tissues is essential, as it is required for maintenance of plasma membranes of all cells, as a precursor for the synthesis of steroid hormones and vitamin D, as well as for production of bile acids. Cholesterol is derived from *de novo* synthesis and is also absorbed from the diet and delivered to the liver via chylomicron remnants. The liver is the major source of *de novo* synthesized cholesterol. Cholesterol is packaged into very low-density lipoproteins (VLDL) by hepatocytes and these are secreted into the systemic circulation. As TG is progressively removed by the action of various lipases, VLDL become increasingly dense and are described as intermediate-density lipoprotein (IDL) and then low-density lipoprotein (LDL). A specific receptor (LDL-R) mediates the uptake of LDL into peripheral cells, where cholesterol is released for use or storage. Surplus LDL can be returned to the liver, where it is taken up by hepatocytes via the LDL-R. Cholesterol can only be eliminated from the body via the liver, where it can be utilized to synthesize bile salts or simply secreted into the bile as free cholesterol. However, up to 95% of the bile salts that enter the small intestine are reabsorbed from the ileum and returned to the liver by the process of entero-hepatic recycling (Cohen, 1999, Redinger, 2003, Rhainds and Brissette, 1999).

Cholesterol that is surplus to the requirements of extra-hepatic tissues can be returned to the liver by a process known as reverse cholesterol transport (RCT). The liver synthesizes and exports empty lipoprotein shells that consist of phospholipids and apoprotein (designated nascent HDL). These “empty” lipoproteins acquire free and esterified cholesterol (CE) from the peripheral tissues. In humans, CE can be transported from HDL to the liver directly or it can be transferred to VLDL, IDL or LDL through the action of cholesterol ester transfer protein (CETP), thus returning to the liver via LDL-R mediated uptake. In rodents, CETP is absent and RCT occurs solely via HDL. The uptake of CE from HDL is also receptor mediated, although mechanistically the process is quite different from that mediated the LDL-R. The class B scavenger receptor SR-BI is believed to be the main receptor mediating RCT from HDL, although both SR-BII and CD36 also bind HDL with high affinity (Gu et al., 1998, Connelly et al., 1999). The action of SR-BI as a mediator of CE uptake and potential importance of CD36 are addressed later (Section 1.4.3.4). In the following

sections, interactions between CD36 and modified-LDL are discussed in the context of hypercholesterolemia and the pathogenesis of atherosclerosis.

1.4.2.2 Background: Modified-LDL

A break through in research on atherosclerosis was the discovery that modification of LDL by acetylation resulted in lipoproteins that induced formation of foam cells *in vitro*. However, there was no evidence that ac-LDLs occur *in vivo*. An impressive body of work performed by Steinberg et al in the early 1980's provided the missing link between foam cell formation and physiological modification of LDL. They found that incubation of LDL with human umbilical vein endothelial cells resulted in oxidation, which rendered the particles subject to increased uptake and degradation by macrophages. Most importantly, these lipid-laden macrophages resembled the foam cells associated with atherosclerosis (Henriksen et al., 1983, Steinbrecher et al., 1984). This work led to the isolation and characterization of the "scavenger receptors" that were responsible for uptake of modified LDL. The first, a receptor on macrophages that bound ac-LDL, was designated SR-A (Kodama et al., 1990). Soon after, two receptors were cloned which were capable of mediating the binding and uptake of Ox-LDL with high affinity, namely CD36 (Endemann et al., 1993) and SR-BI (Acton et al., 1994). Since that time, a very large amount of research has been performed with the goal of uncovering the properties of modified LDL, their interactions with various receptors and their role in the initiation and perpetuation of atherosclerosis.

1.4.2.3 CD36 and Modified Low-Density Lipoproteins

Modification renders LDL both antigenic and pro-inflammatory. Even mild modification results in a lipoprotein that has significantly decreased affinity for the LDL-R and an increased propensity for uptake by macrophages. Oxidation of LDL can be achieved in vitro by numerous methods, such as exposure to copper or iron ions, the release of hydrogen peroxide and reactive oxygen species by enzymes (lipoxygenase, myeloperoxidase, superoxide dismutase), and by reactive nitrogen species (Marsche et al., 2003, Mertens and Holvoet, 2001). The biochemical consequences of LDL oxidation involve both the lipid and protein components. The particles display increased electrophoretic mobility, extensive fragmentation of apoB-100 and cross-linking of lysine residues. Phosphatidylcholine can be converted to lysophosphatidylcholine and

unsaturated FAs in the lipoprotein undergo peroxidation, which can degrade to shorter chain aldehyde products and ultimately result in adduct formation (Endemann et al., 1993, Podrez et al., 2000). Other processes such as enzymatic modification (Kapinsky et al., 2001), glycation and lipoprotein aggregation (Hoff et al., 1992) can also have significant effects on the affinity of binding of LDL to the LDL-R, or to SRs such as SR-A or CD36.

The initial studies of Endemann et al (1993) reported that 293 cells transfected with mouse CD36 bound copper-oxidized LDL with high affinity (KD ~ 1.5μg/mL). Attempts to identify a putative binding site(s) have yielded contradictory results. A number of the functions of CD36 (including oxLDL binding) can be blocked by mAbs that bind to the immuno-dominant domain that spans amino acids 155-183 (Puente Navazo et al., 1996, Yamaguchi et al., 2000). However, in view of the diversity of ligands that bind to CD36 it seems unlikely that there will be a single binding site. A question that has been raised is whether binding of mAb induces a conformational change that interferes with the structures of a number of ligand-binding sites. To address this question, Pearce et al (1998) employed CD36-GST fusion products that spanned the entire protein and identified a region (residues 28-93) that displayed affinity for oxLDL that was comparable to that displayed by the intact molecule. However, oxLDL binding can be disrupted by deleting a single amino acid – the intra-cellular C-terminal lysine residue (Malaud et al., 2002). How the C-terminus affects binding of extra-cellular ligand is unknown but the C-terminus cytoplasmic CD36 is known to mediate the activating effects of oxLDL via NF-κB (Lipsky et al., 1997). These disparate findings will not be resolved until it is known which constituent(s) of oxLDL bind to CD36. Curiously, after separation, both the protein and lipid components of oxLDL were found to bind to CD36. Monoclonal antibody EO6, produced by immunization of apoE deficient mice, binds to oxidized but not native LDL and is able to prevent binding of oxLDL to CD36. It recognizes oxidized phospholipids specifically, but it also blocks binding of protein component of oxLDL to CD36 (Boullier et al., 2000). This finding suggests that CD36 may recognize oxLDL via oxidized phospholipid components of the particles or by phospholipid attached to apoB. A recent study suggests that the phosphocholine headgroup mediates binding to CD36 when phosphatidylcholine is modified by oxidation, and only when it is presented in the conformational context of a carrier such as an oxLDL particle or cell membrane (Boullier et al., 2005).

Although copper oxidation yields a CD36-binding lipoprotein, the level of oxidation may be in excess of the minimally-modified LDL that is associated with the pathogenesis of atherosclerosis. However, LDL that have been oxidized by exposure to biologically relevant agents such as peroxynitrite (Guy et al., 2001) and hypochlorite (Marsche et al., 2003), or enzymatically modified by exposure to cholesterol esterase (Kapinsky et al., 2001) exhibit similar binding affinities for CD36/FAT. These techniques yield mildly modified lipoprotein particles in which the apoB-100 is oxidized but there is little lipid peroxidation. Indeed, greater LDL modification is not necessarily correlated with increased affinity for CD36. Although mildly modified (and thus more heterogeneous) LDL are less well characterized and more difficult to work with, it is anticipated that they will provide information about the specific conditions in the bloodstream and/or arterial intima that lead to an inflammatory state of the sort observed in individuals predisposed to atherosclerosis.

The most important pathological consequences of uptake of OxLDL by macrophages appears to be the formation of foam cells, especially in the context of atherosclerosis where infiltration of the arterial intima with lipid-rich macrophages leads to the formation of 'fatty streaks'. Uptake of native LDL does not appear to generate foam cells because expression of the LDL-R on macrophages is subject to feedback inhibition by intracellular cholesterol levels. In contrast, exposure of macrophages to oxLDL results in increased expression of CD36 and further cholesterol accumulation (Han et al., 1997). This appears to be due to the effect of oxLDL in activating the peroxisome proliferator activating receptor-γ (PPARγ), a nuclear transcription factor that up-regulates expression of CD36 (Nagy et al., 1998, Tontonoz et al., 1998). The resulting increase in oxLDL uptake results not only in accumulation of intracellular cholesterol by macrophages but also production of pro-inflammatory cytokines and other inflammatory mediators (Janabi et al., 2000). Circumstantial evidence for a role of CD36 in the pathogenesis of foam cell formation comes from observations on human aorta. Foam cells from atherosclerotic plaques express high levels of CD36, while very little is detected on those from unaffected intima (Nakata et al., 1999). Furthermore, higher levels of CD36 were detected on foam cells from advanced plaques, compared to those from less severe lesions (Nakagawa-Toyama et al., 2001).

In addition to mediating uptake of oxLDL by macrophages, CD36 expressed by endothelial cells and platelets could also contribute to plaque formation. A recent study indicated that the increased adhesion of phagocytes to endothelial cells that is observed in the presence of oxLDL may be mediated by CD36 (Kopprasch et al., 2004). In this phenomenon, oxLDL particles may act as a multivalent ligand that forms an adhesive bridge between cholesterol laden monocytes and vascular endothelial cells. A similar mechanism could engage platelets, which are known to bind OxLDL via CD36 (Endemann et al., 1993). However, although OxLDL appears to affect platelet adhesion and aggregation, it is not known whether CD36 plays a direct role in this process. An additional role of oxLDL, acting via CD36 on human micro-vascular endothelial cells, may be to promote efflux of cholesterol from the caveolae to the lipoprotein, as well as internalization of endothelial nitric oxide synthase (eNOS) and caveolin-1. The resulting lack of nitric oxide would produce a pro-thrombotic environment that could accelerate vascular dysfunction (Uittenbogaard et al., 2000).

In the context of this Thesis, it is noteworthy that detoxification is one of the major functions of the liver. It cannot be ignored that one function of CD36/FAT in the liver might be removal of oxLDL from the circulation. Thus if hepatocytes express CD36, and CD36 is a potent receptor for removal of oxLDL from the circulation, and a normal function of hepatocytes is excretion and/or recycling of cholesterol, the liver might have a major cardio-protective role by detoxifying pro-atherogenic oxidation products of lipoproteins. This mechanism would be more effective in females, consistent with the lower incidence of cardiovascular disease observed in pre-menopausal women (Kalin and Zumoff, 1990, Barrett-Connor, 1997).

1.4.2.4 Background: Selective Uptake of Cholesteryl Ester from Lipoproteins

As discussed earlier, CD36 has affinity for native lipoproteins, as well as modified LDL. One of these ligands is HDL, the lipoprotein involved in the vital process of reverse cholesterol transport that maintains cholesterol balance in the body. Although the weight of contemporary opinion does not support a major role for CD36 in reverse cholesterol transport, such a role cannot be excluded with certainty. The following section introduces the concept of reverse cholesterol transport, selective uptake of CE from HDL and the role of SR-B1 in these processes.

Reverse cholesterol transport, initially proposed by Glomset in the 1960's, required a mechanism by which HDL-CE could be delivered to the liver (Glomset, 1968). Studies using radio-isotope-labeled HDL in vivo and binding and uptake assays *in vitro*, revealed that the fractional rate of uptake of cholesteryl ester (CE) was greater than that of the HDL apoprotein component ApoA-1 (Glass et al., 1983, Stein et al., 1983). Although different mechanisms were hypothesized to account for this observation, it was highly reproducible and indicated that HDL uptake was an entirely different process to holo-particle uptake of LDL mediated by the LDL-R (Brown and Goldstein, 1986). In the delivery of cholesterol via HDL, CE transport was facilitated specifically, while other lipids and apoproteins were excluded. For this reason, the process has been termed 'selective uptake'.

Acton et al (1996) showed in COS cells transfected with mSR-BI that SR-B1 conferred selective uptake of [^{3}H]cholesterol oleate from HDL in which the apoproteins were labeled with ^{125}I. Subsequent reports showed that the human orthologue of SR-BI (CLA-1) conferred these properties to HEK 293 cells (Murao et al., 1997) and that uptake of [^{3}H]CE by Y1-BS1 adrenocortical cells expressing endogenous SR-BI could be inhibited by anti-mSR-BI antibody (Temel et al., 1997). However, the identification of SR-B1 as a receptor that mediates selective uptake of CE has not resolved the long-standing question as to whether CE is delivered from HDL by a non-endocytic mechanism (Oram et al., 1987, Pittman et al., 1987) or whether HDL are endocytosed, depleted of CE in an intracellular compartment and then re-secreted (DeLamatre et al., 1990, Takata et al., 1988). In the first mechanism, it is envisioned that HDL are bound by the extra-cellular loop(s) of one or more SR-BI molecules, which form a non-aqueous channel that allows the hydrophobic CE to diffuse down the concentration gradient from the HDL to the cytoplasm (Rodrigueza et al., 1999). The endocytic model has been less popular, but it has gained support from recent studies in polarized cells, where SR-BI and labeled HDL have been co-localized in intracellular recycling compartments, with subsequent return of SR-B1 to the cell membrane (Silver and Tall, 2001, Silver et al., 2001). The discovery that SR-BI binds HDL and facilitates CE transport has launched an enormous research effort that has the promise of filling long-standing gaps in understanding of reverse transport of cholesterol.

1.4.2.5 SR-BI as a receptor for HDL

Calvo and Vega (1993) identified a novel 509 amino acid human protein by virtue of its sequence similarity to CD36. The protein was designated “CD36 and LIMP-II Analogous-1”, or CLA-1. Subsequently, orthologues of CLA-1 were identified in hamster-derived CHO cells and mouse-derived 3T3-L1 cells (Acton et al., 1996, Acton et al., 1994). Because CLA-1 and CD36 shared a number of ligands, as well as sequence and structural similarities, Krieger and colleagues named the family Class B Scavenger receptors and the novel protein was re-named Scavenger Receptor Class B-1 (SR-BI). This name is now widely applied to the corresponding proteins of all species. *Sr-b1* has now been cloned also from bovines (Rajapaksha et al., 1997), rats (Johnson et al., 1998, Mizutani et al., 1997) and rabbits (Ritsch et al., 2003). SR-BI has been detected in the liver in all of these species, as well as in chickens, frogs, goldfish, sharks and turtles (Duggan et al., 2002). All of the SR-BI orthologues share approximately 75-80% similarity at the amino acid level (Krieger, 1998, Ritsch et al., 2003).

High expression of SR-BI has been reported in the adrenal, ovary and liver of humans and rodents (reviewed in Azhar et al., 2003). As such, the distribution of SR-BI coincided with tissues that receive CE from HDL *in vivo*. Lower levels of SR-B1 have been detected in testis, kidney, and small intestine (Acton et al., 1996, Cao et al., 1997, Landschulz et al., 1996), endothelial and vascular smooth muscle cells of the aorta (Hirano et al., 1999, Yeh et al., 2002), cultured keratinocytes (Tsuruoka et al., 2002) and epithelial cells of the thymus (Imachi et al., 2000, Johnson et al., 2002), gallbladder (Johnson et al., 2002) endometrium (Ramachandran et al., 2001), lung (Kolleck et al., 2002) and retina (Duncan et al., 2002). SR-B1 is also expressed by monocyte-derived macrophages (Hirano et al., 1999) and by tissue macrophages from the lung (Kolleck et al., 2000) and liver (Fluiter et al., 1998).

As discussed in Section 1.2.2.1, SR-B1 shares topological and structural characteristics with the other Class B SRs, in particular with CD36. However, SR-B1 has a number of features that are unique and relevant to its role as a HDL receptor. Human *Cd36* and *Sr-bI* genes share similar intronic structures and both undergo alternative splicing at their 3’ ends (Noguchi et al., 1993, Webb et al., 1998). However, while *Cd36* alternative transcripts differ only in non-coding regions, differential splicing of *Sr-bI* results in 3 isoforms that differ in the C-terminal cytoplasmic domain. These are designated *Sr-bI*, *II* and *III*. Although *Sr-bII* mRNA can account for as much as 50% of the total *Sr-b*

transcripts (depending on species and tissue), under basal conditions the relative abundance of SR-BII protein is much lower (12% in murine liver and 5% in murine adrenal and testes) (Webb et al., 1998). The third and newly identified isoform, SR-BIII, has not been characterized with respect to relative abundance or function (Svensson et al., 2005). As the extra-cellular domains are identical, the ligand binding affinities of the isoforms are identical. Nevertheless, SR-BII appears to be considerably less efficient than SR-B1 in mediating selective CE uptake. The reasons for this difference are unclear; as studies performed using SR-BI/CD36 chimeric proteins indicate that the extracellular loop, rather than the C-terminal cytoplasmic tail, is the domain that is critical for CE uptake (Connelly et al., 2001, Connelly et al., 1999, Gu et al., 1998). Another consequence of the altered C-terminus is that the adapter protein PDZK1, which has been shown to interact with the terminal 15 amino acids of SR-BI (Ikemoto et al., 2000), does not associate with SR-BII. PDZK1 is believed to stabilize SR-BI in the plasma membrane (Silver, 2002) and indeed a recent study found that the majority of SR-BII in transfected CHO cells had an intra-cellular location. Furthermore, whereas HDL bound by SR-BI remained at the surface, HDL particles that were bound by SR-BII were internalized rapidly and became co-localised with SR-BII in the endosomal recycling compartment (Eckhardt et al., 2004). A tempting speculation is that the two isoforms perform distinct roles in HDL cholesterol transport. If they have unique functions, this could explain earlier conflicting kinetic data obtained using anti-SR-B1 antibodies (discussed in section 1.4.2.4), because frequently α-SR-BI antibodies do not discriminate between the two isoforms.

SR-BI appears to bind HDL via apoprotein (Xu et al., 1997). The affinity of SR-BI for apoproteins apoA-I, apoA-II and apoC-III was similar to the binding of either lipid-free HDL, soluble HDL proteins, or reconstituted lipoprotein particles. Mutational analysis of mouse SR-BI revealed that replacement of two glutamine residues in the extracellular region (Gln 402 and Gln 418) with positively charged residues led to a significant reduction in binding of native HDL and of selective cholesterol uptake, without a commensurate loss of LDL binding activity (Gu et al., 2000). It is likely therefore, that charge is an important factor in apoprotein recognition by SR-BI, although it was not immediately clear why some apoproteins could bind and not others. In order to dissect the recognition motif/s, apoA-1 peptides were tested for affinity for SR-BI. Both N- and C- terminal fragments bound with high affinity and were able to cross-link SR-BI molecules, indicating that there are multiple recognition sites on the HDL receptor

(Williams et al., 2000). Significantly, these researchers also described binding of a model class A amphipathic α -helix molecule (37pA) to SR-BI. Many apoproteins contain multiple repeating units of amphipathic α -helices containing a number of negatively charged amino acids clustered in the centre of each polar face. Comparing reconstituted HDL particles of different sizes and apoA-1 conformations, de Beer et al (2001) found that larger HDL particles, which have a higher α-helical content and a larger negative charge, bind to SR-BI with much higher affinity than smaller particles. Thus SR-BI recognition of apoproteins may occur via a secondary structural feature, independent of the precise amino acid sequence.

However, high affinity binding of apoprotein to SR-BI does not necessarily correlate with efficient selective transport of CE. In fact, HDL from which ApoA-I was displaced by ApoA-II in vitro displayed increased affinity for SR-BI, but was a less efficient donor of CE for selective uptake (Pilon et al., 2000). Mice deficient in ApoA-I have HDL that are large and CE-rich. ApoA-I-/- HDL are still bound efficiently by SR-BI but CE uptake is attenuated (Temel et al., 2002). Likewise, a small chemical inhibitor that blocks CE selective uptake (BLT1-5) actually enhances binding of HDL to SR-BI (Nieland et al., 2002). These findings support a two-step mechanism for non-endosomal selective uptake of CE via SR-B1. It is proposed that productive HDL binding to SR-BI is followed by a lipid transfer step (Gu et al., 1998). However, it is still not evident why only SR-BI, but not other high affinity HDL binding proteins such as SR-BII or CD36, is able to perform the later process of lipid transfer efficiently. A clue comes from a recent study in which mutation of two glycosylation sites (Asn108 and Asn173), neither of which is present in CD36, resulted in the loss of selective uptake function (Vinals et al., 2003).

In summary, there is abundant evidence that SR-BI is a functional HDL receptor and that it participates in selective uptake of CE by cells. Furthermore, *in vivo* experiments in mice suggest that SR-BI is physiologically significant in reverse cholesterol transport from HDL to cells in the liver and adrenal cortex, thus contributing to maintenance of cholesterol homeostasis and to the delivery of cholesterol for steroidogenesis. The importance of this route in humans is less clear, because the presence of cholesterol ester transport protein (CETP) allows utilization of LDL rather than HDL for reverse transport of cholesterol (Cuchel and Rader, 2003). A further function of SR-B1 that has not been reviewed here is in facilitating the afferent phase of reverse cholesterol

transport. There is considerable evidence that the molecule has a function in mediating efflux of free cholesterol from cells into HDL (de La Llera-Moya et al., 2001, de la Llera-Moya et al., 1999, Ji et al., 1997, Kellner-Weibel et al., 2000). This places SR-BI in a unique position to mediate reverse cholesterol transport at both the afferent (pick up of cholesterol from the periphery) and efferent (delivery of cholesterol to the liver) stages of this process.

1.4.2.6 CD36 as a Receptor for Native Lipoproteins

The binding of native HDL and LDL by CD36 has been assessed, using transfected cell lines (Calvo et al., 1998). Expression of human CD36 in COS cells or Sf9 insect cells (which have negligible background binding of human lipoproteins) resulted in high affinity binding of HDL, LDL and VLDL, as well as AcLDL. The apparent Kd values for binding of LDL, HDL, VLDL and oxLDL were 6.3μg/mL, 2.9μg/mL, 1.2μg/mL and 1.7μg/mL respectively (Calvo et al., 1998). Slightly higher Kd values have been calculated subsequently for binding of rat CD36 to human HDL (Connelly et al., 1999), and mouse CD36 to mouse HDL (de Villiers et al., 2001). Furthermore, the affinity of CD36 for HDL compares favourably with the binding of either mouse or human SR-BI to human HDL, with apparent Kd values between 10-35μg/mL (Acton et al., 1996, Connelly et al., 1999, Murao et al., 1997, Webb et al., 1998). At this time, the physiological significance of CD36 binding to native lipoproteins is unknown.

The finding in this laboratory that CD36 is expressed by hepatocytes (Zhang et al., 2003) raised the question of whether it has a significant role as an accessory receptor for reverse cholesterol transport. CD36 has been found to mediate selective uptake of CE in transfected cell lines but with an efficiency estimated at approximately 10-14% of SR-BI (Connelly et al., 1999, de Villiers et al., 2001, Gu et al., 2000, Gu et al., 1998). In addition, it has been reported that CE absorbed from HDL by CD36 transfectants is hydrolysed more slowly and less efficiently than by cells that express SR-B1 (Connelly et al., 2003). This evidence, combined with lack of evidence that CD36 was expressed by tissues that participate in selective uptake of CE (liver and adrenal gland), led to the view that the molecule had no physiological relevance to selective cholesterol uptake or reverse cholesterol transport. However, a very recent study has found that binding of HDL to CD36 is sensitive to the ApoA-1/ApoA-II composition of the lipoprotein. Furthermore, although uptake of CE was relatively low when concentration of HDL was

low (5μg/mL), at higher ligand concentrations (60μg/mL) it was equivalent to that of SR-B1 (De Beer et al., 2004). If confirmed, this raises important questions about the relevance of CD36 in reverse cholesterol transport under physiological or pathological conditions where lipoprotein composition may be altered.

As described above, a major function of HDL binding to SR-BI is to facilitate flux of free cholesterol from cells to HDL. The ability of CD36 to perform this function is controversial. *In vitro* studies using transfected cell lines suggest that CD36 is a comparatively poor mediator of free cholesterol efflux (Connelly et al., 2001, Parathath et al., 2004). However, other evidence indicates that CD36 is as active as SR-B1 in promoting efflux of cholesterol from intestinal epithelial cells to HDL (van Bennekum et al., 2005, Werder et al., 2001).

Although the binding affinity of native LDL to CD36 and SR-BI is lower than for native HDL, both receptors can mediate selective uptake of CE from LDL and they do so with equivalent efficiency in transfected COS cells (Connelly et al., 2003). However, attempts to block endogenous CD36 in HepG2 hepatoma cells with antibody had no effect on either LDL binding or LDL-CE uptake, suggesting that the molecule is not important in interactions with native LDL, at least in cells derived from hepatocytes (Rhainds et al., 2003). Furthermore, it is not yet established whether LDL-CE is a significant contributor to reverse cholesterol transport *in vivo,* in either rodents or humans.

Finally, while it is recognized that VLDL can bind CD36 with high affinity (Calvo et al., 1998), no functional consequences of this ligand-receptor interaction have been reported until recently. A recent study, using human platelets, suggests that VLDL binding to CD36 results in enhanced thromboxane A2 production and accelerated collagen-mediated platelet aggregation (Englyst et al., 2003). This may represent an additional mechanism by which high levels of triglyceride-rich lipoproteins contribute adversely to the pathogenesis of atherosclerosis.

1.4.2.7 Summary of CD36 and Lipoproteins

CD36 can bind both native and modified lipoproteins with high affinity. Expression of CD36 appears to confer on cells the ability to internalize oxLDL and it also appears to

facilitate the processes of free cholesterol flux and selective uptake of CE from native lipoproteins, albeit with low efficiency. Overall, the *in vitro* assays using transfected cell lines have failed to establish a definitive role for CD36 in mediating lipid uptake from native lipoproteins. In fact, the only function of the molecule in lipoprotein metabolism that has been demonstrated unequivocally is its role of inducing foam cell formation when macrophages are incubated with oxLDL. Nevertheless, *in vitro* experimental data is not always an accurate predictor of physiological function. There is sufficient uncertainty to leave open the possibility that CD36 may function as a physiological mediator of CE uptake from either HDL or LDL, especially in cells where the ratio of SR-BI to CD36 is low, under conditions of high ligand concentration, or where the composition of the lipoprotein is changed.

1.4.3 States of Altered CD36 Expression

1.4.3.1 Spontaneously Hypertensive Rats and their Transgenic Counterparts

Spontaneously hypertensive rats (SHR) have well characterized traits that include; hypertension, hypertriglyceridaemia, defective adipose metabolism of fatty acid, hyperglycaemia and insulin resistance. During efforts to map these characteristics by quantitative trait locus analysis, a peak of linkage to the defects in FA and glucose metabolism was identified on chromosome 4 in the same region that encodes CD36 (Pravenec et al., 1999). Further analysis revealed that some strains of SHR rats have a truncated copy of *Cd36* gene (Section 1.3.3) and are CD36-deficient (Aitman et al., 1999). When this region of chromosome 4 was replaced with the corresponding region from Wistar Kyoto (WKY) rats (the strain from which SHR were originally derived), insulin-mediated glucose uptake was partially restored and the lipogenic activity of adipocytes was corrected completely. However, elucidating the relationship between defective CD36 and the SHR phenotype has been complicated by two factors. Firstly, neither the SHR strain, nor the WKY strain from which it was derived, were inbred prior to distribution from the original breeding colony in Tokyo. Secondly, the SHR strain itself has been divided into two sub-strains one of which is stroke-prone (SHR-SP) while the other is not (SHR). Gotoda et al reported that neither SHR nor SHR-SP that have been maintained since their development in Japan carry the mutated copy of *Cd36*. The defective CD36 observed in SHR sourced from NIH is believed to have arisen as a

new mutation in the founders used to establish the NIH colony. No metabolic differences could be identified between SHR from either source (Gotoda et al., 1999).

Therefore, lack of CD36 is not the sole cause of the metabolic defects observed in SHR rats. The evidence suggests either that gene(s) other than *Cd36* are responsible for the metabolic defects observed in SHR rats or that the phenotypes that are shared by all SHR strains are mediated by different genetic mechanisms that have accrued independently. To investigate whether there is a causative link between defective CD36 and the metabolic traits displayed by SHR rats, Pravenec et al (2001) produced a transgenic line of SHR that expresses wild type CD36 constitutively under the control of the EF-1α promoter. Although the levels of *Cd36* mRNA and CD36 protein were low relative to the WKY strain, they were sufficient to improve glucose tolerance, serum FA levels and insulin-responsive glucose utilization by muscle tissue. It has also been shown that these metabolic parameters can be normalised by feeding SHR with a diet rich in short-chain fatty acids, presumably because short chain fatty acids enter cells passively and do not require a protein transporter. Indeed this difference in handling of short- and long-chain FAs supports the case that CD36 has an important physiological role in uptake and metabolism of long-chain FAs (Hajri et al., 2001). The hypertensive phenotype in SHR rats was not affected by CD36 rescue or feeding short-chain FAs, while the low plasma cholesterol levels found in SHR rats does not map to chromosome 4 (Kato et al., 2000). Thus the genetics that underlie insulin resistance, its accompanying metabolic disturbances and associated disease risk factors appear to be multi-factorial in rats, as in humans.

1.4.3.2 CD36 Null and Transgenic Mice

Homozygous gene knockout of *Cd36* produces profound effects on glucose, fatty acid and cholesterol metabolism and homeostasis. While the gross appearance and lifespan of CD36 null mice was normal, in the fasted state they had raised serum levels of total cholesterol, free FA and TG and decreased plasma glucose compared with wild-type controls. At the cellular level, peritoneal macrophages displayed reduced ability to internalize oxLDL, while adipocytes lacked the saturable component of long-chain FA uptake. Compared with wild-type mice, HDL from CD36 null mice were larger and contained more phospholipid and cholesterol. VLDL particles contained higher than normal levels of TG, whereas IDL/LDL particles were relatively TG poor (Febbraio et

al., 1999). These authors interpreted the differences in cholesterol metabolism in the null mice as secondary to the defect in long chain fatty acid uptake, rather than a direct effect of altered handling of lipoproteins (although this possibility could not be dismissed).

In CD36 null mice, tissues with a high metabolic demand for FA display reduced uptake of FA, providing strong support for a physiological role of the molecule in FA transport. However, in addition to raising plasma FA concentration, impaired uptake of FA in CD36 null mice produced a number of other outcomes. Thus, while it was hypothesized that increased plasma TG levels resulted from increased VLDL-TG synthesis by the liver (Hajri et al., 2002), recent experiments indicate that high levels of plasma FFA can inhibit lipoprotein lipase and have downstream effects by decreasing release of TG from lipoproteins (Goudriaan et al., 2005). Furthermore, decreased FA uptake results in lower levels of acyl-CoA, causing accumulation of 1,2-diacylglycerol (DG) and depletion of intracellular TAG stores in muscle and adipose tissue (Coburn et al., 2000).

CD36 null mice display similarities with NIH strain SHR rats, such as decreased FA uptake in skeletal, heart and adipose tissue. However, the metabolic phenotypes of these models of CD36 deficiency are not identical. Under normal conditions (chow diet), CD36 null mice exhibit evidence of enhanced insulin responsiveness. They have rapid glucose clearance, increased glucose uptake by muscle, decreased glycogen storage in muscle and liver, and levels of triglyceride that are lower in muscle but higher in the liver than in wild-type mice (Hajri et al., 2002). In contrast, SHR rats display insulin resistance. Despite these apparently advantageous metabolic characteristics, CD36 null mice are less able to adapt to dietary challenge and assume a SHR-like phenotype when stressed by a high fructose diet, developing glucose intolerance, reduced glucose uptake by muscle and hyper-insulinemia (Hajri et al., 2002, Pravenec et al., 1999).

To test the role of CD36 in uptake of FA by muscle, the molecule was over-expressed in transgenic mice under the control of the muscle specific creatine-kinase (MCK) promotor. These mice had leaner body mass, lower levels of plasma TG and FA, and reduced VLDL-associated TG. Although these mice had lower levels of plasma cholesterol than controls, the cholesterol content of lipoprotein fractions was unchanged (Ibrahimi et al., 1999). This suggests that decrease in FA consumption by peripheral

tissues may not be the only mechanism responsible for the changes in cholesterol homeostasis observed in CD36 null mice.

Finally, studies in CD36 null mice provide support for a pro-atherogenic role of CD36. When CD36 deficiency is superimposed on the pro-atherogenic background of ApoE deficiency, the double knockout mice had fewer and less severe aortic lesions when fed a high fat diet than the mice with ApoE deficiency alone. This was paradoxical because the CD36-ApoE double knockout mice had higher total plasma cholesterol, particularly in the LDL, IDL and VLDL lipoprotein fractions, than the single ApoE knockout mice (Febbraio et al., 2000). Studies using bone marrow chimeras showed that the cardio-protective effects were due to bone marrow-derived cells and were attributed to reduced oxLDL uptake by CD36-deficient macrophages (Febbraio et al., 2004).

1.4.3.3 CD36 Deficiency in Humans

CD36 deficiency in humans can be divided into 2 groups. The Type I phenotype is characterized by the absence of CD36 on both platelets and monocytes/macrophages (and all cell types), while in the type II phenotype, expression of CD36 by cells of the monocyte/macrophage lineage is near normal (Yamamoto et al., 1990). Approximately 0.3% of the Japanese population has type I CD36 deficiency and 4% has the type II CD36 deficiency. At least five mutations have been identified that can result in these phenotypes (Kashiwagi et al., 2001).

Although many CD36 deficient individuals display no obvious phenotypic abnormalities, generation of anti-CD36 antibodies following either pregnancy or transfusion can lead to life-threatening thrombocytopenia (Bierling et al., 1995, Curtis et al., 2002, Kashiwagi et al., 2001, Morishita et al., 2005). CD36 deficiency has also been identified in patients with Takayasu arteritis, a form of systemic vasculitis of unknown pathogenesis (Yagi et al., 2004). Most importantly, type I CD36 deficiency has also been associated with defective myocardial LCFA uptake and insulin resistance/metabolic syndrome phenotypes. Tanaka et al (2001) reported that of 6970 patients who underwent myocardial ^{123}I-BMIPP scintigraphy, 33 (0.47%) displayed complete deficiency in myocardial LCFA uptake. All of these patients carried genomic *Cd36* mutation/s and expressed no CD36 protein on either platelets or blood monocytes. A study conducted on 26 individuals with CD36 deficiency patients found increased

plasma triglyceride and fasting plasma glucose levels, decreased HDL cholesterol and higher blood pressure than normal individuals (Miyaoka et al., 2001). In a larger study, 61 patients with Type I CD36 deficiency were divided into diabetic and non-diabetic groups and compared with a non-diabetic control group that was matched for age, sex and body mass index (Furuhashi et al., 2003). Oral glucose tolerance tests showed that the percentage of patients with insulin resistance was not significantly different between the CD36 deficient group and the control group, suggesting that human CD36 deficiency is not directly associated with insulin resistance. However, the later study confirmed that serum triglycerides were raised in both CD36 deficient groups compared to the control group. Interestingly, in direct contrast to the study of Miyaoka et al (2001), both of the CD36-deficient groups had higher HDL cholesterol levels than controls. It is difficult to reconcile the data in these studies, especially as age, gender ratio and BMI in the sampled groups are almost identical and the mean HDL-C levels reported for the CD36-deficient patients are also very similar (1.24 mmol/L compared with 1.26-1.33 mmol/L). The differences between the two studies arise from the mean HDL-C levels that are reported for the control groups (1.01 mmol/L compared with 1.61 mmol/L). Further studies are required to characterize the lipoprotein profile in type I CD36 deficiency, which appears to be moderate rather than severe.

Studies *in vitro* using human monocytes suggest that CD36 deficiency might have a cardioprotective effect. Monocyte-derived macrophages from CD36-deficient donors take up 60% less radiolabelled ox-LDL than cells from normal individuals (Nozaki et al., 1995). Furthermore, induction of NF-κB activity and secretion of pro-inflammatory cytokines in response to oxLDL is reduced in macrophages from CD36-deficient donors compared with normal donors (Janabi et al., 2000). It appears, therefore, that Cd36/ApoE double knockout mice and CD36-deficient humans may share a reduction in susceptibility to atherosclerosis. However, the effect of CD36-deficiency in humans is less dramatic than that observed in CD36-deficient mice, perhaps because other scavenger receptors can facilitate oxLDL uptake in humans (Nozaki et al., 1995).

1.4.4 Summary of CD36 Functions

Overall, the results of studies in SHR rats, CD36 transgenic and null mice and in CD36-deficient humans clearly indicate a physiological role for CD36 in lipid metabolism. However, it is difficult to encompass all of the effects of CD36 in a single explanation.

This is not surprising, given the range of ligands that have affinity for the molecule and its relatively broad tissue distribution. The finding that deficiency of CD36 and over-expression of CD36 have complementary effects on long-chain FA uptake by skeletal muscle provides strong support for an important role of the molecule in FA transport. In addition, the role of CD36 in oxLDL uptake by macrophages is well supported and the importance of this process in the pathogenesis of atherosclerosis is becoming clearer. However, the impact of CD36 (or its absence) on either glucose or cholesterol homeostasis is less clearly defined. With one exception (de Villiers et al., 2001), all of the studies reviewed here were conducted and interpreted without consideration of expression of CD36 in the liver. Given the central role of the liver in many metabolic processes, the significance of CD36 in FA, cholesterol and lipoprotein metabolism cannot be understood completely without an understanding its function in that organ.

1.5 Factors Regulating Expression Of The Class B Scavenger Receptors CD36 and SR-BI

Much of this project focuses on factors regulating expression of CD36 (and to a lesser extent, SR-BI) in the liver. In contrast, most of the research in this field has investigated regulation of CD36 in adipocytes, macrophages and myocytes, or SR-B1 in macrophages and steroidogenic cells of the adrenal cortex, ovary or testis. As discussed below, regulation of the two scavenger receptors appears to be quite tissue-specific and can be influenced at a number of levels including gene expression, post-transcriptional events, post-translational modifications, protein trafficking and the presence of additional stabilizing anchor proteins at the plasma membrane. Cellular events such as activation, differentiation or cell-to-cell adhesion may also affect levels of CD36 and/or SR-BI at the cell surface. In this section, the promoter structures of *Cd36* and *Sr-bI* are discussed, followed by a summary of the agents that have been demonstrated to modify class B scavenger receptor expression *in vitro* and *in vivo*.

1.5.1 Promoter Structure

1.5.1.1 The Promoter Structure of *Cd36*

Analysis of the 5' untranslated region of *Cd36* in humans revealed the presence of binding sites for a number of regulatory factors. These include; two NFI sites, the phorbol ester responsive elements AP-2 and AP-3, a NF-κB/rel site, interferon-gamma (IFNγ) response elements and a sterol response element (SRE) (Armesilla and Vega, 1994). Subsequently, promoter analysis by Tontonoz et al (1998) demonstrated binding of PPARγ-RXR (peroxisome proliferator-activated receptor – retinoid X receptor) heterodimers to a DR-1 (direct repeat with one nucleotide spacer) motif located at position -273 to -260. PPARγ ligands significantly induced both a transfected *Cd36* promoter-reporter construct containing the DR-1 element and endogenous *Cd36* expression in primary human monocytes. Polyomavirus enhancer-binding protein 2/core-binding factor (PEBP2/CBF) is also a potentially important regulator of *Cd36* in monocytes and a PEBP2/CBF binding site was identified at position –103 (Armesilla et al., 1996). Human *Cd36* promoter activity was preserved in macrophage-derived cell lines within a segment containing the first 158 bp upstream of the start site. In contrast, promoter activity was not detected in the segment –273 to +19 bp when human *Cd36* constructs were transfected into mouse C2C12N cells. Analysis of the murine *Cd36* upstream region revealed promoter activity in two separate elements, located in the fragments –309 to –170 and –170 to –81. Both of these segments are required for high-level expression of *Cd36* in adipocytes. Binding sites for Oct-4, core-binding factor (CBF) and PPARs were identified within these regions. Further, although a CCAAT motif was located approximately 375 bp upstream of exon I, no TATA box was identified in the 5' flanking region of mouse *Cd36.* A SP-1 site and a GC-rich sequence were observed, which may serve as the promoter in the case of murine *Cd36* (Teboul et al., 2001). Very recently, a DR-3 type pregnane X receptor response element (Zhou et al., 2006) and a DR-7 type Liver X receptor response element (Zhou et al., 2008) have also been identified in the murine *Cd36* promoter, located between -1134 and -251. The contribution of nuclear hormone receptors to the transcriptional regulation of *Cd36* in the liver and other tissues is discussed in Section 1.5.4.1.

Detailed analyses have been recently performed on the human and murine *Cd36* gene sequences further upstream to the regions initially investigated. This revealed a complex multi-promoter structure that is present in both species. Three independent and

conserved promoter sequences have been identified that span approximately 20kb and can result in at least five mRNA isoforms. The various isoforms are generated via alternative splicing of short exonic sequences, upstream of exon three (which contains the translational start site). Analysis of the promoter selectivity in various mouse tissues (liver, intestine, kidney, spleen, heart, skeletal muscle, brown adipose tissues and tongue) indicated that the alternative isoforms are differentially expressed and further, that the respective promoter units responded differently to PPARα and PPARγ ligands (Sato et al., 2007). The rat *Cd36* promoter sequence was not specifically investigated, however alignment with a single published sequence revealed homology with the murine 1A isoform. It is not known whether multiple transcripts are also present in this species (Sato et al., 2002).

Collectively, these studies suggest that the regulation of the *Cd36* gene is unusually complex, involving tissue-specific promoter isoform expression, the potential action of many transcription factors as well as species-specific traits. Currently, there is a paucity of information regarding either the nature of the *Cd36* promoter in rats, or about the regulatory elements that are important in regulating expression of *Cd36* in the liver.

1.5.1.2 The Promoter Structure of *Sr-bI*

The *Sr-bI* promoter region has been cloned and partially characterised in both human (Cao et al., 1997) and rat (Lopez and McLean, 1999, Mizutani et al., 2000). The promoter regions from these species are not identical, suggesting that control of *Sr-bI* expression may not be the same in all species. Sequence analysis of the region upstream of the human *Sr-bI* revealed an AT-rich sequence (TATA-box) 30 bp upstream of the transcription start site, located 140 bp upstream of the start codon (assigned as +1). No CAAT box was identified, although the sequence from +1 to -440 bp was considered to be GC rich (Cao et al., 1997). A number of additional putative sequence elements were also reported, including five SP-1 sites, two C/EBP sites and one each of NF-Y, AP-1, SF-1 and SREBP binding sites. Of particular interest was the SF-1 site located at -210 to -217 bp. SF-1 is a member of the orphan nuclear receptor gene family and it is expressed constitutively in steroidogenic tissues such as the adrenal cortex, Leydig cells of the testes, and granulosa, thecal and corpus luteal cells of the ovary. *In vitro* studies using a human *Sr-bI* promoter/reporter construct indicated that the region containing this SF-1 binding site was required for transcriptional activity in Y1 adrenocortical cells

and gel shift assays showed that purified SF-1 protein bound to this sequence (Cao et al., 1997). However, SF-1 is not expressed in liver, mammary gland or human placenta, suggesting that another transcription factor or factors must be involved in regulation of *Sr-bI* in these tissues. With regard to the liver, the liver receptor homolog -1 (LRH-1) protein was shown subsequently to bind to the SF-1 site, and may contribute therefore, to *Sr-bI* transcription in these non-steroidogenic tissues (Schoonjans et al., 2002). Finally, having demonstrated that agonists of both peroxisome proliferator-activated receptors (PPAR) α and γ were able to enhance SR-BI protein levels in human hepatoma cell lines, Malerod et al (2003) (identified a PPAR response element (PPRE) in the human *sr-bI* promoter at -459 to -472 bp.

Analysis of the 5' un-translated region of rat *Sr-bI* by two independent groups has resulted in some contrasting observations. Lopez and McLean (1999) cloned backwards to position -2228 (relative to the ATG start codon). These researchers identified a transcriptional start site at -107 and a putative TATA box between postitions -178 to -187. Like the human *Sr-bI* promoter, no CAAT sequence was located but a number of other putative regulatory elements were described including; 11 E boxes, 7 Sp1 sites, 4 estrogen receptor response element (ERE) half sites, 2 AP-1 sites, 2 SREBP-1c binding sites and 1 SF-1 site. SREBP-1c and SF-1 were shown to act synergistically to induce expression of rat *Sr-bI* reporter plasmid in co-transfection studies in both HepG2 and Y1 murine adrenal cells. In contrast, an independent study, investigating the rat *Sr-bI* promoter mapped the transcriptional start site to position -128, and described different sequence beyond -1517bp (relative to the ATG start codon) (Mizutani et al., 2000). Three Sp1/Sp3 binding sites were identified in a region proximal to the start site (-141 to -1) and all three of these were shown to be important for high level expression of *Sr-bI*. However, in this study no effect of SF-1 on rat *Sr-bI* expression was observed in co-transfection experiments in HeLa cells. The reasons for the discrepancy in sequence and activity of the promoter between these two studies are not clear. In summary, analysis of the human, murine and rat *Sr-bI* has provided some mechanistic insights towards explaining the regulation of *Sr-bI* by hormones such as hCG and ACTH and by changes in intracellular sterol levels that occurs in steroidogenic tissues. However, the factors that are important for regulation in the liver require further investigation.

1.5.2 Regulation by Cytokines and other Immunoregulatory Molecules

Many cytokines have been shown to exert a regulatory effect on *Cd36* transcription, particularly by blood monocytes and peritoneal macrophages. This is of particular interest with respect to the inflammatory microenvironment that is believed to precede atherosclerotic lesion development in the arterial intima. The effects of some cytokines vary considerably between tissues. For example, mouse *Cd36* mRNA expression is up-regulated in the liver, down regulated in adipose tissue (Memon et al., 1998) and is unaffected in monocytes (Nakagawa et al., 1998, Yesner et al., 1996) by tumour necrosis factor-α and interleukin-1.

A negative regulatory effect of interferon γ (IFNγ) on *Cd36* transcription has been reported in both human monocytes (Nakagawa et al., 1998) and murine foam cells (Panousis and Zuckerman, 2000). Given that the human *Cd36* promoter contains putative consensus sequences of IFNγ regulatory elements (Armesilla and Vega, 1994) it is possible that this cytokine is capable of directly modulating *Cd36* expression. In contrast, the effect of other cytokines on *Cd36* expression such as interleukin-4 (Feng et al., 2000, Yesner et al., 1996), transforming growth factor-β (Han et al., 2000) and interleukin-10 (Rubic and Lorenz, 2006) appears to be secondary to their effect on PPARγ (either via transcriptional activation or phosphorylation of the receptor). The regulation of *Cd36* by PPARγ is discussed in detail in section 1.5.4.1.

The capacity of cytokines to regulate expression of SR-BI has not been extensively investigated. In one study, analysis of liver tissue from hamsters suggested that *Sr-b1* mRNA and protein levels were reduced in the liver following treatment with TNFα or Il-1 (Khovidhunkit et al., 2001). Neither IFNγ nor IL-10 appear to affect transcription of *Sr-b1* (Panousis and Zuckerman, 2000, Rubic and Lorenz, 2006) and transforming growth factor-β exposure to murine macrophage-derived foam cells resulted in only a modest (~25%) decrease in transcript levels (Zuckerman et al., 2001).

Although there is some evidence for gender differences in the expression of certain cytokines, their effect on gene transcription is by nature, acute and short-lived. Therefore they are unlikely to play a role in maintaining a gender bias in scavenger receptor expression in the liver or elsewhere.

1.5.3 Regulation of Hepatic Class B Scavenger Receptor Expression by Metabolic Ligands

As discussed previously (Section 5.1), the promoter regions of both *Cd36* and *Sr-bI* contain regulatory sequences that can bind hierarchical transcription factors such as PPARs and SREBPs that are expressed in the liver and are important for the maintenance of metabolic homeostasis. The activity of these transcription factors is highly responsive to the metabolic state of the cell and can be modulated by fatty acids and sterols respectively. It is possible that cells and/or tissues have the capacity to regulate the expression of CD36 and SR-B1 according to their metabolic requirements, to sense the availability of nutrients in the extracellular mileu, or in order to respond to homeostatic demands of the body. For example, *in vivo* studies have shown that feeding rodents a diet that is enriched with specific fatty acids or cholesterol results in modulation of SR-BI expression in a cell-specific and species-dependent manner. Male rats that were fed a high cholesterol diet exhibited a modest decrease in SR-BI expression by hepatic parenchymal cells, but significantly increased expression by Kupffer cells (Fluiter et al., 1998, Hoekstra et al., 2005). However, no changes in hepatic SR-BI expression were observed in response to an increased dietary cholesterol intake by mice (Mardones et al., 2001) or hamsters (Woollett et al., 1997).

In humans, dietary fatty acids are known to regulate lipoprotein concentrations. Isocaloric exchange of polyunsaturated FA in place of saturated FA is associated with decreased LDL and, consequently, total plasma cholesterol concentrations (Mensink and Katan, 1992). As very high intake of polyunsaturated FA may also result in decreased HDL cholesterol (Mattson and Grundy, 1985), it was hypothesised that the underlying mechanism may involve the regulation of hepatic SR-BI (Spady et al., 1999). In a study conducted in hamsters, these authors showed that supplementation of the control diet with polyunsaturated FA compared with saturated FA (30% of digestible energy) increased SR-BI transcript and protein expression by approximately 50%. Furthermore, Loison et al (2002) demonstrated that the saturated FA myristic acid reduced expression in SR-BI protein at physiological concentrations (2.4% of digestible energy) in male hamsters. In the context of this study, it is interesting to note that recent data suggests the effect of dietary FA on lipid metabolism in hamsters is also associated with gender. Morise *et al* (2006) reported no specific changes in hepatic SR-BI protein expression between animals fed a diet with polyunsaturated FA, compared with those fed a dietary saturated FA. However, in animals fed the polyunsaturated FA diet the

gender difference in hepatic SR-BI expression (lower in females and higher in males) appeared to be more pronounced. A decrease in plasma HDL cholesterol associated with the polyunsaturated FA diet was observed in males only. In contrast to the studies perfomed in hamsters, isocaloric exchange of n-6 or n-3 polyunsaturated fatty acids for carbohydrate (10% of digestible energy) produced only a modest reduction of plasma HDL cholesterol and did not alter SR-BI protein expression at all in livers from LDL receptor knock-out mice (Vasandani et al., 2002). The effect of dietary fatty acids on SR-BI expression in the livers of rats has not been investigated to date.

In C2C12 muscle cells, treatment with oleic acid resulted in increased ubiquitination of CD36 protein and decreased total CD36 levels. In contrast, the addition of insulin had an opposite effect. The rate of fatty acid uptake was also modulated, proportionally, by the amount CD36 protein that was present in these cells under either treatment. These experiments demonstrated that *in vitro*, CD36 turnover can be directly regulated by high levels of ligand (Smith et al., 2008).

1.5.4 Regulation of CD36 and SR-BI by Members of the Nuclear Hormone Receptor Superfamily

The nuclear hormone receptors are a superfamily of DNA-binding transcription factors, many of which play important roles in lipid and glucose metabolism. Within this superfamily, are steroid hormone receptors such as the estrogen and androgen receptors, receptors which have no known endogenous ligands (known as orphans) and receptors previously classified as orphans for which ligands have now been assigned (referred to as adopted). Classically, nuclear hormone receptor action is mediated by binding of the activated receptor to a specific hormone response element in the promoter of the target gene (Mangelsdorf et al., 1995).

1.5.4.1 Orphan and Adopted Nuclear Receptors

The peroxisone-proliferator-activated receptor (PPARs) subgroup has three isoforms (PPARα, PPARγ and PPARδ) that are expressed at different levels and perform distinct roles in various tissues. PPARs are activated by endogenous FA and FA derivatives, in addition to many isoform selective pharmacological agonists and regulate processes such as FA oxidation, adipogenesis, insulin sensitivity and lipid deposition. *Cd36* is a

recognized target gene for both PPARα (Motojima et al., 1998) and PPARγ (Tontonoz et al., 1998). Although PPARγ-regulated expression of CD36 has been demonstrated to be critical in adipocyte development and the progression of macrophages into foam cells, in the liver, induction of CD36 is controlled by PPARα. Administration of fibrates (PPARα agonists) to either diet-induced obese C57Bl/6 mice, or obese Zucker rats increased levels of *Cd36* mRNA expression in the liver but not white adipose tissue in both models (Guerre-Millo et al., 2000). Recently, examination of the multiple promoter structure of the *Cd36* gene has revealed that responsiveness to PPARα agonists is conferred by the most distal promoter isoform (1A) (Sato et al., 2007). Although significant levels of induction of *Cd36* were observed, this promoter isoform displayed a considerably delayed response in human, rat and mouse liver cells, compared with other PPARα target genes. The delayed onset of induction was not observed in other tissues in which the 1A isoform is present and responsive to PPAR activation (Sato et al., 2002, Sato et al., 2007). The reason for this is not understood, however it underscores the cell-type specific nature of CD36 regulation. Of particular interest to this study, was the observation that levels of PPARα expression varied considerably between males and females in both murine and rat liver. However, in contrast to our observation of CD36, expression of hepatic *Pparα* mRNA and protein was higher in males compared to females (Jalouli et al., 2003). In addition, PPARα signaling was also impaired in female mouse liver (Ciana et al., 2007). Therefore although it may be important for acute induction of CD36 in the presence of specific PPAR activators, it appeared unlikely that PPARα would be involved in establishment or maintenance of the female predominant pattern of CD36 expression in the liver. For this reason, PPARα was not considered for further investigation in this study.

Like *Cd36*, *Sr-bI* is a well documented target gene for a number of nuclear hormone receptors. For example, upregulation of both promoter activity and SR-BI protein expression has been demonstrated in murine and human hepatocytes cell lines in response to treatment with the liver X receptor/retinoid X receptor (LXR/RXR) agonist 22-R-OH-cholesterol (Malerod et al., 2002b). This is significant as LXR/RXR has been previously shown to regulate the expression of a number of genes that are crucial for reverse cholesterol transport i.e. cholesterol 7α-hydroxylase and the cholesterol transporters ABCA1 and ABCG1 (Edwards et al., 2002). Conversely, treatment of primary rat hepatocytes with bile acids resulted in down regulation of SR-BI protein expression via the action of the farnesoid X receptor/retinoid X receptor (FXR/RXR)

nuclear cascade. Although no FXR response elements were identified in the *Sr-bI* promoter, downstream effectors in the FXR/RXR pathway were shown to interfere with liver receptor homologue-1 (LRH-1) induced transactivation (Malerod et al., 2005). Down-regulation of SR-BI by rifampicin, an activator of pregnane X receptor (PXR) in rat liver cells has also been observed. PXR is a xenobiotic receptor that can also be activated by endogenous steroids such as progestins and estrogen. In the context of cholesterol metabolism, PXR acts to prevent the toxic accumulation of bile acid in the liver (Sporstol et al., 2005). Finally, PPARα and PPARγ activation by agonists such as fibrates and thiazolidinediones is believed to enhance the clearance of cholesterol by the liver, contributing to their athero-protective effects. The expression of SR-BI protein was increased by both PPARα and γ agonists in rat liver cells, however in contrast to *Cd36*, the γ isoform of PPAR was shown to be the strongest inducer of *Sr-b1* promoter activity (Malerod et al., 2003). Thus, the expression of SR-BI in the liver is closely coupled to cholesterol metabolism and bile acid synthesis via common nuclear receptor regulatory pathways.

1.5.4.2 Steroid Hormone Receptors

The steroid hormone receptors are ligand-dependent transcription factors that regulate the expression of hormone target genes. Although the roles of the sex steroids estrogen, progesterone and testosterone are best characterized in gonadal and associated reproductive tissues, it is now well established that their respective receptors are expressed and active in a wide variety of extra-gonadal tissues e.g. central nervous system, skeletal muscle, bone, endothelium, liver and haematopoetic cells (Wierman, 2007). Notably, the expression of the low-density lipoprotein receptor (LDL-R) in the liver has been demonstrated to be positively regulated by estrogen (Windler et al., 1980), via the action of the estrogen receptor (ER) which forms a complex with another transcription factor (Sp1) to bind to the LDL promoter (Li et al., 2001). The regulation of SR-BI by estrogen was first described by Landschulz et al. (1996). In this study, administration of high dose estrogen to male rats was shown to result in reciprocal regulation of SR-BI in the liver (decreased expression) and adrenal gland (increased expression). Furthermore compared to male rats, females had higher steady state levels of SR-BI in the adrenal and lower levels in the liver. In contrast, expression of SR-BI in other rat tissues i.e. kidney, heart, testes, skeletal muscle and adipose tissue was unaffected by estrogen. The regulation of SR-BI by estrogen appears to be not only

tissue, but cell type specific, as opposing effects were observed by thecal compared to granulosa cells in the ovary (Landschulz et al., 1996) and parenchymal versus Kupffer cells in the liver (Fluiter et al., 1998).

Evidence from several subsequent studies has suggested that the effect of estrogen on SR-BI expression may not be due to direct regulation via the estrogen receptor. Firstly, Graf et al (2001) demonstrated that although steady-state levels of SR-BI were lower in female compared to male rats and gonadectomy caused a detectable increase in SR-BI protein in livers from the females, no changes in the levels of *Sr-bI* mRNA were observed. Interestingly, they also described a reciprocal change in the expression of the alternative isoform, *Sr-bII*. Expression of this splice variant was elevated in female rat liver compared to males, reduced by gonadectomy and restored following estrogen treatment. In the liver at least, estrogen may be influencing expression of SR-BI/SR-BII by a post-transcriptional process that involves regulation of isoform switching. Secondly, hypophysectomy of male rats abrogated estrogen suppression of SR-BI protein in the liver and induction in the adrenal gland, indicating the involvement of one or more secondary factors. Co-treatment with dexmethasone and estrogen implicated ACTH as an intermediary factor regulating SR-BI expression in the adrenal gland, however no affect of dexmethasone treatment was observed in the liver (Stangl et al., 2002). Although a number of studies have demonstrated modest reductions in *Sr-bI* mRNA in rat liver in response to estrogen treatment (Srivastava, 2003, Stangl et al., 2002) the magnitude of this change does not appear to be commensurate with the observed changes in SR-BI protein expression and in cholesterol ester uptake. In summary, modulation of SR-BI expression in response to estrogen appears to be species, tissue and cell-type specific. Evidence suggests that regulation is occurring post-transcriptionally and indirectly (via ACTH in the adrenal gland), although the mechanism by which this occurs in the liver has not yet been described. A single study described upregulation of SR-BI expression in human monocyte derived-macrophages and HepG2 hepatoma cells in response to supraphysiological doses of testosterone (Langer et al., 2002) but this has not been confirmed, or the subject of any further investigation.

In contrast to the extensive investigations of steroid hormone regulation of SR-BI, there is very little information regarding the effect of these hormones on expression of CD36, particularly in the liver. Sporadic reports have indicated that CD36 expression is down-

regulated by estrogen in hormone-dependent breast cancer cell lines (Uray et al., 2004), or by tamoxifen-treatment in normal and neoplastic human breast-tissue (Silva et al., 1997). Estrogen has also been shown to block ritonavir –induced CD36 expression in macrophages in an ERalpha dependent manner (Allred et al., 2006). However, detailed investigation of the expression of CD36 in response to changes in systemic steroid hormone levels has not been performed for any of the metabolic or steroidogenic tissues.

1.5.5 Summary of SR-B Regulation

The regulation of SR-BI and CD36 expression has been the subject of considerable research, focused primarily on transcriptional regulation by nuclear hormone receptors and inflammatory cytokines. The expression of both of these class B scavenger receptors is responsive to the activity of numerous orphan and adopted nuclear hormone receptors, although they do not appear to be involved in the establishment or maintenance of sexually dimorphic expression of either SR-BI or CD36. Rather, they may be important for acute induction of expression in response to inflammatory or metabolic stimulus. Estrogens may be key negative regulators of SR-BI expression in the liver although further investigation is required to clarify the involvement of the ER and to resolve inconsistencies in the reported data. The role of estrogens in the regulation of CD36 in the liver has not been studied, despite some evidence for involvement in other tissues. Similarly, the effect of testosterone on the expression of either SR-BI or CD36 has not been explored.

Finally, although not discussed here in detail, independent factors can exert a further level of control by influencing the cell surface localisation of both receptors. In the skeletal muscle CD36 protein is stored in endosomal compartments which are available for rapid translocation to the cell membrane, in response to insulin signalling or contraction. In contrast, the surface localisation of SR-BI is controlled by physical association with an adapter protein named PDZK-1. It is therefore relevant to consider not only total protein levels but the localisation of CD36 within the cell when examining aspects of regulation.

1.6 Potential Mechanisms Of Gender Regulation Of Hepatic Gene Expression

There are a number of factors that must be considered when commencing the evaluation of gender differences in general. First, is the possibility of innate chromosomal sexual dimorphism of cells which could potentially persist, irrespective of differences in the hormonal mileu. This idea is supported by the fact that sex-related differences can be observed in non-sexual tissues (eg in heart, O'Connell et al., 2003). Unfortunately a clear hypothesis has not yet been proposed for how this might be achieved, however, there is evidence that cells derived from male or female embryos, prior to any sex hormone exposure still exhibit profoundly different responses to some physiological challenges (Penaloza et al., 2009). Thus, the gender and consequently, gender-dependent characteristics are imprinted in these cells a long time before sexual differentiation of the organism occurs. If this were the case with regard to the expression of CD36 and SR-BI in the liver, it would be anticipated that sexually dimorphic hepatic expression of these genes would be present in the neonate, and would not be subject to the influences of sex hormones during puberty or in the adult.

However, the most profound influences on sexually dimorphic gene expression result from the action of hormones. The sex steroids (estrogens and progestins produced predominantly by the ovaries in females, and androgens produced by the testes in males) exert differentiating effects on both sexual and non-sexual tissues. Classically, the action of the sex steroids have been ascribed to the transcriptional effects on target genes, mediated by the appropriate nuclear hormone receptor i.e. ER, PR and AR respectively (for reviews see Fannon et al., 2001, Guiochon-Mantel et al., 1996). The expression of these hormone receptors and their various isoforms in tissues is another level at which the expression of target genes can be regulated (Pelletier, 2000). In addition, a growing body of evidence suggests that the steroid hormones have non-genomic mechanisms of action, which are hormone receptor-independent and operate at a post-transcriptional level (Luconi et al., 2002, Falkenstein et al., 2000, Bjornstrom and Sjoberg, 2005). Furthermore, pituitary hormones such as LH, FSH, TH and GH display gender-specific patterns of production, secretion and actions. These hormones interact with the sex steroids via a complex feedback system referred to as the hypothalamo-pituitary-gonadal axis.

In this study, the primary aim is to better understand the mechanism/s that underlie sexually dimorphic expression of two class B scavenger receptors in the liver. To this end, the relationship between gonadal and/or pituitary hormones and the expression of CD36 and SR-BI will be examined *in situ*, using a number of models of hormone modulation. The first task of this study is to establish the extent of sexually dimorphic scavenger receptor expression in adult rats, using a number of laboratory strains and surveying a wide representation of cell and tissue types. Although the primary focus of the study is the regulation of CD36 in the liver, knowledge of the comparative expression of both receptors in other tissues by males and females may provide insight into the factors that contribute to hepatic gender differences. The expression of the scavenger receptors will then be assessed at critical stages of postnatal development in order to investigate the effect of normal physiological changes in sex-related hormones in both genders. Finally; surgical, pharmacological and genetic interventions will then be employed to study in closer detail the role played by gonadal and pituitary hormones in the regulation of the class B scavenger receptors.

1.7 Project Hypotheses

To investigate the sexually dimorphic expression of the class B scavenger receptors, CD36 and SR-BI, the following hypotheses will be tested.

In rodents, sexually dimorphic (female-predominant) expression of CD36 occurs only in the liver. SR-BI expression occurs in an opposite pattern (male predominant).

Prior to puberty, hepatic expression of both scavenger receptors is equivalent in males and females.

The sex steroids estrogen and testosterone are required for the establishment and maintenance of the sexually dimorphic CD36 pattern of expression in the adult rat liver.

Sexually dimorphic expression of CD36 in the rat liver persists following the disruption of pituitary hormone signalling.

Chapter 2 MATERIALS AND METHODS

2.1 Experimental Animals

The strain and sources of all rodents used in this study are listed in Table 2.1. The animals were either housed in barrier conditions in the Laboratory Animal Services facility (Medical School North, 6th Floor, University of Adelaide) or in conventional conditions in the IMVS animal facility (1st Floor, IMVS). All animals were raised on a 12-hour light-dark cycle with *ad libitum* access to water and normal rat chow. Unless specifically stated, animals were young adults (8-10 weeks of age) at the time experiments commenced. All animal experimentation described in this study was performed with approval from the University of Adelaide Animal Ethics Committee and/or Royal Adelaide Hospital Animal Ethics Committee.

Table 2.1 List of Rodents used in this study

STRAIN	ABBREVIATION	BREEDING STATUS	SOURCE
RATS			
Dark Agouti	DA	inbred	Gillies Plains, IMVS
Dwarf	DW	inbred	University of Queensland
Lewis	LEW	inbred	ARC, Perth
Spontaneously Hypertensive Rat – Non-Stroke	SHR-N	inbred	ARC, Perth
Spontaneously Hypertensive Rat – Stroke Prone	SHR-SP	inbred	Gillies Plains, IMVS
Sprague Dawley	SD	outbred	Gillies Plains, IMVS
Wistar Kyoto	WKY	inbred	ARC, Perth
MICE			
BalbC	-	inbred	Gillies Plains, IMVS
C57Bl/6	-	inbred	Gillies Plains, IMVS
CBA	-	inbred	Gillies Plains, IMVS

2.2 Surgical Procedures - Gonadectomy

Throughout the surgical procedures, rats were anaesthetised using isoflurane (David Bull Laboratories, Vic, Australia) delivered with O_2 and NO_2. Male rats were castrated or sham-castrated via a midline incision in the lower abdomen. Each testis was drawn into the abdomen and after a silk ligature was tied around the spermatic cord, the testis was removed together with the epididymis and the testicular fat pad. In the sham operation, a ligature was tied distal to the testis and only the fat pad was removed. Oophorectomy was performed via an abdominal incision. A ligature was tied around the junction of the uterine horn and the Fallopian tube, allowing the ovary, the Fallopian tube and the attached adipose tissue to be removed. In the sham-operation, only the ovarian fat was removed. After gonadectomy, or the sham-operations, the abdomen was closed with silk sutures and the rats were housed normally until required.

2.3 Isolation of Primary Rat Hepatocytes

The procedure for isolation of primary rat hepatocytes was performed by Dr. Peter Coyle, (IMVS) using a technique used routinely in their laboratory (Rofe et al., 1980). An abdominal laparotomy was performed on a rat anaethetised with 0.3mL of sodium pertobarbitone (Nembutal, Abbott Laboratories, Sydney Australia). Five hundred units of heparin was injected into the sub-hepatic vena cava and the portal vein was then cannulated using a 22G I.V. catheter, secured tightly with silk suture and connected with tubing to an inflow perfusion reservoir. The thoracic superior vena cava was also cannulated with a 16G I.V. catheter, secured and connected to tubing. The inflow was driven by a peristaltic pump at a constant rate of 40mL/minute. Before reaching the rat, the perfusion buffer flowed through a 37^0C water incubator and was gassed with 5%CO_2 in oxygen. Initially, the liver was washed with perfusion buffer (see appendix) for one minute, then 0.5mmol/L sodium EDTA in perfusion buffer for 3 minutes. The liver was then perfused with recirculating perfusion buffer containing 50mg/100mL of Collagenase H (Roche Diagnostics Australia, Castle Hill, NSW) and $CaCl_2$ (59mg/100mL) for approximately 20 minutes. At the end of the digestion, the entire liver was excised and the parenchymal cells were released by incision of the capsule. The cell preparation was passed through a 250μm stainless steel sieve to remove the undigested tissue. Cells were suspended in William's Media E (W4125, Sigma, St Louis,

Missouri. see appendix for specific details), centifuged at 100 x g for 5 minutes and then the supernatant was decanted. This step was repeated three times differentiating hepatocytes that pellet at this speed, from other hepatic cell types that remain in the supernatant. Cells to be maintained in tissue culture were then seeded into collagen-coated flat-bottom 35mm culture wells at a density of 1 x 10^6 cells/2mL of William's Media E. Cells were allowed to adhere at 37^0C for 2 hours (5%CO_2), and then washed with fresh media prior to the commencement of experiment.

2.4 Hormone Treatments

Rats that received hormone supplementation were injected subcutaneously on the flanks with 1μL per gram of body weight of either E2 (estradiol-17-β, Sigma, St.Louis, USA) dissolved in absolute ethanol and then diluted to 10μg/mL in peanut oil, progesterone (75 μg/mL in peanut oil) or Sustanon (Organon (Aust.), Lane Cove Australia. The latter contains the testosterone esters propionate (20mg/mL), phenylpropionate (40mg/mL) and isocaproate (40mg/mL) and it was diluted 1:15 in peanut oil prior to injection. Rats that received E2 or progesterone were injected daily whereas rats that received the slow-release formulation of testosterone were injected every second day. Sham-treated animals received equal volumes of peanut oil, or were untreated (as specified). Zoladex 3.6 mg (GnRH superagonist) was provided by Zeneca (Macclesfield, Cheshire, UK), through the courtesy of Professor Brian Setchell (University of Adelaide). A single pellet was administered subcutaneously to each rat, under anaesthesia using the syringe and introducer provided by the manufacturer.

2.5 Neonatal MSG Treatment

For each experiment, 2 pregnant rats were delivered to the medical school animal house between 3 and 6 days prior to giving birth. The litter from one rat received 4mg/10μL/gram of body weight sodium monosodium glutamate (MSG) dissolved in MilliQ H_2O by subcutaneous injection on days 2, 4, 6, 8 and 10 post-partum. The other litter was given 10μL/gram of body weight 10% NaCl, under the same protocol (Zelena et al., 1998). Pups were weaned and sexed at 21 days and then raised in standard barrier conditions to maturity.

2.6 Tissue Collection

Animals were exsanguinated by cardiac puncture under isoflurane anaesthesia. Collected blood was transferred to Vacuette Serum Gel Tubes, allowed to clot for 30 minutes at room temperature, spun for 15 minutes at 18 x g and the resulting serum was transferred to eppendorf tubes and stored at -20^{0}C until required. Tissues harvested for RNA extraction *i.e.* liver, adrenal gland and heart were cut into slices (less than 5 mm width) and placed immediately into RNAlater (Qiagen) solution to stabilize. Within one hour samples were placed into -800C freezer for storage until needed. Tissues required for preparation of membrane proteins was sliced, rinsed briefly in 1 x PBS (see appendix), patted dry on filter paper and then snap frozen in liquid nitrogen and stored at -80^{0}C. Fresh frozen liver sections for immunohistochemistry were prepared from the caudal margin of the middle lobe of the liver. From this, wedge-shaped blocks were oriented in OCT medium (Sakura, Tokyo, Japan) to present cross-sections to the microtome blade. The OCT-embedded tissue was snap frozen in liquid nitrogen-cooled isopentane and stored at -80^{0}C until required. Heart, testis, ovary, spleen, tongue, trachea, kidney, small intestine, soleus (red skeletal muscle) and gastrocnemius (white skeletal muscle) and adrenal gland tissues were also prepared in this manner.

2.8 Antibodies

The primary antibodies used in this study are listed in Table 2.2. For immunohistochemistry using the direct technique, the primary antibodies were detected using horseradish peroxidase (HRP) conjugated to either immunopurified F(ab')2 sheep anti-mouse immunoglobulin (Amersham Pharmacia Biotech, Buckinghamshire, England) or affinity purified donkey anti-rabbit IgG (Rockland, Pennsylvania, USA). Unless specifically stated, these secondary conjugates were used at a dilution of 1:20 and 1:50 respectively. For the Western Blot technique, bound antibody was detected with HRP conjugated to goat anti-rabbit IgG at a dilution of 1:10000 (Pierce Biotechnology, Rockford, Il.)

Table 2.2 Primary antibodies used in this study

Specificity	Name	Nature	Dilution	Manufacturer/Reference
CD36 (Rat)	UA009	mAb (IgG1) hybridoma S/N	Neat	(Zhang et al., 2003)
CD36 (mouse, human)	MO25	Purified mAb (IgG1) (~1.6mg/mL)	1:2000 (WB)	(Tandon et al., 1989)
CD36 (mouse, rat)	JC63.1	Purified mAb (IgA)	various	Cayman Chemical
Giardia Negative isotype control	1B5	mAb (IgG1) hybridoma S/N	Neat	G.Mayrhofer (unpublished)
SR-BI	Anti-SR-BI	Purified (IgG) rabbit polyclonal	1:2000	Novus Biologicals (NB 400-104)
Giardia	R127B8	Purified (IgG) rabbit polyclonal	1:2000	P.Ey (unpublished)

2.8 Radioimmunoassays for Serum Hormones

A competitive radioimmunoassay was used to measure testosterone in rat serum. This assay was a commercial kit (Spectria Testosterone [^{125}I] Coated Tube Radioimmunoassay, Orion Diagnostica, Espoo, Finland) that was generously supplied by Dr Peter O'Loughlin, IMVS. This assay accurately measures testosterone in serum at concentrations between 0.1 and 50 nmol/L and has a cross-reactivity of less than 5% for 5α-Dihydrotestosterone and less than 0.1% for all other steroids tested. 17β-estradiol (E2) was measured in serum using the Ultra-Sensitive Estradiol RIA kit (DSL, Texas, USA). This assay has a linear range between 0.0185 and 2.775 nmol/L and a cross-reactivity that is less than 3% with estrone and negligible with the other estrogenic metabolites and steroids that were tested. Prior to testing, stored rat serum samples were thawed, spun at 14, 000 rpm for 5 minutes and the resulting supernatant was then used immediately. Both assays were performed according to the manufacturers instructions and all samples were tested in duplicate. The amount of ^{125}I present in each tube was counted for 60 seconds using a Packard AutoGamma Counter.

2.9 Preparation of Cell Smears

Smears of primary hepatocyte cells were prepared manually because smears prepared using the cytospin had a low proportion of intact cells. Single cell suspensions were washed in 1 x PBS and then pelleted in a 1.5mL microfuge tube. A fine glass pipette was used to remove as much supernatant as possible. Cells were then gently resuspended in 1 volume of 50% FCS in 1 x PBS. Between 20 and 50 μL of cell suspension is then placed on a glass slide and spread thinly using a pipette tip. Excess liquid was then removed by flicking slide vigorously 2 or 3 times. Slide was then air-dried for 30 minutes and then stored with desiccant at -20^0C until needed.

2.10 Immunofluorescent labelling of Primary Hepatocytes

Single cell suspensions (prepared as described in 2.9) were labelled by indirect immunofluorescence for flow cytometry. Hepatocytes were enumerated using trypan blue exclusion and then transferred to a FACS tube (Becton Dickinson, San Jose, CA, USA). Cells were pelleted by centrifugation at 200 x g for 10 minutes and the cell pellets were then washed in 3mL of cold FACS wash buffer (see appendix). Following re-centrifugation, cells were resuspended in 50μL primary antibody (with 10% NRS) and incubated for 1 hour on ice. Cells were then washed twice in cold FACS buffer and then resuspended in FITC-conjugated secondary antibody (FITC-goat anti-mouse Ig, BD Pharmingen, 12064D) diluted 1:100 in 1 X PBS with 10% NRS. After incubation on ice for one hour in the dark, the cells were washed twice and then resuspended in 0.5ML of FACS buffer and analysed by flow cytometry immediately.

2.11 Flow Cytometric Analysis

Flow cytometric analysis of labelled cells was performed using a FACScan flow cytometer (Becton Dickinson), equipped with Cell Quest software (versions 3.1 onwards). Intact cells were gated based on their forward-scatter (FSC, size) and side-scatter (SSC, complexity) characteristics. Subsequent analysis of fluorescence involved only those cells within the gated population. The 1B5 negative control antibody was used to optimise instrument settings and background fluorescence levels. Background fluorescence was defined as that part of the negative peak which contained 98-99% of the recorded events falling in the gate.

2.12 Indirect Immunohistochemistry

Six-micron thick sections were cut from tissue samples embedded in OCT, using a Bright cryostat, at -24^{0}C and then air-dried onto glass slides at room temperature for 30 minutes. Unless specifically stated, sections were fixed in absolute ethanol for 10 minutes at 4^{0}C. Following re-hydration in 1 x PBS, each section was covered with approximately 50μL of primary antibody (with 10 % normal serum) and incubated for one hour in a humid chamber at 4OC. Unbound primary antibody was rinsed off in three changes of 1 x PBS and incubation was repeated with the HRP-conjugated secondary antibody (affinity purified F(ab')2 sheep-anti-mouse Ig-HRP). After washing, antibody bound to the tissue was detected using DAB (SigmaFast). The sections were then counterstained with Gill's hematoxylin (see appendix) and mounted in DePeX medium (BDH, Poole, UK). Air-dried cell smears prepared from single cell suspension were also labelled and stained using this protocol.

2.13 Video Image Analysis

Areas of liver sections stained by mAb UA009 were measured by video-image analysis, using an Olympus BX40 microscope equipped with a video camera. Specimens were inspected using the Immuno Stain Analysis application and the data was analysed with VideoPro32 software (Leading Edge, Adelaide, Australia) as described (Coventry et al., 1995). Thresholds for specific staining were set on sections stained with the isotype control mAb 1B5 and lightly counterstained with haematoxylin. Immunoperoxidase staining (brown) was discriminated from the haematoxylin counterstain (blue) and the detection thresholds were then kept constant for the complete analysis. Using a X 4 objective lens (4-6 fields per section), vascular structures were excluded and measurements were made of the total area of parenchyma and the area occupied by stained hepatocytes. The area of immunoperoxidase staining was expressed as a percentage of the total area of the frame accepted for analysis and the values for the fields from each section were averaged.

2.14 Preparation of Total Membrane Proteins

Snap-frozen slices of liver were ground to a powder under liquid nitrogen in a mortar and pestle and then lysed in a 10-fold volume of ice-cold Homogenization Buffer (see appendix) supplemented with 5µL/mL Mammalian Protease Inhibitor Cocktail P8430 (Sigma, St.Louis, USA) Samples were then passed through a 21-gauge needle 5 times, centrifuged (3000 x g) for 10 minutes, and the resulting supernatant was re-centrifuged for 45 minutes at 100,000 x g (Jokinen et al., 1994). The final pellet (containing the membrane fraction) was washed and then resuspended in 1 x TBS (see appendix) and the total protein concentration of the sample was determined using a Bradford-based assay (Biorad Protein Assay, Biorad, Hercules, CA, USA).

2.15 SDS-Polyacrylamide Gel Electrophoresis

Individual samples were adjusted so that the desired amount of total protein was contained in 16µL. Four microlitres of 5 x Sample Buffer (see appendix) was added to each sample and these were then boiled (96^0C for 5 minutes) prior to loading into a discontinuous polyacrylamide gel (stacking gel 4%, resolving gel 12%). The loaded gel was electrophoresed at 200V for 45-60 minutes in 1 x Running Buffer (see appendix) using the Mini-Protean II unit (Biorad). A coomassie pre-stained broad-range protein marker (New England Biolabs, 6-175 kDa) was included on every gel.

2.16 Western Blot

Following electrophoresis the gel was equilibrated in 1 x Transfer buffer (see appendix) for 30 minutes. The separated proteins were then transferred onto PVDF membrane using a wet transfer system (100mA, 70 minutes). The membrane was then blocked using 5% skin milk powder in TBS containing 0.1% Tween (TTBS) and probed with primary antibody overnight at 4^0C. Membrane was then washed for five minutes each in five changes of TTBS then incubated for 1 hour with HRP-conjugated anti-rabbit secondary antibody. Bound antibody was visualised by SuperSignal West Femto ECL detection substrate (Pierce Biotechnology, Rockford, Il). Finally, the membrane was

exposed to X-Ray film for 1-4 minutes. Densitometry was performed on the films using an FX scanner and analysed using QuantityOne software (Biorad).

2.17 RNA Extraction

Tissues stored in RNAlater were thawed, weighed and then homogenized in buffer RLT from the RNeasy Mini Kit (Qiagen, GmbH, Germany), using a motorized pellet pestle (Sigma), followed by 5 passages through a 21 Gauge needle. Total RNA was extracted on RNeasy columns according to the manufacturer's instructions. During extraction, a 15-minute treatment with DNase (RNase-free DNase, Qiagen) was included to remove any residual genomic DNA from the preparation. The total yield of RNA was determined by measuring the absorbance of each sample at 260nM using a spectrophotometer (SmartSpec, Biorad).

2.18 Reverse Transcription

Complementary DNA (cDNA) was prepared using the Superscript II reverse transcription system (Invitrogen, Groningen, Netherlands). First strand synthesis was performed for each sample according to the manufacturer's instructions using 1mM oligo dT and 2μg of total RNA. The cDNA was then diluted 1:4 in MilliQ water and stored at -20^0C until required.

2.19 Oligonucleotide Design

Oligonucleotides pairs that specifically amplify the target gene were designed using Primer Designer software (Scientific and Educational Software) for endpoint PCR or using Primer Express Software (Applied Biosystems) for real-time PCR. The sequence and position of all the primers used in this study is listed in Table 2.3. In all cases, the amplicons were designed to cross intron-exon boundaries so that amplification from contaminating genomic DNA could be detected. Short sequence BLASTn searches were performed to ensure that the oligonucleotides were specific for their respective target

genes. The oligonucleotides were purchased from Geneworks (Adelaide, Australia), dissolved in MilliQ water to give 5nM working solutions and stored at -20^{o}C.

Table 2.3 List of Oligonucleotide sequences used for Reverse Transcription PCR

NAMES	SEQUENCE 5'→3'	PRODUCT SIZE	GENBANK SEQUENCE/ REFERENCE
PRIMER PAIRS USED IN END-POINT PCR EXPERIMENTS			
mCD36 FP1 mCD36 RP1	CCAATGGTCCCAGTCTCATT CCTTAAAGGAATCCCCGTGT	417 bp	NM_007643
mGAPDH FP1 mGAPDH RP1	TGATGACATCAAGAAGGTGGTGAAG TCCTTGGAGGCCATGTAGGCCAT	240 BP	(Kohler et al., 2003)
SR-BI/FP3 SR-BI/RP1	GCACGGTTGGTGAGATCCT GGTGGATGTCTAGGAACAAGG	625 bp	D89655.1
β-ACTIN FP1 β-ACTIN RP1	CTGGAGAAGAGCTATGAG AGGATAGAGCCACCAATC	330 bp	(Nudel et al., 1983)
PRIMER PAIRS USED IN REAL-TIME PCR EXPERIMENTS			
SR-BI/II RT FP1 SR-BI/II RT RP1	AGGGAGTTCAGACAAAAGGTTAACA AAACGAAGGCTTCGGTTCTCT	80 bp	U76205
SR-BI RT FP2 SR-BI RT RP2	TAGTAAAAAGGGCTCGCAGGAT GGCAGCTGGTGACATCAGAGA	70 bp	U76205
CD36 RT FP1 CD36 RT RP1	TGCTGCACGAGGAGGAGAAT GCACCAATAACGGCTCCAGTA	68 bp	AF113914
36B4 FP 36B4 RP	AGATGCAGCAGATCCGCAT GGATGGCCTTGCGCA	81 bp	(Singh Ahuja et al., 2001), X15096
PRIMER USED FOR REVERSE TRANSCRIPTION			
Oley80 – Oligo dT	GCGTTTAAA (T)30 (A/G/C) (A/G/C/T)	N/A	Dr P. Ey unpublished

2.20 End-Point PCR

Hot-start PCR was performed on cDNA samples produced from heart or liver total RNA. The reactions had a total volume of 25µL, comprised of: 2.5µL each of forward and reverse primers, 12.5µL of Amplitaq Gold Master Mix (Applied Biosystems, Foster City, CA), 7µL MilliQ H_2O and 2µL of cDNA. The reactions were performed in a PTC-100 Thermocycler (MJ Research, Watertown, MA) using the following thermal protocol: i) enzyme activation at 95^{0}C for 10 minutes, ii) N cycles of 95^{0}C for 30 seconds (strand separation), 57-64^{0}C for 30 seconds (primer annealing) and 72^{0}C for 2 minutes (extension). The precise PCR conditions *i.e.* number of amplification cycles,

the starting concentration of cDNA and the annealing temperature for primer pairs was optimized for each unique experiment.

2.21 Gel Electrophoresis and Densitometric Estimation of Relative mRNA Levels

Five microlitres of 6 x Orange G loading buffer (see appendix) was added to each PCR tube and the products obtained from end-point PCR reactions were resolved by electrophoresis through a 1.5% agarose gel in 1 x TAE (see appendix). A marker lane containing a 100bp DNA ladder (Geneworks, Adelaide) was included on each gel. Following electrophoresis, the gel was placed in SYBR gold (Molecular Probes, Eugene, USA) diluted 1:10000 in 1 x TAE staining solution for between 10-25 minutes. Bound SYBR gold was detected as a fluorescent signal using the FX scanner (Biorad). Densitometric analysis of the resulting bands was performed using QuantityOne software (Biorad).

2.22 Real-Time PCR

Semi-quantitative real-time PCR was performed on an ABI Prism 5700 Sequence Detection System, using SYBR Green PCR Master Mix reagents (Applied Biosystems). Reactions were made to a final volume of 25µL, consisting of 12.5µL of SYBR Green PCR Master Mix, 2.5µL of cDNA, 1.5µL each of 5µM forward and reverse primers and 7.5µL of MilliQ water. Amplification was initiated by a 10-minute incubation at 95^0C, followed by 40 repetitions of a two-step cycle (95^0C for 15 seconds and 60^0C for one minute). Melt curve analysis was performed at the conclusion of the amplification protocol. The relative initial levels of transcripts were calculated using the comparative CT method. Optimization of reactions and an assessment of efficiency of amplification of the target and housekeeping genes over a range of cDNA template dilutions were performed according to the manufacturer's instructions. This is discussed in detail in Chapter 5. Each sample was performed in duplicate and levels of target genes were expressed relative to the levels present in cDNA prepared from normal male liver.

2.23 Statistical Analysis

Numerical data are presented as mean ± standard deviation or mean ± standard error as indicated in the figure legend. The significance of differences between means of experimental groups was tested using one-way analysis of variance, and Tukey's post-test was used for real-time PCR and video-image analysis. Western analysis of SR-BI expression was assessed using a two-tailed Student's T-test. Significance was accepted at $P<0.05$. Statistical calculations were performed using GraphPad Prism (GraphPad Software, San Diego, California, USA).

Chapter 3 GENDER DIFFERENCES IN EXPRESSION OF CLASS B SCAVENGER RECEPTORS

3.1 Introduction

The work presented in this chapter was performed to assess the general hypothesis that expression of CD36 and SR-BI in the liver is different between males and females of the same species. This hypothesis was derived partly from an initial observation made by Dr. Xingqi Zhang, in her doctoral thesis. Dr. Zhang characterized a new monoclonal antibody (mAb) and showed that it recognized rat CD36. This was determined by using the antibody to immunoprecipitate a protein from female rat liver and analysis of the N-terminal amino acid sequence. Contrary to earlier reports on the distribution of CD36 in the rat (Abumrad et al., 1993, Van Nieuwenhoven et al., 1995) Dr Zhang's investigations in a number of rat strains revealed that the protein was expressed extensively in the liver (Zhang et al., 2003). With the exception of this apparent anomaly and the absence of the protein on platelets, the distribution of the UA009 antigen (CD36) was consistent with that described in rats and other species. It was found subsequently, that the level of expression of CD36 varied considerably between males and females of the DA strain (Zhang et al., 2003). It was suggested that the low levels of CD36 in liver from male rats and the use of male rats in most studies might account for the reported absence of CD36 in the liver.

In contrast to the paucity of studies investigating hepatic expression of CD36, the presence of the Scavenger Receptor Class B - I (SR-BI) in rat liver is well documented. Furthermore, a number of studies have reported that SR-BI also displays sexually dimorphic expression in the liver (Graf et al., 2001, Landschulz et al., 1996, Stangl et al., 2002). However, unlike CD36, expression of SR-BI is reportedly biased towards greater expression in male rats compared with females. While the primary focus of this project was to investigate hepatic expression of CD36, the possibility that the expression of these related receptors might be co-ordinated in the liver merited inclusion of SR-BI in the study. Information was also sought on the comparative distributions of the two

scavenger receptors in hepatic and extra-hepatic tissues. Four hypotheses are examined in this chapter.

1) Adult male rats of various strains express less CD36 protein in the liver than age-matched females.
2) Adult male rats express more SR-BI in the liver than age-matched females.
3) Gender dimorphism in expression of CD36 and/or SR-BI is not found in extra-hepatic tissues.
4) Gender bias in expression of CD36 in the liver is also present in mice.

3.2 Gender Dimorphism in Expression of Hepatic CD36 in the Rat

3.2.1 Background

The initial studies in DA rats were immunohistochemical, examining frozen sections of liver and freshly prepared primary hepatocytes. Other strains were also examined to determine whether the gender difference observed in DA rats was of general significance. The Sprague Dawley strain was chosen because it is used frequently in studies on lipid metabolism. Two sub-strains of Spontaneous Hypertensive Rats (SHR), the stroke-prone (SHR-SP) and the normotensive sub-strain derived from NIH (SHR-N), are used as models for type 2 diabetes. The SHR-SP and the wild-type Wistar Kyoto strain are CD36 replete, while the SHR-N sub-strain has a defective copy of the *Cd36* gene (Pravenec et al., 1999). Lewis rats, the parent strain of growth hormone deficient dwarf (dw/dw) rats, were studied for reasons discussed in detail in Chapter 6.

3.2.2 Effect of Gender on Expression of CD36 by Hepatocytes in the Liver of DA Rats

Expression of CD36 in the liver of young adult male and female DA rats (n=5 per group) was examined by staining frozen sections with mAb UA009, using indirect immunohistochemistry. MAb 1B5 (anti-giardia) was used as an isotype-matched negative control. As shown previously (Zhang et al., 2003), CD36 was expressed within the hepatic lobule in a centrilobular pattern. Staining of hepatocytes was most intense

surrounding the central vein, with intensity diminishing in a gradient towards the portal triad. In liver from female rats, the majority of hepatocytes were stained, and only relatively few parenchymal cells in the peri-portal region were unstained. In contrast, in liver from males, only the hepatocytes closest to the central vein in each lobule expressed CD36 (Fig. 3.1A and B).

In order to determine whether CD36 is expressed by the hepatocytes, or by the sinusoidal endothelial cells, primary hepatocytes were prepared by perfusing the liver with collagenase (section 2.3). The centrifugation protocol used to prepare primary hepatocytes is reported to yield samples with greater than 90% purity. When cell smears were stained with mAb UA009, the majority of hepatocytes from female liver were stained, while only a small percentrage of male hepatocytes were stained. (Fig. 3.1 D and E). Neither the frozen liver sections, nor the smears of primary hepatocytes, were stained by incubation with mAb 1B5 (Fig. 3.1C and F).

3.2.3 Expression of CD36 by Hepatocytes in Liver from Male and Female Rats from Several Laboratory Strains

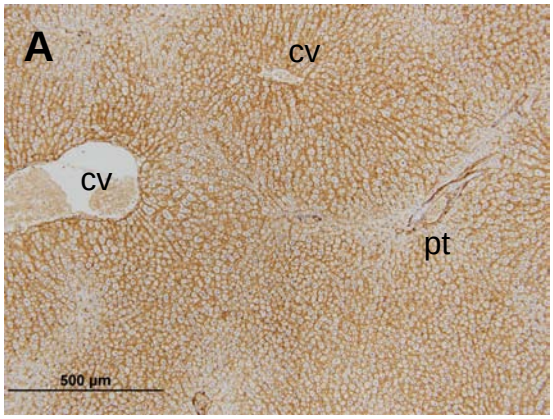
As shown in Fig. 3.2A and B, mAb UA009 did not stain liver sections prepared from either male or female rats of the SHR-N sub-strain. In all of the other strains, the expression of CD36 was greater in sections prepared from females. (Fig. 3.2C-H). Furthermore, the relative staining intensities and the relative areas of the hepatic lobules stained by mAb UA009 in females and males of these strains followed the same pattern as that observed in DA rats. However, in females of some strains (SHR-SP and WKY), the area of unstained or weakly stained cells in the periportal regions was greater than in female DA rats (Fig. 3.2E and G), while very few hepatocytes were stained in males of these strains (Fig. 3.2F and H). In contrast, in the Lewis strain, essentially all hepatocytes in the hepatic lobules of female rats were stained intensely by mAb UA009 (Fig. 3.2I). In males of the Lewis strain (Fig. 3.2J), the area of hepatocytes stained was comparable to male DA rats, but there was high intensity of staining.

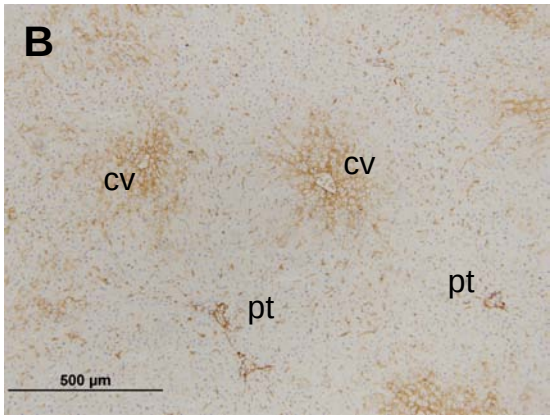
Figure 3.1: Detection of CD36 in liver sections and isolated hepatocytes from adult DA rats, using the indirect immunoperoxidase technique.

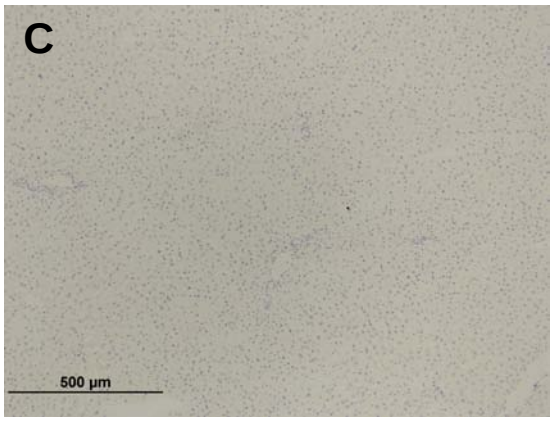
Female liver, showing staining of hepatocytes by mAb UA009 in a zone extending from the central vein (cv) almost to the portal tract (pt) (A). In comparison, mAb UA009 stained only a small number of cells localised around the central vein in liver from male rats (B). In cell smears of freshly isolated hepatocytes, most cells were stained by mAb UA009 in preparations from female rats (D) whereas only a minority of cells were stained in preparations from male rats (E). Liver sections (C) and hepatocyte cell smears (E) were not stained by mAb 1B5 (anti-giardia, negative isotype control).

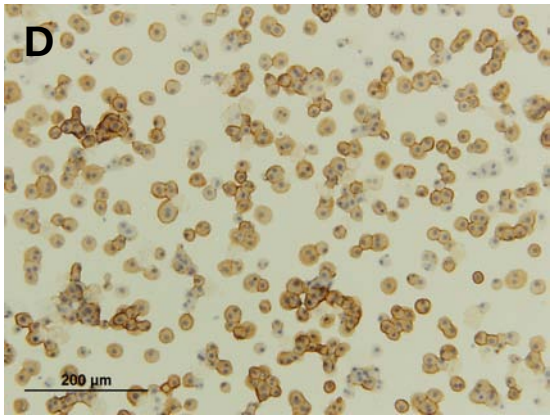
A-C, photographed using a 10 X objective lens.

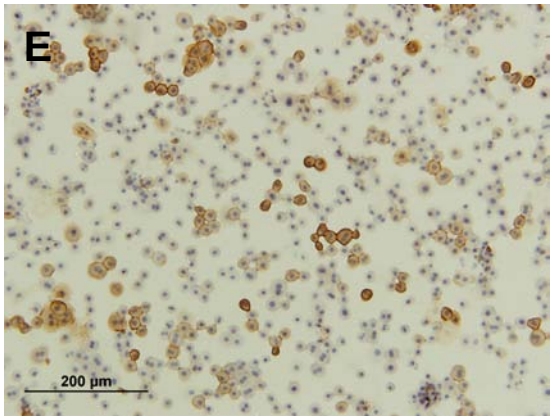
D-F, photographed using a 20 X objective lens.


A
cv
cv
pt
500 µm


B
cv
cv
pt
pt
500 µm


C
500 µm


D
200 µm


E
200 µm

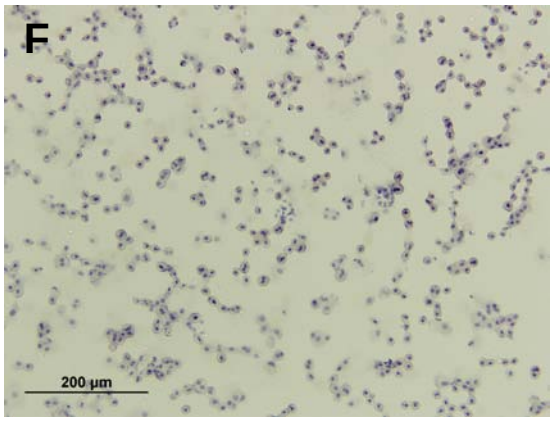
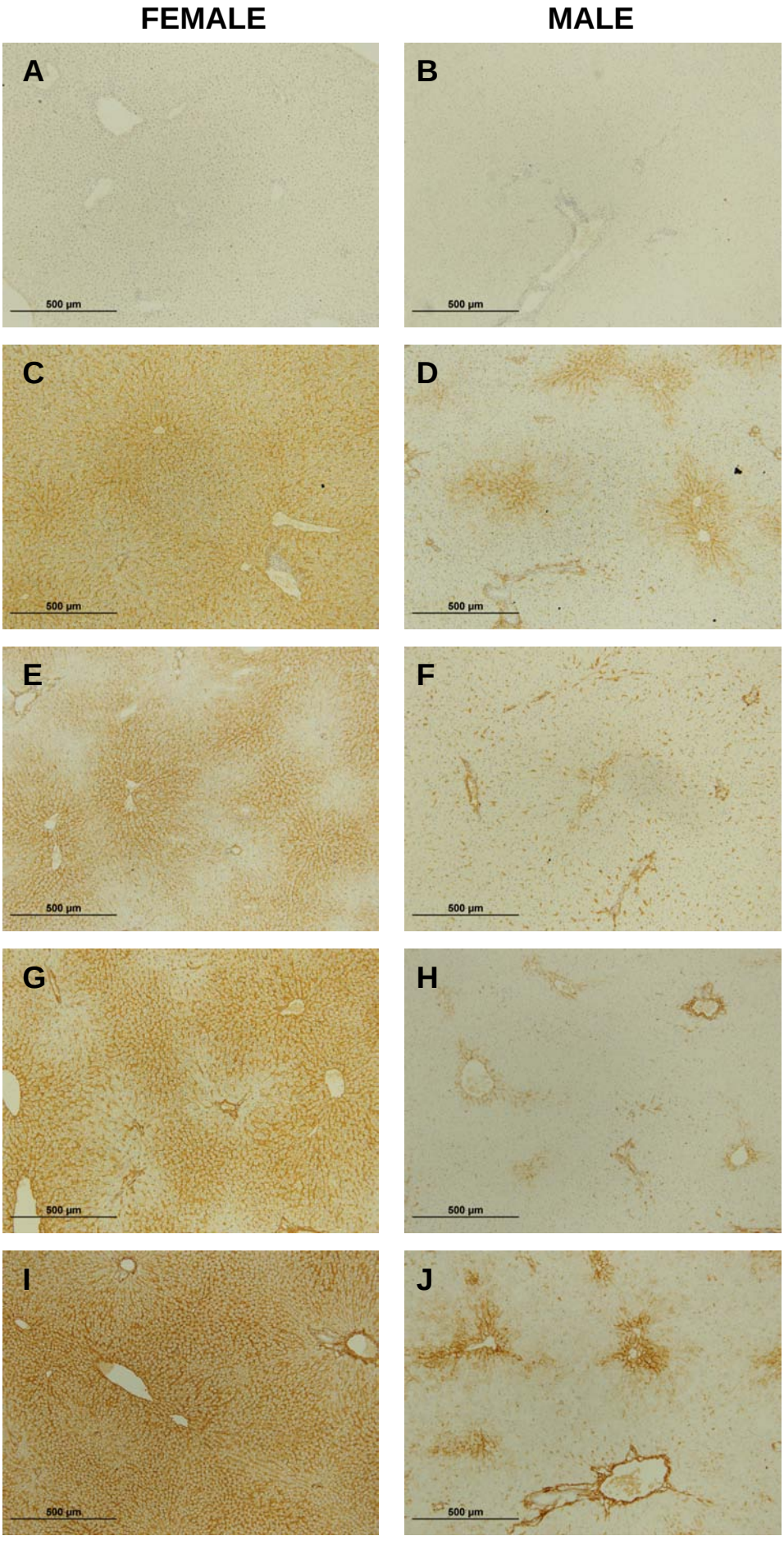

F
200 µm

Figure 3.2: Hepatic expression of CD36 in several laboratory rat strains.

No staining was observed in sections of liver from either female (A) or male (B) rats of the CD36-deficient SHR-N strain. In liver sections from female SD (C), SHR-SP (E), WKY (G) or Lewis (I) rats the majority of hepatocytes were stained by mAb UA009. In contrast in sections of liver from the male SD (D), SHR-SP (F), WKY (H) and Lewis (J) strains, only a small proportion of hepatocytes were stained, localised around the central vein.
Photographed using x10 objective.


FEMALE
MALE
A
B
C
D
E
F
G
H
I
J
500 µm

3.3 Effect of Gender on Hepatic Expression of SR-BI

3.3.1 Background

Because of the recognised role of SR-BI as a receptor for HDL, its regulation in the liver has been studied with considerable interest. Landschulz et al (1996), using SD rats, was the first to report a gender difference in hepatic expression of SR-BI. These authors reported greater expression in males, and reduction of expression when intact male rats were treated with pharmacological doses of estradiol. Other studies (Graf et al., 2001) have demonstrated a similar effect of estrogen in human hepatoma cells. Stangl et al (2002) showed that the effect of estradiol administration to male rats on expression of both *Sr-bI* transcripts and SR-BI protein is dependent on the presence of pituitary hormones. This suggests that the effect of estrogen may be indirect, rather than being due to direct engagement of estrogen/estrogen receptor dimers with the *Sr-bI* promoter. Interestingly, the reported gender difference in hepatic expression of SR-BI is biased in the opposite direction to that observed by us for CD36. This suggests that hormonal status may have a very significant influence on the ratio of CD36 to SR-BI expression by hepatocytes. It is noteworthy that there have been no studies, on the effects of estrogen at doses in the physiological range on expression of SR-BI by hepatocytes either *in vivo* or *in vitro*.

It was of interest, therefore, to re-examine the effects of gender on expression of SR-BI, in conjunction with the studies on CD36 expression in the liver. Furthermore, all of the studies described above compared levels of protein by Western Blot. The following experiments describe the expression of SR-BI in the liver and specifically, the distribution within the hepatic lobule in male and female rats of the DA and SD strains.

3.3.2 Immuno-Histochemical Detection of SR-BI In Rat Liver

Expression of SR-BI was examined in frozen sections of liver from male and female rats, using a commercial polyclonal rabbit antibody raised against a peptide from the C-terminus of the human SR-BI protein. This antibody cross-reacts with rat SR-BI. Reactivity with rat adrenal gland was used as a positive control for detection of antigen by the antibody, while an immuno-purified rabbit polyclonal antibody against a giardia surface protein was used as a negative control. The polyclonal anti-SR-BI antibody

stained the adrenal cortex when used at 1-5 µg/mL (Fig. 3.3A and section 3.4.2), with no staining of the parenchyma of the adrenal medulla. Furthermore, there was no staining of the adrenal cortex by the negative control antibody R127B8 (Fig. 3.3B).

When used to stain liver sections, employing identical conditions to those that demonstrated SR-BI in the adrenal cortex, the antibody produced only faint staining of frozen sections of liver from male and female rats. There was no staining of hepatocyte cell membranes and only weak punctate staining within the cytoplasm of the cells (Fig. 3.3C and D). This staining was considered to be specific, because it was not present in sections stained with the negative control antibody. However, it did not conform to the expected cellular distribution of SR-BI. Extending incubation times, increasing antibody concentration, or modifying the tissue fixation technique (methanol, acetone, and paraformaldehyde) did not affect either the pattern or the intensity of staining.

3.3.3 Detection and Comparison of SR-BI Expression in Male and Female Liver by Western Blot

Western blot was used to verify that the liver contains SR-BI that is detectable using the polyclonal anti-SR-BI antibody. Membrane proteins were extracted from snap-frozen DA rat liver and equal amounts of protein from male and female liver (25µg) were separated by reduced SDS-PAGE, transferred to PVDF membrane and probed with 1µg/mL polyclonal anti-SR-BI antibody. Bound antibody was detected using an ECL detection kit, as described in Methods (section 2.14).

Fig. 3.4A shows a representative Western blot. The predominant band, present in all liver samples had an apparent molecular weight of approximately 82 kDa, which is the reported size of glycosylated monomeric SR-BI (Cao et al., 1997). The smaller bands present (~62 kDa) may be partially-glycosylated or unglycosylated forms of the molecule. Upon visual inspection, the lanes containing membrane proteins from male liver did appear to have bands with more intense staining than those in lanes containing female liver samples. The 82 kDa bands in the lanes from all liver samples (5 males and 5 females) were subjected to semi-quantitative analysis by densitometry. Although there was a higher amount of immuno-reactive SR-BI detected in male compared with female liver (Fig. 3.4B), this did not reach statistical significance.

Figure 3.3: Detection of SR-BI using indirect immunoperoxidase technique.

Staining of adrenal gland sections with polyclonal rabbit anti-SR-BI antibody (A) resulted in strong staining of cells in the rat adrenal cortex (ac) and the absence of reactivity in the adrenal medulla (am). No staining was observed in adrenal gland sections stained with polyclonal rabbit anti-giardia ab R127B8 (B). Staining of liver sections with anti-SR-BI resulted in weak, punctate staining by parenchymal cells that was evenly distributed across the lobule in both females (C) and males (D). No staining was observed in liver sections stained with the negative control antibody R127B8 (E).

Photographed using 10X objective.

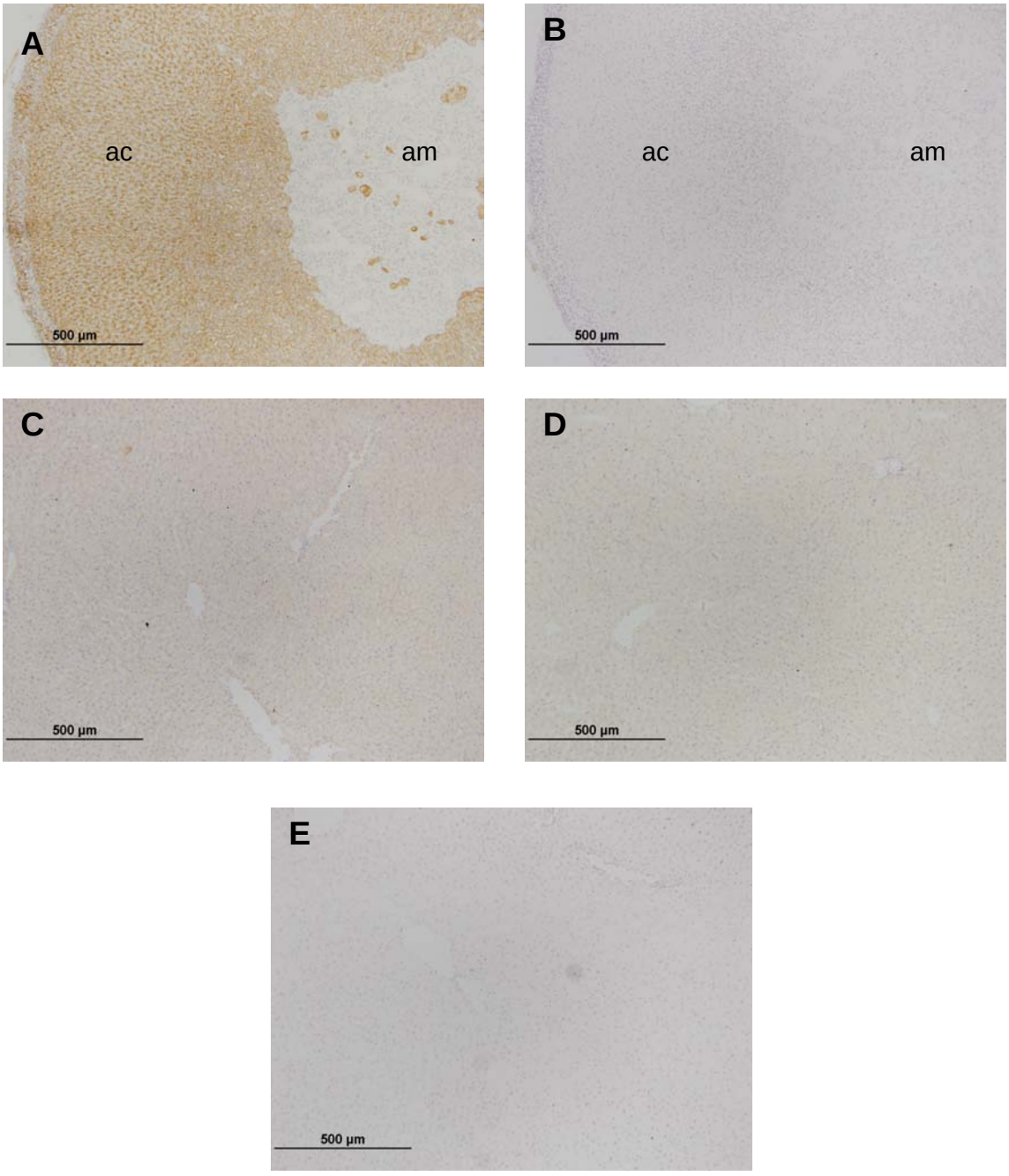
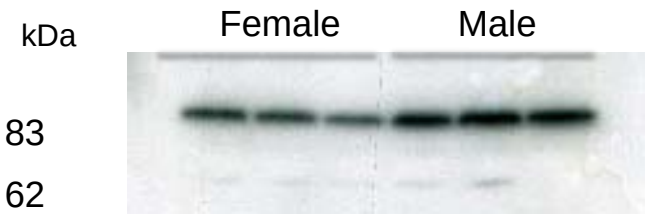

A
ac
am
500 µm
B
ac
am
500 µm
C
500 µm
D
500 µm
E
500 µm

Figure 3.4 Detection and measurement of SR-B1 in liver and adrenal tissue from female and male rats by Western analysis.

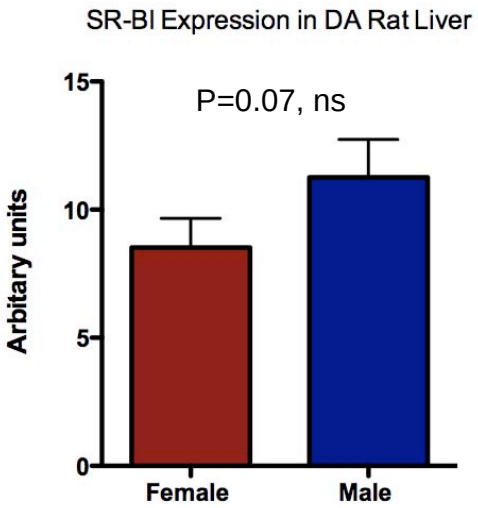
Equal amounts of membrane proteins from liver samples (25µg) obtained from five male and five female adult DA rats were subjected to reduced SDS-PAGE (12%) and electro-blotted to PVDF membrane. The figure shows the result of probing with a rabbit anti-SR-B1 antibody. A stained band of approx. 82kD is resolved in samples. Stained bands were detected by ECL. A representative blot is shown in (A). A second faint band of approx 62kDa was present in liver samples and may represent under-glycosylated species of SR-BI. Quantitation of the 85kD bands from liver samples in A by densitometry (mean +/- SD, n = 5 rats per group). The results are expressed in arbitary units and there was no significant difference between male and females (B). Protein samples from female and male SD rats (3/gp) were subjected to Western analysis under identical conditions to those described above (C). Lane 1, marker. F, female; M, male. Adrenal samples were from a pool of three rats. Each liver sample is from an individual rat. Graph showing densitometric analysis of 82 kDa band from liver proteins (D).

FA – female adrenal, MA- male adrenal, M-Liver – male liver, F-Liver – female liver.

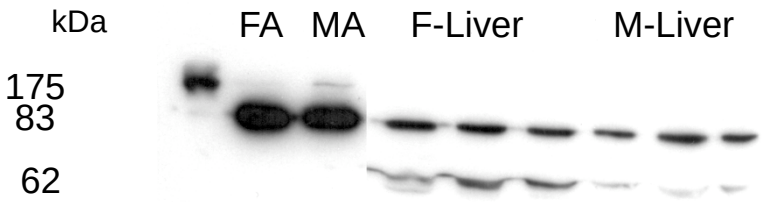
A


kDa
Female
Male
83
62

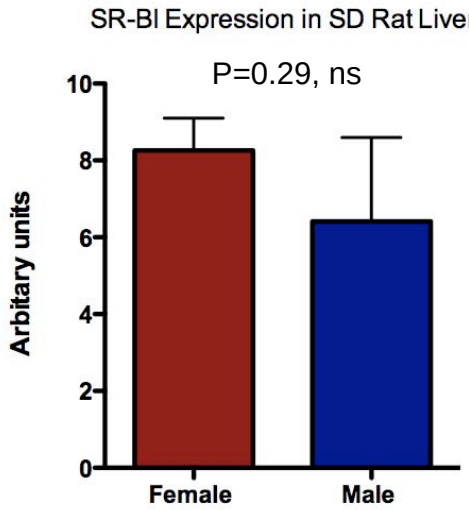
B


SR-BI Expression in DA Rat Liver
P=0.07, ns
Arbitary units
15
10
5
0
Female
Male

C


kDa
FA
MA
F-Liver
M-Liver
175
83
62

D


SR-BI Expression in SD Rat Liver
P=0.29, ns
Arbitary units
10
8
6
4
2
0
Female
Male

As the majority of studies of SR-BI expression in the liver have been performed using rats of the SD strain, the analysis was repeated using male and female rats of this strain. Membrane proteins extracted from the adrenal gland of male and female SD rats were pooled (n=3 each gender), and included as a positive control. The 82 kDa band was present in all samples whilst the adrenal samples also had a faint, larger band of apparent molecular weight ~160 kDa, which probably represents the dimeric species of SR-BI reported by Reaven et al. (2003). There was no consistent difference in the intensity of staining of the 82 kDa bands in the lanes from the male or female rats and this was confirmed using densitometry (Fig. 3.4C and D). The amount of SR-BI in lanes containing adrenal proteins, relative to the total amount of membrane protein applied, was much greater than in liver. Contrary to reports by others (Graf et al., 2001, Landschulz et al., 1996, Stangl et al., 2002), no gender difference was observed in hepatic expression of SR-BI in this strain. Consistent with the aforementioned studies, there did however appear to be more SR-BI in the adrenal samples from females compared with males (Fig. 3.4C).

3.4 Effect of Gender on Expression of Scavenger Receptors in Extra-Hepatic Tissues

3.4.1 Background

Other than our description of CD36 expression in the adrenal cortex, testicular leydig cells and ovarian follicles of the rat (Zhang et al., 2003), there has been only limited research on CD36 expression in the steroidogenic tissues (Abumrad et al., 1993, Petrik et al., 2002, Zibara et al., 2002). In comparison, a landmark study on SR-BI distribution by Landschulz et al. (1996) reported that the adrenal gland and ovary contain approximately 25 fold higher levels of the protein (per milligram of tissue) than the liver. In addition, they observed that the steady state levels of SR-BI expression in the adrenal glands of female SD rats were higher than in males and that treatment with pharmacological doses 17α-Ethinyl estradiol induced a 4 fold increase in expression in this tissue. In contrast, estradiol did not induce any change in ovarian expression of SR-BI. Subsequent studies have suggested that in general, SR-BI expression in steroidogenic tissues is upregulated by tissue-specific trophic hormones. (Azhar et al., 2002, Cao et al., 1999, Mizutani et al., 1997). Functionally, this would ensure that cells

in the steroidogenic target tissues could respond rapidly to acute biosynthetic demands for cholesterol (Azhar et al., 2003, Plump et al., 1996, Temel et al., 1997). Outside of the steroidogenic tissues, there do not appear to have been any reports on the influence of gender on the expression of either CD36 or SR-BI in other extrahepatic tissues.

The following experiments investigate whether gender-related dimorphic expression of CD36 is unique to the liver. A tissue survey was also performed to compare CD36 and SR-BI expression in a variety of tissues in male and female rats, with a particular reference to the adrenal gland. Furthermore, a comparison was made of CD36 and SR-BI distribution in the ovary and the testis.

3.4.2 Expression of CD36 And SR-BI in the Adrenal Gland

Indirect immunohistochemistry was performed using mAb UA009 on frozen tissue sections of adrenal glands obtained from male and female DA rats. In both males and females, sinusoidal endothelium in the cortex and the medulla expressed CD36. However, parenchymal cells of the medulla were not stained. In sections from females there was no staining of parenchymal cells in the zona glomerulosa and only patchy staining in the zona fasiculata. However, cells in the zona reticularis were stained more intensely (Fig. 3.5A). In contrast, most of the staining in the cortex of adrenals from male rats appears to be associated with endothelium and there was only weak staining of parenchymal cells in the zona reticularis (Fig. 3.5B). Staining of the cortical sinusoids in both sexes was most intense in the zona reticularis and it became progressively weaker in a gradient towards the superficial layers of the zona fasiculata. There was no staining of adrenal tissues by the isotype matched control mAb. (Fig. 3.5C).

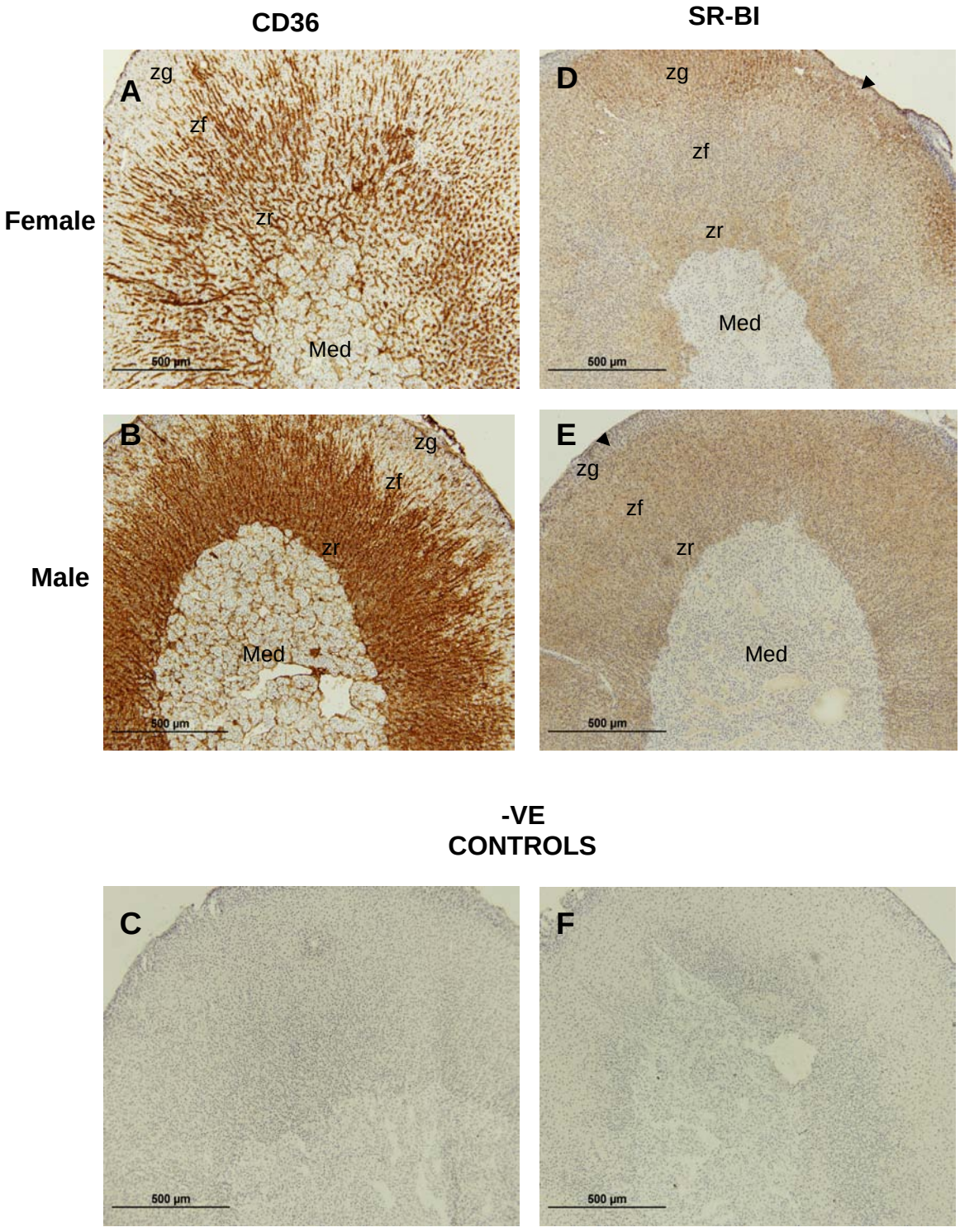
As described briefly in section 3.3.2, immunohistochemical staining of adrenal sections from DA rats with polyclonal anti-SR-BI antibody revealed the presence SR-BI in the cortex of the gland. When tissues from adult male and female rats were examined, there was staining of all parenchymal cells in the adrenal cortex, with the exception of a thin rim of cells in the zona glomerulosa (Arrows, Fig. 3.5D and E). The pattern of staining was similar in tissues from female and male rats but staining in the zona glomerulosa appeared to be more intense in females. This is consistent with the gender difference

Figure 3.5: Photomicrographs showing expression of CD36 and SR-BI in adrenals from male and female DA rats.

CD36 was detected using the indirect immunoperoxidase technique. There is intense staining on the cortical and medullary sinusoids in sections from male (A) and female (B) rats. Parenchymal cells in the zona reticularis and to a lesser extent the zona fasciulata were also stained in male but not female sections. Staining of SR-BI protein was detected on most parenchymal cells in the adrenal cortex in both male (D) and female (E) sections except for a thin rim of cells immediately below the capsule (arrows). No staining was detected on male adrenal sections incubated with the negative control anti-giardia antibodies mAb 1B5 (C) and polyclonal Ab R127B8 (F).

Photographed using x10 objective.

Abbreviations: zona glomerulosa (zg), zona fasiculata (zf), zona reticularis (zr), medulla (Med).


CD36
SR-BI
Female
Male
A
zg
zf
zr
Med
500 µm
D
zg
zf
zr
Med
500 µm
B
zg
zf
zr
Med
500 µm
E
zg
zf
zr
Med
500 µm
-VE
CONTROLS
C
500 µm
F
500 µm

observed in the amounts of SR-BI detected in the adrenal by Western blot analysis (section 3.3.2). There was no staining of adrenal tissue by the control polyclonal antibody (Fig. 3.5F).

3.4.3 Expression of CD36 and SR-BI in the Steroidogenic Tissues of the Testis and Ovary

Frozen sections of adult testis were stained by the indirect immuno-peroxidase technique to identify CD36 and SR-BI. Monoclonal antibody UA009 stained interstitial cells intensely and also the endothelium of vessels in the interstitial spaces (Fig. 3.6A). Previous studies have shown that the intensely stained interstitial cells are Leydig cells and not CD36-positive interstitial macrophages (Zhang et al., 2003). In contrast, polyclonal rabbit anti-rat SR-B1 stained fewer Leydig cells and the staining was near the limits of detection by immuno-histochemistry (Fig. 3.6B). There was no staining by either of the negative control antibodies (Fig. 3.6 C and D).

In the ovary, mAb UA009 stained granulosa cells in the more mature follicles, but there was no staining of the luteinised granulosa cells in the corpus luteum. The extensive vascular endothelium in the corpus luteum was stained intensely. Monoclonal antibody UA009 did not stain the steroidogenic thecal cells in the interstium of the ovary (Fig. 3.6E).

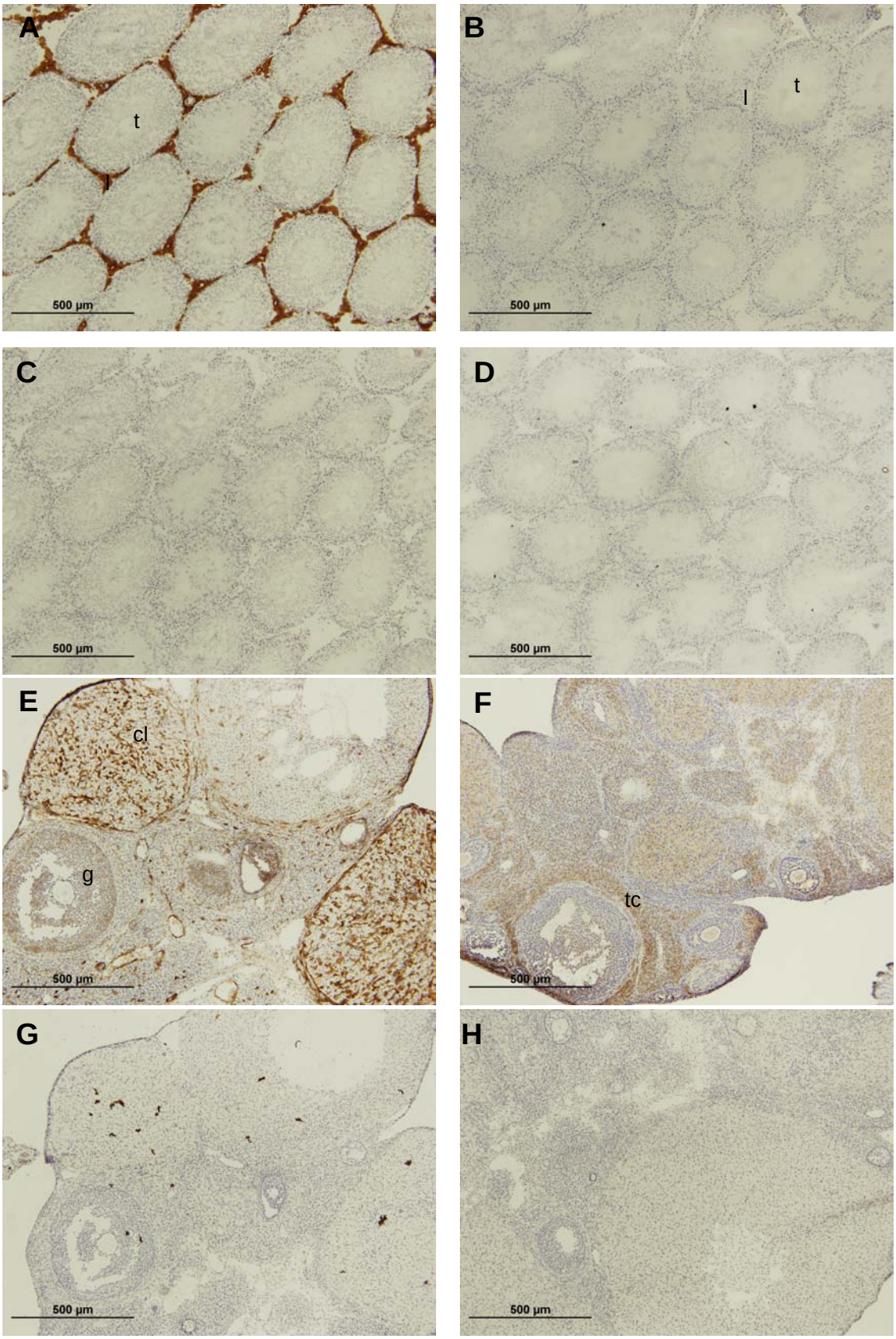
In comparison, SR-BI was expressed by the thecal cells that surround the immature follicles and was also expressed strongly by luteinized granulosa cells of the corpus luteum. However, granulosa cells in follicles were unstained (Fig. 3.6F). Thus, CD36 and SR-BI have quite distinctive and different patterns of expression in the steroidogenic tissues of the ovary.

3.4.4 Effects Of Gender On Expression Of CD36 and SR-B1 in Other Extra-Hepatic Tissues

A broad survey was performed to examine whether gender affects expression of CD36 and SR-BI in tissues other than the liver and adrenal glands. Consistent with the

Figure 3.6: Photomicrographs showing Expression of SR-BI and CD36 in DA rat Gonads, detected by the indirect immunoperoxidase technique.

Abundant CD36 protein was detected on Leydig cells (l) and vascular tissue of the testes but the seminiferous tubules (t) were unstained (A). SR-BI protein was only barely detectable on the Leydig cells using this technique, and was absent from the tubules of the testes (B). In the ovary, CD36 protein was detected on the granulosa cells (g) in the follicles (f), as well as by macrophages and vascular endothelium in the corpus lutea (cl)(E). In contrast SR-BI protein was absent from Granulosa cells, but detected on the thecal cells (tc) surrounding the follicles and on luteal cells (F). No staining was detected on sections of ovary or testes with the negative control antibodies 1B5 (C and G) or polyclonal R127B8 (D and H).
Photographed using x10 objective.


A
t
l
500 µm
B
l
t
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C
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D
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E
cl
g
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F
tc
500 µm
G
500 µm
H
500 µm

distribution of CD36 reported by Zhang et al. (2003) and others (Abumrad et al., 1993), strong staining by mAb UA009 was observed in frozen sections from heart, red skeletal muscle, adipose tissue, spleen and on specific cell types in sections from small intestine, tongue, trachea, and kidney. CD36 was not detected in sections from brain, white skeletal muscle or resting mammary tissue. Fig. 3.7A-B shows a longitudinal cross-section of small intestine from a male and female rat in which the pattern and intensity of CD36 staining is indistinguishable. Similarly, none of the other tissues examined displayed gender-biased CD36 expression. Expression of SR-BI was restricted to the steroidogenic tissues and the liver (described above), with the exception of low levels detected in the small intestine (Fig. 3.7 C-D) and on mucosal cells lining the trachea. The distribution of CD36 and SR-BI in male and female DA rats is summarized in Table 3.1 below.

Table 3.1: Immunohistological detection of CD36 and SR-BI protein in frozen tissue sections from male and female 10 week old DA rats

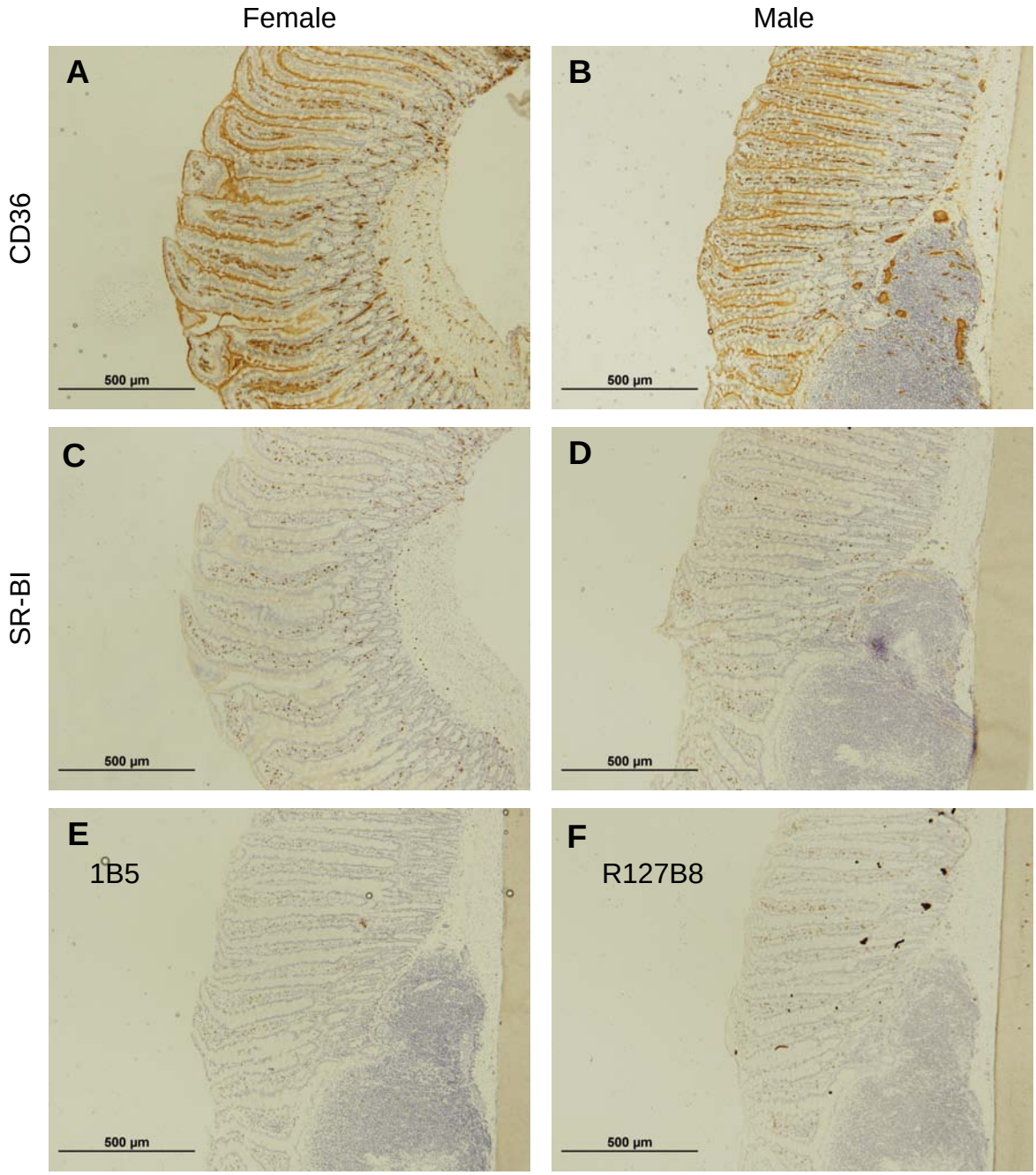
TISSUE/ CELL TYPE	CD36		SR-BI	
	FEMALE	MALE	FEMALE	MALE
Hepatocyte	++/+++	+	+	+
Adrenal cortex	+	++	++	+
Cardiomyocyte sarcolemma	++	++	-	-
Skeletal muscle sarcolemma (red muscle)	+++	+++	-	-
Skeletal muscle sarcolemma (white muscle)	-	-	-	-
Enterocyte brush border	++	++	+	+
Adipocyte cell membrane	+++	+++	-	-
Spleen – marginal zone cells	++	++	-	-
Renal tubule epithelium	+	+	-	-
Brain	-	-	-	-
Tracheal Epithelium	+	+	+	+
Vascular Endothelium	++	++		

Extensive staining of entire section is represented by +++, strong staining of some cell types within the slide is represented by ++ and sparse staining across the section or on specific cells only is represented by +. The absence of any specific staining on the slide is represented by -.

Figure 3.7: Photomicrographs showing Expression of CD36 and SR-BI by enterocytes in the small intestine of DA rats.

CD36 protein was detected on the brush border of enterocytes and on vascular tissue present in the lamina propria. High endothelial venules in Peyer's patches were stained strongly. The intensity and distribution of staining was equivalent in samples from male (A) and female (B) adult rats. Columnar absorptive cells in the small intestine express SR-BI weakly in both male (C) and female (D) rats. Small numbers of intensely stained cells scattered through the lamina propria stained with the negative control mAb (E) and polyclonal antibody (F) are eosinophils containing endogenous peroxidase.

Photographed using x10 objective.


Female
Male
CD36
SR-BI
A
B
C
D
E
1B5
F
R127B8
500 µm
500 µm
500 µm
500 µm
500 µm
500 µm

3.5 Expression of CD36 in the Liver in Mice

3.5.1 Background

Historically, the possibility that CD36 is expressed in mouse liver has been ignored, due to the failure of Abumrad et al. (1993) to detect *Cd36* mRNA in rat liver and of Greenwalt et al. (1995) to detect CD36 on sinusoidal endothelium in mouse liver. Recent interest in the regulation of gene expression in the liver by peroxisome proliferator-activated receptors (PPAR) has re-kindled interest but has yielded inconsistent results with respect to CD36. As in other tissues, expression of *Cd36* in the liver can be induced by treatment of mice with ligands for both PPARα and PPARγ. (Memon et al., 2000, Motojima et al., 1998, Yu et al., 2001, Yu et al., 2003). These authors reported that the levels of *Cd36* transcripts in untreated C57Bl/6 mice were at the limits of detection by Northern blot. However using an RNase protection assay, *Cd36* mRNA has been detected in hepatocytes, Kupffer cells and endothelial cells isolated from livers of MFI mice (Malerod et al., 2002a).

The following experiments were designed to investigate whether *Cd36* mRNA and CD36 protein is present in the livers of normal male and female mice from three commonly used inbred mouse strains (CBA, C57Bl/6J and Balb/C). They were undertaken to re-examine the question of whether expression of CD36 is expressed in mouse liver and to investigate whether it displays gender regulation in a manner similar to that observed in rats.

3.5.2 Detection of *Cd36* mRNA in Mouse Liver

Reverse transcription PCR was performed on cDNA prepared from male and female liver obtained from CBA, C57BL/6 and BALB/c mice. Separate samples of liver were prepared from each of five mice per group, while cDNA was prepared from the heart of an individual mouse from each group (to serve as positive control cDNA, containing *Cd36* transcripts).

In a pilot experiment, PCR amplification was performed on one liver and one heart sample from each of the six groups, using primers mCD36-FP1 and mCD36-RP1.

Additionally, to provide a reference for the amounts of cDNA added to the PCR reactions, equal quantities of cDNA from each sample were used in separate reactions to amplify a segment from transcripts encoding the house keeping enzyme GAPDH (primers GAPDH-FP and GAPDH-RP). The PCR products from these reactions were resolved using agarose gel electrophoresis, stained with SYBR gold and visualised using the Biorad FX scanner (Fig. 3.8A). A single band of the appropriate size (417bp, *Cd36* amplicon and 240bp, *Gapdh* amplicon) was observed for each sample. The intensity of the *Gapdh* amplicons was quite consistent for all samples, whereas the intensity of the *Cd36* bands varied considerably. In each of the groups, the *Cd36* amplicons appeared more intense in reactions using heart cDNA as template, compared with those using liver cDNA template. Significantly, in the samples using cDNA prepared from C57BL/6 and BALB/c liver, the *Cd36* amplicons appeared to be stained more intensely when the template was prepared from female liver.

Optimization experiments were undertaken to optimize the PCR reaction, test the effect of cycle number and primer/template dilution on the yield of product. Figure 3.8B shows a representative gel, in which PCR products amplified from C57Bl/6 mouse liver cDNA were sampled at various cycles. This showed that product could be detected in reactions using female cDNA as template at an earlier cycle number than in reactions where the template cDNA was prepared from males. This occurred in each of the three mouse strains, although product appeared at later cycles when the cDNA was prepared from BALB/c, compared with cDNA prepared from other the other strains.

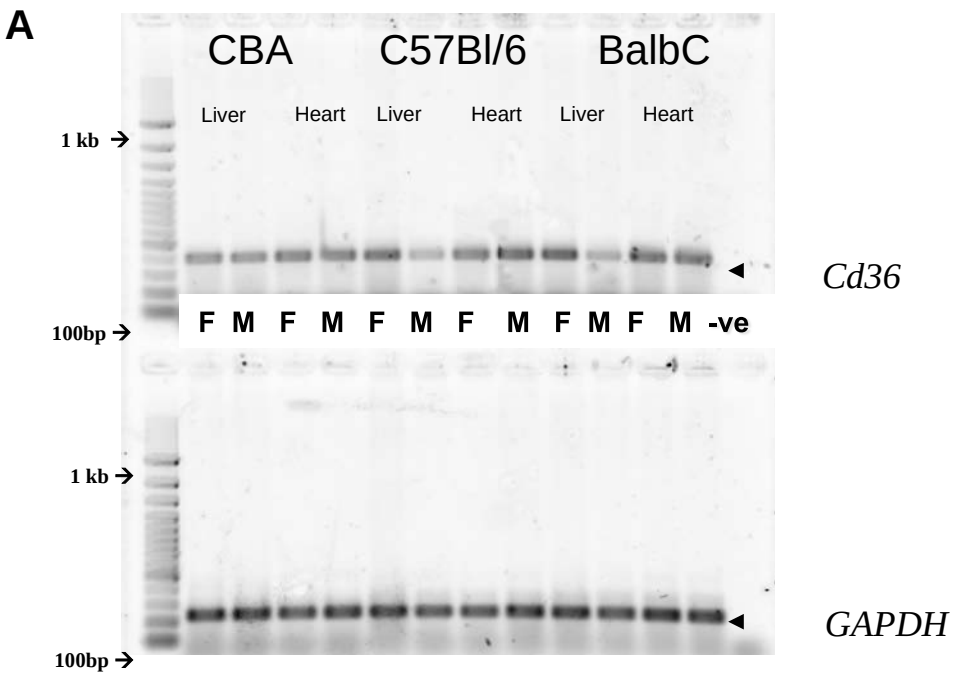
Based on this pilot data, semi-quantitative RT-PCR analysis was performed on all samples, using the following conditions. CDNA was prepared as described in section 2.16 and diluted 1:4 for use as template in the PCR. Both sets of primers were used at a concentration of 0.5μM. The products were visualised after either 26 (C57Bl/6 and CBA) or 28 (BALB/c) cycles. The intensity of each specific amplicon band was measured using the Biorad FX scanner and QuantityOne software and expressed as a ratio compared with the density of the Gapdh band for each sample.

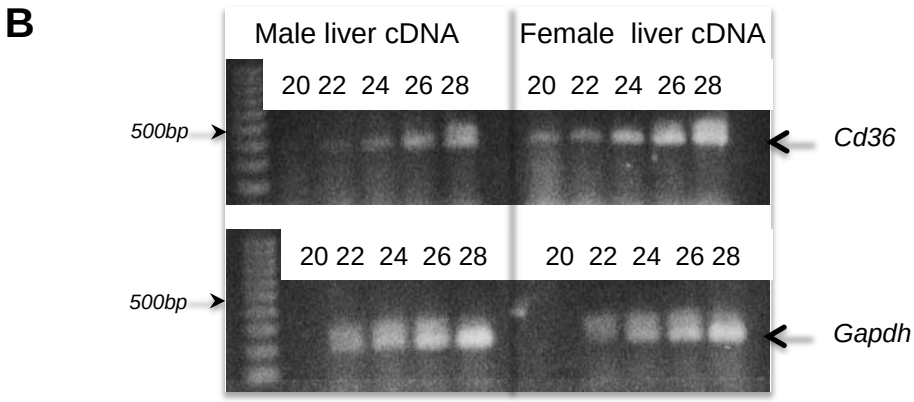
Figure 3.8C displays the mean *Cd36/Gapdh* ratio for the products amplified from liver cDNA prepared from five animals in each group. The results show that in each strain, the ratio is approximately one third lower in males than in females. The difference between genders within each strain was significantly different when tested using a two-

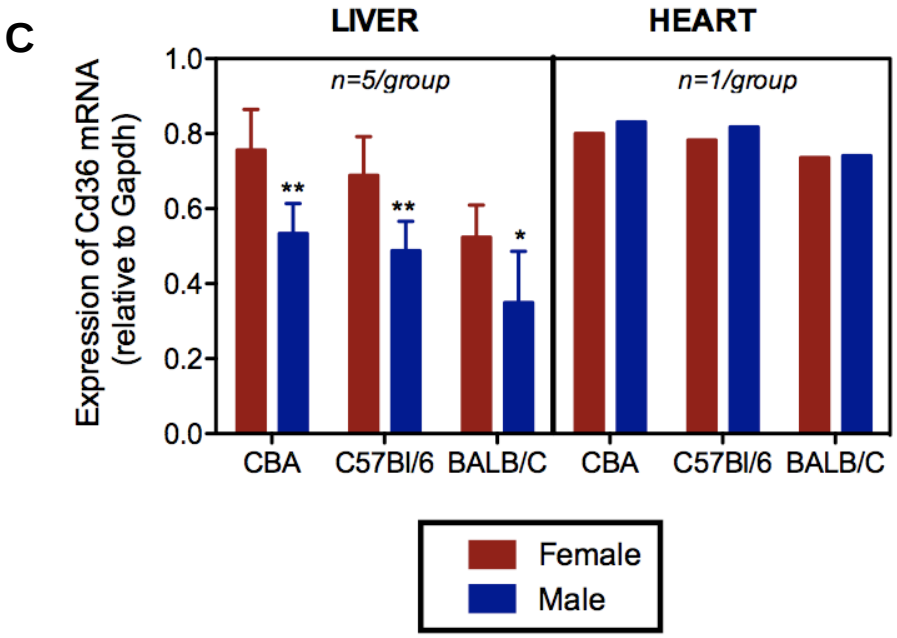
Figure 3.8: Semi-quantitative measurement of *Cd36* mRNA transcript expression in mouse liver.

Gel electrophoresis of PCR products obtained using the primer pairs mCD36FP1/RP1 and GAPDHFP1/RP1 to amplify mouse cDNA obtained from the liver or hearts of male and female mice (A). Cycle sampling was performed on each of the three mouse strains. Representative gel photograph of PCR products obtained from liver cDNA of male and female C57Bl/6 mice. PCR products were sampled between cycles 20 and 28 during the amplification reaction (B). The intensity of resolved Cd36 PCR products prepared from cDNA of the three mouse strains was measured by densitometry and expressed as a ratio compared with the Gapdh band for each sample. The graph displays mean +SD for liver samples from males and females of each strain (n=5/group) and a single positive control reaction (heart cDNA) for each gender and strain (C). Females of each strain had significantly higher levels of *Cd36* than their male counterparts.

**$p<0.01$, * $p<0.05$


A
CBA
C57Bl/6
BalbC
Liver
Heart
Liver
Heart
Liver
Heart
1 kb
100bp
F M F M F M F M F M F M -ve
Cd36
1 kb
100bp
GAPDH


B
Male liver cDNA
Female liver cDNA
20 22 24 26 28
20 22 24 26 28
500bp
Cd36
20 22 24 26 28
20 22 24 26 28
500bp
Gapdh


C
LIVER
HEART
n=5/group
n=1/group
Expression of Cd36 mRNA (relative to Gapdh)
1.0
0.8
0.6
0.4
0.2
0.0
**
**
*
CBA
C57Bl/6
BALB/C
CBA
C57Bl/6
BALB/C
Female
Male

tailed t-test ($p>0.05$). In contrast, the ratio in the heart was very similar in the males and females of each strain.

3.5.3 Attempted Detection of CD36 in Mouse Liver by Immunohistochemistry

As *Cd36* mRNA could be detected easily in liver by RT-PCR, an attempt was made to detect CD36 protein in frozen sections of liver by immunohistochemistry. A commercial mouse anti-mouse CD36 mAb (IgA isotype) was obtained and tested on frozen sections of liver and heart. In the control tissue (heart), staining of CD36 was clearly visible on cardiac myocytes and there was no background staining by the secondary antibody conjugate when used in conjuction with the isotype-matched control mouse mAb. However, on frozen sections of liver, it was not possible to reduce the non-specific background staining to an acceptable level irrespective of primary antibody concentration (dilutions ranging from 1:50 to 1:1000 were tested). To address this, the anti-mouse CD36 mAb was biotinylated using a biotin-X-NHS kit and bound protein was detected using a streptavidin-HRP conjugate. A commercial mouse-on-mouse Ig blocking reagent (VectorStain) was also employed, however these steps failed to resolve the high levels of background staining. The problems with background were related to binding of the secondary sheep anti-mouse HRP conjugate to endogenous mouse IgA in the liver, non-specific binding of the primary antibody to IgA receptors in the liver and binding of Streptavidin to endogenous biotinylated proteins in the liver. In the presence of a high level of background staining, it was not possible to distinguish difference in staining between sections incubated with the anti-CD36 antibody and those incubated with the isotype-matched negative control antibody.

3.6 Discussion

3.6.1 Sexually Dimorphic Expression of CD36 and Liver Zonation

The gender difference in hepatic expression of the class B scavenger receptor CD36 in DA rats (Zhang et al., 2003) was confirmed by immunohistochemical staining of liver sections and isolated hepatocytes. The distinctive centrilobular distribution of CD36 provides some suggestions regarding its function, because cells within hepatic lobules

are functionally heterogeneous. Most of the cholesterol synthesis, β-oxidation of fatty acids, ketogenesis, and gluconeogenesis in the liver is performed by the hepatocytes in the periportal regions of the lobule. In contrast, the centrilobular hepatocytes are responsible for the majority of the gluose utilisation, bile acid synthesis from cholesterol, triglyceride synthesis and metabolism of xenobiotics (reviewed in Jungermann and Kietzmann, 1996). Patterns of gene expression within the liver lobule reflect this zonality of function. A similar centrilobular pattern of expression has been reported for a variety of important liver enzymes, including cholesterol 7α-hydroxylase (Massimi et al., 1998), estrogen sulfotransferase (Mancini et al., 1992, Fischer et al., 1982), glucokinase (Fischer et al., 1982) and numerous cytochrome P450 isoenzymes (Oinonen and Lindros, 1998).

CD36 protein (especially in male rats) is expressed in greatest amounts by those hepatocytes that are exposed to blood that has already been exposed to the sinusoids of the peri-portal regions of the lobule. These cells are, therefore, exposed to lower oxygen tensions and lower levels of some dietary nutrients than the peri-portal hepatocytes, as well to lower concentrations of metabolites and toxins that are delivered to the liver via the portal blood. They may also be exposed to lower concentrations of growth factors and hormones, compared with hepatocytes located closer to the portal triads. Given the range of ligands that have been reported to bind to CD36, the molecule could contribute to a number of the physiological functions of the centrilobular hepatocytes. These may include uptake of cholesterol from HDL and or LDL for disposal by direct excretion and via bile acid synthesis, scavenging of oxidized LDL, uptake of FA for triglyceride synthesis, or signalling in response to binding of some of these ligands. Although individual hepatocytes appear to express more CD36 protein in female rats than males, there is an obvious difference between the genders in the greater proportion of cells in the intermediate zone of the lobules that express the molecule in females. In females, CD36-expressing cells extend well into the periportal zone in strains such as DA rats (Zhang et al, 2003) and in others i.e. SD and Lewis (shown here), they include most of the hepatocytes in the lobules.

3.6.2 Inter-Strain Differences In Hepatic CD36 Expression

The gender differences in hepatic CD36 expression were observed in both the inbred and random bred rat strains, despite strain differences in CD36 expression. The physiological consequences of the gender difference in hepatic expression of CD36 await elucidation. Furthermore, the significance of the strain differences in CD36 expression are not clear. There may be genetic polymorphism in regulation of the *Cd36* gene, or alternatively it may be a response to differences in basal metabolism between the strains. It seems unlikely that the strain differences reflect known minor polymorphisms within *Cd36* that affect immuno-reactivity with mAb UA009. The staining of other CD36-positive cells such as endothelium, is of similar intensity in all strains.

3.6.3 Expression Of CD36 In Mouse Liver

There was a greater abundance of *Cd36* transcripts in C57Bl/6 and Balb/C mice than in CBA mice, and in females compared with males in each strain. This indicates that sexually dimorphic expression of CD36 extends also to mice and complements findings in rats (Zhang et al., 2003) and humans (Stahlberg et al., 2003). Unfortunately, for technical reasons, it was not possible to examine the distribution of CD36 expression within the hepatic lobules in mice. Furthermore, there have been no studies of the lobular distribution of the CD36 in humans. Further studies, using quantitative Western analysis, are needed to confirm the immuno-histochemical studies in rats and the analysis of transcripts in mice. This is particularly pertinent for mice, where due consideration of a role for hepatic CD36 has not hitherto been afforded when interpreting the metabolic phenotypes of the *Cd36* and *Sr-b1* global knock-out mice.

It was a matter of surprise that SR-B1 was not detected easily by immuno-histochemistry in the livers of male or female rats, despite its ready detection in adrenal and ovary. Despite its importance in reverse cholesterol transport and the detection of *Sr-b1* transcripts in rat (Landschulz et al., 1996), mouse (Babitt et al., 1997) and human (Cao et al., 1997) liver, there have been few immuno-histochemical studies of SR-B1 in the liver, and no reports of how it is zonally distributed within the hepatic lobule. Levels of SR-B1 expression by hepatocytes rat liver are at the detection limits of using the methods employed in the present studies. Landschulz et al. also reported similar

difficulties using immunoflourescence on liver sections (1996). It is noteworthy, however that low levels of expression in a large organ such as the liver may still confer important functional capacity to the organ. It is interesting to note that (Ritsch et al., 2003) have reported that SR-BI expression is concentrated around the central vein in Chinchilla Bastard rabbits, providing some evidence that SR-BI and CD36 may share a similar zonal distribution. Unfortunately, the gender of the rabbits used in this study was not specified.

Despite low levels of expression by individual hepatocytes, Western analysis using the antibody employed for immuno-histochemistry detected SR-BI in membrane preparations of rat liver. The levels detected were considerably lower than those observed in lower amounts of adrenal membrane proteins. Although there appeared to be more SR-BI protein in samples from males compared to females, no significant differences were detected in preparations of liver membranes in either the DA or the SD strains. Thus, unlike CD36, the magnitude of the difference in expression of SR-BI appears to be quite small. It is possible that with a larger group size this may achieve statistical significance, as reported by (Graf et al., 2001, Landschulz et al., 1996, Stangl et al., 2002). The same workers observed greater expression of SR-B1 in the adrenals of female SD rats, and this observation was confirmed in the present study. Although effects of diet on expression of SR-BI in the liver have not been completely characterized, there is some evidence that variation in the lipid and/or cholesterol content of the food can influence expression fo SR-BI protein in the liver (Fluiter and van Berkel, 1997, Hoekstra et al., 2005). It is possible that the differences between the present findings and those of earlier studies are due to the composition of food rations and this is worthy of further investigation.

3.6.4 CD36 and SR-BI Expression in Extra-Hepatic Tissues

Higher expression of CD36, in females compared to males did not occur in any tissues that were surveyed, apart from the liver. In the adrenal gland, expression of both CD36 and SR-BI was sexually dimorphic although in opposite directions. CD36 was higher in male rats compared to females, whereas higher expression of SR-BI was observed in females. Comparisons of the cell-specific distribution by rat steroidogenic tissues revealed that these two related scavenger receptors display quite distinct patterns of expression.

3.6.4.1 The Adrenal Gland

Expression of CD36 in the rat adrenal cortex was described first by Zhang et al (2003). The present investigation shows that CD36 is expressed by more cortical parenchymal cells in males than in females. Thus, the gender difference in CD36 expression in the adrenal gland is reversed compared with the liver. CD36 is expressed heterogeneously in the parenchyma; primarily by cells of the zona reticularis and the inner zona fasiculata. SR-BI is also expressed in the zona reticularis and zona fasiculata, with an essentially homogeneous distribution in these layers. However, there appears to be greater expression of SR-BI in females than in males. Cells within these zones in the adrenal cortex are responsible for production of both glucocorticoid and sex steroid hormones and they have a high demand for exogenous cholesterol. Expression of CD36, as well as SR-BI, by these cells raises the possibility that the molecule may play a role in selective uptake of CE from HDL in the adrenal. However, the abundance of SR-BI and its greater capacity to mediate uptake of CE makes a contribution from CD36 less likely. However, the known function of the molecule as a fatty acid translocase raises the possibility that the adrenal cortex may derive some of its cholesterol requirements from endogenous metabolism of fatty acids. The regulatory mechanisms responsible for the gender difference in CD36 expression in the adrenal cortex were not studied further. However, because the gender bias was the opposite of that observed in the liver, it is likely that the mechanisms are different in the two organs.

3.6.4.2 Testis

Examination of CD36 and SR-BI expression in the testis by immunohistochemistry showed that both scavenger receptors were expressed on Leydig cells in the testes, although at greatly different levels. Leydig cells were stained intensely by mAb UA009, whereas staining by the polyclonal anti-SR-BI antibody was barely detectable. This suggests either that CD36 is a major mediator of cholesteryl ester uptake by Leydig cells or that the function of CD36 in these cells is to supply fatty acids for *de novo* synthesis of cholesterol. Interestingly, male SR-BI-/- knockout mice are fertile, while their female littermates are infertile (Trigatti et al., 1999), suggesting that testosterone production by Leydig cells is not dependent on SR-B1-mediated CE uptake.

3.6.4.3 Ovary

CD36 and SR-B1 were not co-localised in the ovary. CD36 was detected only on follicular cells (shown in Fig. 3.6E). However, Petrik et al. (2002) have reported recently that expression of both CD36 and two of its ligands (thrombospondin 1 and 2), varies considerably between the antral and luteal phases of the oestrus cycle. Strong follicular expression was observed in the early antral phase and this was replaced by diffuse luteal staining during the early luteal phase. However, CD36 was not expressed by granulosa cells in the late luteal phase. Thus, the pattern of expression of CD36 described in the present study may represent a snapshot of one phase of the ovarian cycle. CD36 expression in the ovary may be regulated by hormonal changes associated with the oestrus cycle, either directly or indirectly. Petrik et al (2002) hypothesize that interactions between CD36 and thrombospondin may play a role in regulation of angiogenesis during the extensive tissue remodelling that occurs during the ovarian cycle.

Expression of SR-BI in the ovary has been studied much more extensively than CD36. As described by others (Landschulz et al., 1996, Reaven et al., 1998), SR-BI was not detected on granulosa cells of follicles, while it is expressed strongly by thecal cells. Various roles have been postulated for SR-BI in the ovary, including uptake of apoptotic granulosa cells by thecal cells in atretic follicles (Svensson et al., 1999) and uptake of CE from HDL by thecal cells for steroid hormone production (Azhar et al., 1998). As discussed above, female (but not male) SR-BI-/- knockout mice are infertile and these mice display reduced CE storage in the ovary. However, this does not appear to adversely affect female steroid hormone synthesis in these animals, as adequate levels of progesterone and estrogen are produced and the mice ovulate normally. When cultured *in vitro*, pre-implantation embryos harvested from SR-BI-/- females that were mated with wild type males displayed abnormal morphology, reduced viability and developmental arrest compared with embryos from wild type females (Trigatti et al., 1999). This evidence points to an embryonic developmental defect rather than the inability of KO mice to conceive or sustain pregnancy. In contrast, CD36 -/- females retain normal fertility (Febbraio et al., 1999). Thus it appears that the absence of either scavenger receptor can be compensated for with respect to the cholesterol requirements for ovarian hormone synthesis. It is not known whether the two molecules are complementary in achieving this compensation or whether other mechanisms are able to

provide the cholesterol requirements for oestrogen and progesterone synthesis within the ovary.

3.6.5 Conclusions

In summary, the experimental evidence presented in this chapter indicates that sexually dimorphic expression of CD36 in the liver was present in all strains of rat tested and a similar dimorphism is found at the level of transcription in a number of mouse strains. Expression of CD36 appeared to be identical between males and females in all other tissues tested, with the exception of the adrenal gland. Expression of the molecule in the adrenal cortex displayed a gender bias in the opposite direction to the liver. The higher expression of CD36 in the liver of females warrants further investigation in order to understand the physiological significance and the mechanisms responsible for the dimorphic regulation of this protein.

Chapter 4 DEVELOPMENTAL REGULATION OF CD36 IN THE LIVER

4.1 Introduction

It was established in the previous chapter that there was higher expression of CD36 in the liver in adult female rats compared with males. This observation led to the hypothesis that gender-specific hormones such as estrogen or progesterone in females, or testosterone in males, are responsible for establishing and maintaining the gender-specific adult pattern of expression. Gonadal steroid hormones can influence gene expression in hormone-responsive tissues in a number of ways. Following engagement by the appropriate steroidal ligand, nuclear hormone receptors such as the androgen receptor (AR) or estrogen receptor (ER) act directly on the promoters of target genes. If the steroid-responsive gene is itself a regulatory protein, such as a transcription factor, the presence or absence of the relevant steroid hormone can alter the expression of many other genes indirectly.

Alternatively, gonadal steroids interact with the hypothalamo-pituitary axis, allowing them to have indirect influences via other hormones such as LH, FSH or GH (Gatford et al., 1998). Prior to puberty, the serum levels of gender-specific steroid hormones, gonadotropins and growth hormone differ markedly from those found in adult rats (Jansson et al., 1984, Dohler and Wuttke, 1975). One approach to investigating possible roles (direct or indirect) of gonadal steroids in regulating CD36 expression in the liver was to examine whether the gender bias in expression of the molecule is present during the pre-pubertal period, or whether its appearance coincides with puberty.

No studies that investigate expression of CD36 in the liver of juvenile rodents have been reported. However, there is evidence that the amount of CD36 present in some other tissues may change as rats reach maturity. There is an approximately 5-fold increase in levels of *Cd36* transcripts in the heart between birth and age 21 days (Van Nieuwenhoven et al., 1995). Similarly, intestinal enterocytes from adult rats express considerably higher levels of *Cd36* transcripts than those from neonatal intestine (Chen et al., 2001). It was not clear, therefore, whether the gender difference in hepatic

expression of CD36 would be present already in rat pups, or whether it would appear later, perhaps as a response to dietary, environmental or hormonal changes during the juvenile period. Observations in pre-pubertal male and female pups provide, therefore, a baseline against which any changes that occur at puberty can be compared.

Two sub-hypotheses were formulated, namely:

1) Prior to puberty, juvenile rats do not display gender-biased expression of CD36 in the liver.

2) During puberty, increases in serum levels of gender-specific steroid hormones coincide with acquisition of the adult pattern of hepatic CD36 expression.

4.2 Expression of CD36 in the Liver in Juvenile DA Rats

4.2.1 Background

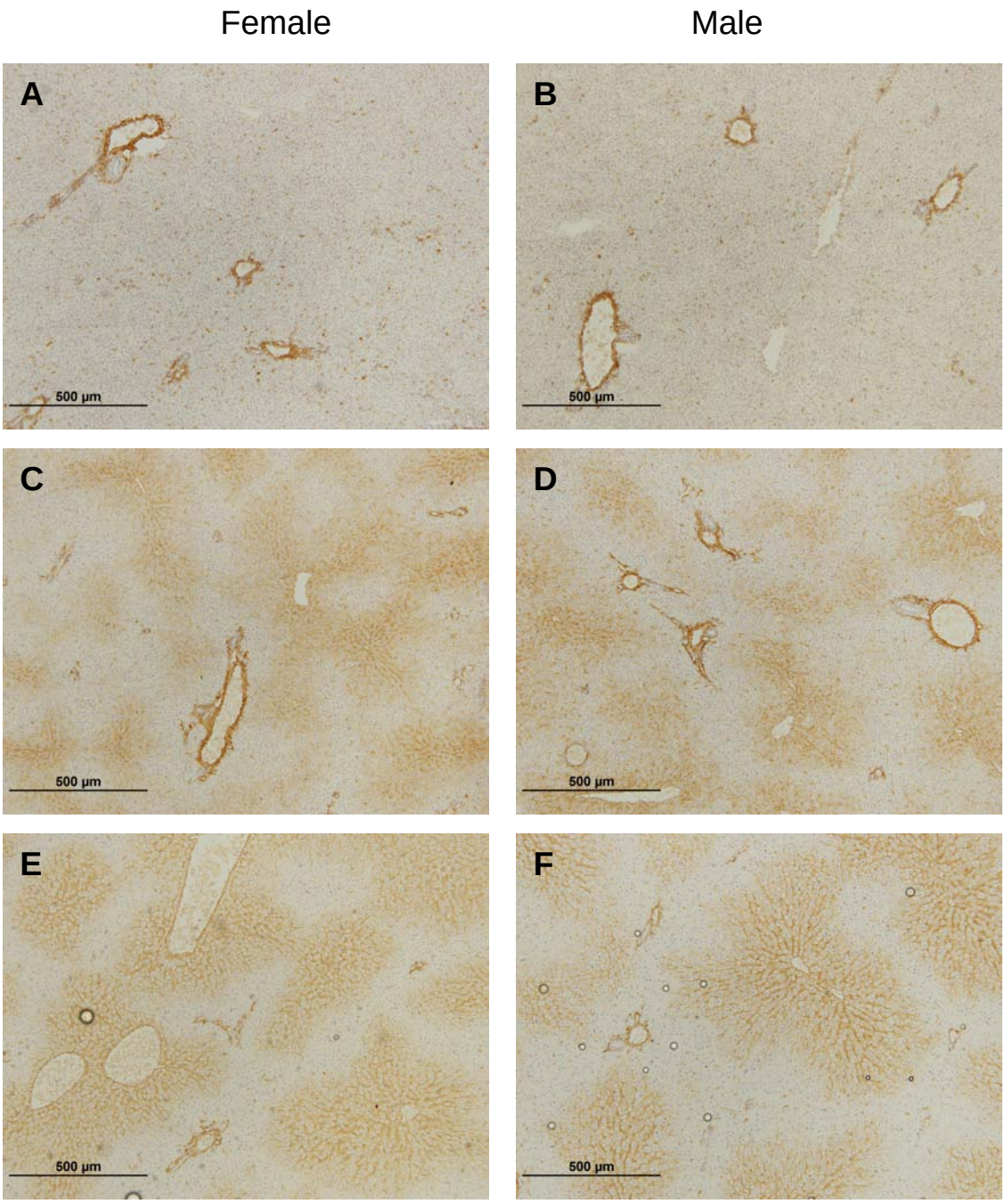
Rats were studied during the pre-pubertal period to examine CD36 expression in the liver prior to the possible influences from gonadal steroids. As this also spanned the weaning period (approximately 3 weeks of age), it allowed observations on CD36 expression during the change from dependence on suckling to an independent conventional solid diet.

4.2.2 Effect of Age on CD36 Expression in the Liver During the Pre-Pubertal Period

Groups of five male or female pre-pubertal rats were examined, at ages 2 weeks (pre-weaning), 4 weeks and 5 weeks (post-weaning). Frozen sections of liver were stained with mAb UA009, using indirect immuno-histochemistry. There was no staining by isotype-matched control mAb 1B5 in sections from any of the groups. Surprisingly, there was no staining of hepatocytes in sections of liver from either male or female 2 week old rats (Fig. 4.1 A&B), although vascular endothelial cells and scattered cells with irregular shape cells within the lobule (presumably Kupffer cells) were stained strongly. In sections of liver from 4 week-old rats, there was diffuse staining of hepatocytes, with more intense staining at the centres of lobules (Fig. 4.1C&D). The greater intensity of staining around the central veins was due predominantly to staining of sinusoidal endothelium. However, there was also weaker staining of the cell

Figure 4.1: Hepatic expression of CD36 in juvenile DA rat during post-natal development.

Liver was obtained at ages two, four and five weeks of age and frozen sections were stained with anti-CD36 mAb UA009 using the immunohistochemical technique. A, C, E; females aged 2, 4 and 5 weeks respectively. B, D and F: males aged 14, 28, 35, days, respectively. Photographed using X10 objective.


Female
Male
A
B
C
D
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500 µm
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500 µm
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500 µm

membranes of some hepatocytes in these areas. The pattern and intensity of staining was similar in sections from all rats within each group and at this age, no difference was discernable between sections from male and female rats.

In liver sections from 5 week-old rats, the centrilobular pattern of staining by mAb UA009 was well established (Fig. 4.1 E&F) and there was distinct hepatocyte cell-surface staining. The proportion of the liver lobule in which CD36 could be detected was intermediate between that observed in adult females and adult males and there was no difference between females and males at this age.

4.3 Effect of Puberty on Expression of CD36 in the Liver in Female and Male DA Rats

4.3.1 Background

In female rats, the onset of puberty can be defined anatomically and by changes in levels of circulating hormones. Although there are differences between rat strains, external vaginal opening coincides with the first ovulation, which occurs at approximately 35 to 45 days of age. These events are precipitated by a cascade of endocrinological changes, culminating with the first surge of LHRH. This surge in turn "activates" competent ovarian cells to commence high-level production and secretion of estrogen. (Ojeda et al., 1986). A regular cyclical pattern of gonadal estrogen and progesterone secretion is established following this first ovulation.

In male rats, the onset of puberty occurs later and is less clearly defined than in females. The first gonadotropin surge (FSH) peaks at approximately 40 days of age and reproductive capacity is acquired shortly afterwards. The first mature sperm are present from approximately 45 days of age (Odell and Swerdloff, 1976). Testosterone can be detected in the serum of male SD rats from birth onwards, and levels fluctuate considerably until approximately day 40. From this time, the concentration of circulating testosterone increases steadily, overshooting normal adult levels by 50 days of age and then settling to adult levels. A similar pattern is observed for total serum androgens. (Dohler and Wuttke, 1975).

At the ages used in the following experiments (6 and 7 weeks of age), both females and males can be considered to be either peri-pubertal or recently post-pubescent respectively. Although patterns of gonadal steroid and pituitary hormone secretion may not have reached adult equilibrium, the endocrinological mileu would be considerably different from those in pre-pubescent rats. As in section 4.2, liver expression of CD36 was examined in groups of five male and five female rats of each age.

4.3.2 Peri- and Post-Pubertal Expression of CD36 in the Liver

Immuno-histochemical staining by mAb UA009 showed increased expression of CD36 by hepatocytes in liver sections from 6 week-old female rats (Fig. 4.2A), compared with that observed in tissue from 5 week-old rats (Fig. 4.1E). Furthermore, the proportion of each lobule that was stained was greater at this age. These changes were evident in liver sections from all members of the group. By 7 weeks of age, these changes were accentuated (Fig. 4.2C), with a large proportion of the hepatocytes in each lobule stained intensely by mAb UA009. Thus, all of the animals in this group had attained the adult phenotype by the age of 6-7 weeks (approximately one to two weeks after puberty).

When liver sections from male rats aged 5 weeks (Fig. 4.1F) and 6 weeks (Fig. 4.2B) were compared, there was no difference in the patterns of staining by mAb UA009. However, there was considerably less staining in sections from 7 week-old male rats (Fig. 4.2D) than in either 6 week-old male rats (Fig. 4.2B), or age-matched female rats (Fig. 4.2C). In tissue from 7 week-old male rats, the majority of hepatic parenchymal cells were unstained and expression of CD36 was confined to a rim of hepatocytes around the central vein in each lobule, thus approximating the pattern observed in adult males. This was not due to a technical failure because other cell types within the lobules, such as vascular endothelial cells and Kupffer cells, were stained intensely by the antibody. Although this reduction in the proportion of stained hepatocytes was observed in all rats within the group, there was some individual variation. Three of the five rats displayed an adult male pattern of expression, whereas the other two displayed staining that was intermediate between that observed in the 6 week-old rats. It appears, therefore, that changes in CD36 expression in the liver occurred at about 7 weeks of age in male DA rats, approximating the time of puberty. These results indicate that the sexually dimorphic pattern of expression of hepatic CD36 in rats occurs at approximately 6 to 7

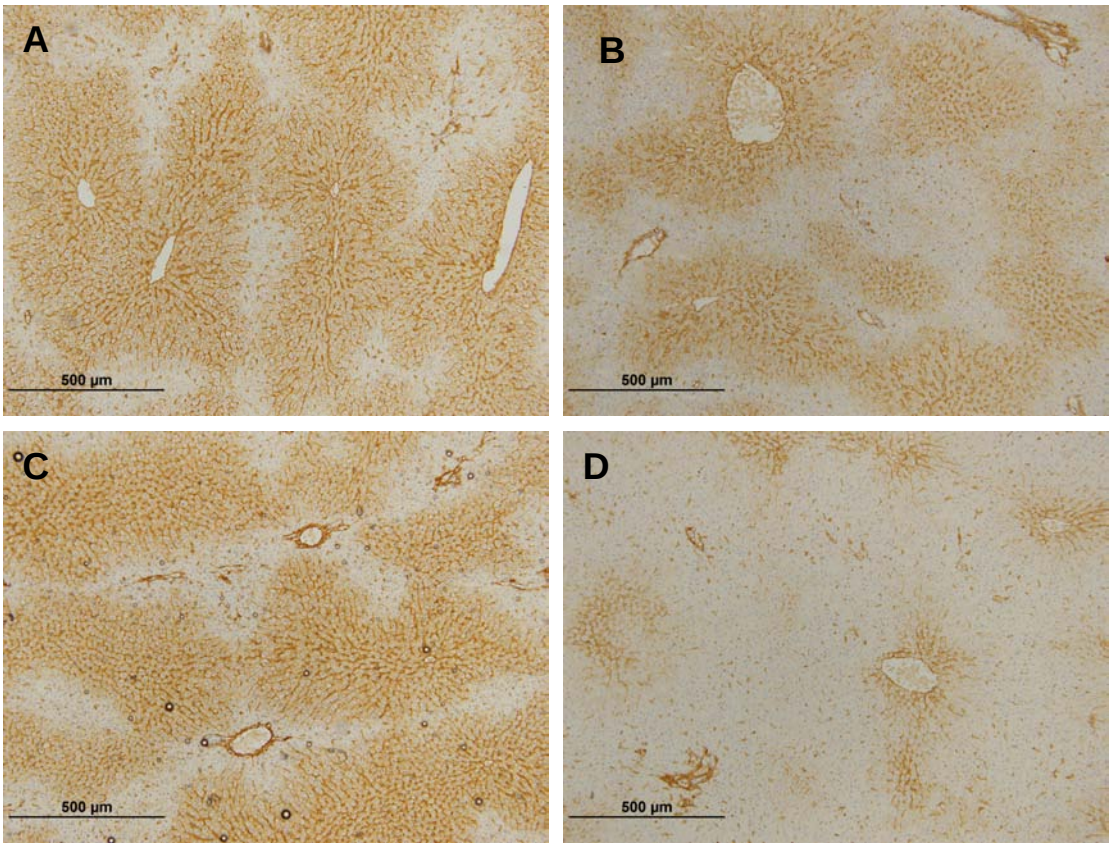
Figure 4.2: Expression of CD36 in DA rat liver during the pubertal period.

Immunohistochemical analysis of CD36 protein in livers of 6 week old (female, A; male, B) and 7 week old (female, C; male, D) rats using mAb UA009. In female rats aged 6 weeks and older, the majority of hepatocytes strongly express CD36 protein (A, C), corresponding to the adult pattern of expression. In male rats at this age, moderate levels of CD36 staining were observed (B). In contrast at 7 weeks of age very few hepatocytes expressed CD36 (D), as is observed in adult male rats.

Photographed using x10 objective.

Female

Male



weeks of age and that it is linked closely to puberty and the establishment of the adult sex hormone status. It involves upregulation of CD36 expression in the liver in female rats and down-regulation in male rats, in both cases resulting in the distinctive gender-specific patterns of expression within lobules that are observed in adults.

4.4 Expression of CD36 in the Liver During Adulthood

4.4.1 Background

Having demonstrated that expression of CD36 in the liver changes during development and again at the onset of puberty, studies were undertaken to compare patterns of expression in young sexually mature rats and mature adults.

4.4.2 CD36 Expression in the Liver in Young and Mature Adult Rats

Frozen sections of liver from groups of post-pubertal (8 week old), young adult (12 week old) and mature adult (24 week old) rats were stained with mAb UA009 to assess expression of CD36 (n=5 of each gender per age-group). In female rats, staining patterns were comparable between all three age groups (Fig. 4.3A, C and E), with the majority of hepatocytes within lobules expressing CD36. In sections from the group of eight week-old male rats (Fig. 4B), all showed the adult male pattern of CD36 expression, indicating that the process of down-regulation observed at 7 weeks of age had become established. This pattern, with staining by mAb UA009 confined to the rim of hepatocytes surrounding the central vein (Fig. 4.3D and F), was maintained in young adults (12 weeks of age) and in mature males (24 weeks of age).

4.5 CD36 Expression and Analysis of Serum Hormone Levels

4.5.1 Background

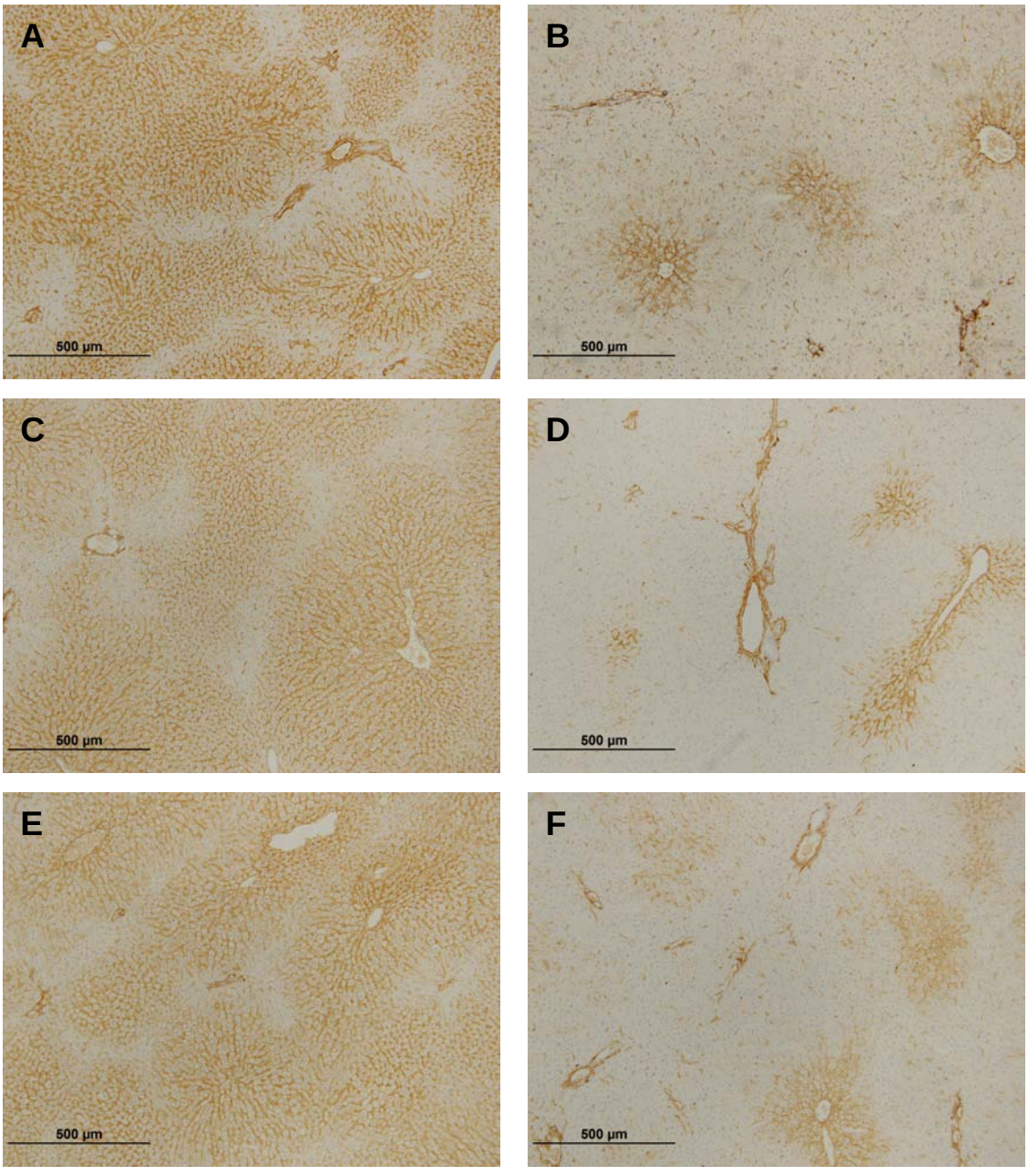
Measurement of sex steroid hormones would provide a more objective indication of the onset of puberty an allow comparison with data in another strain of rat (Dohler and Wuttke, 1975). This is relatively straight-forward in males, where testosterone is the dominant androgen and levels are relatively constant. While comparable studies on

Figure 4.3: CD36 expression in mature adult DA rats.

Expression of CD36 protein was analysed by immunohistochemistry in frozen liver sections from male and female rats aged between 8 and 24 weeks of age. Maturation did not effect hepatic CD36 expression in either males or females. A, C and E; females aged 8, 12 and 24 weeks respectively. B, D and F; males aged 8, 12 and 24 weeks respectively. Photographed using x10 objective.

Female

Male



circulating estrogen could be undertaken in female rats, several factors make analysis of serum 17β estradiol (E2) in young rats more difficult to interpret. Firstly, alpha feto-protein in juvenile rats binds E2 and renders it inactive; making simple measurements of serum levels a poor indicator of the bioavailability of the hormone (Ojeda et al., 1986). Secondly, due to the ovulatory cycle in post-pubertal animals, the serum levels of E2 change cyclically. To be interpretable, levels of E2 must be determined in individual animals throughout the oestrus cycle and compared as integrated "areas under the curve". For these reasons, correlation of expression of CD36 in the liver with serum sex hormones was limited to testosterone in males.

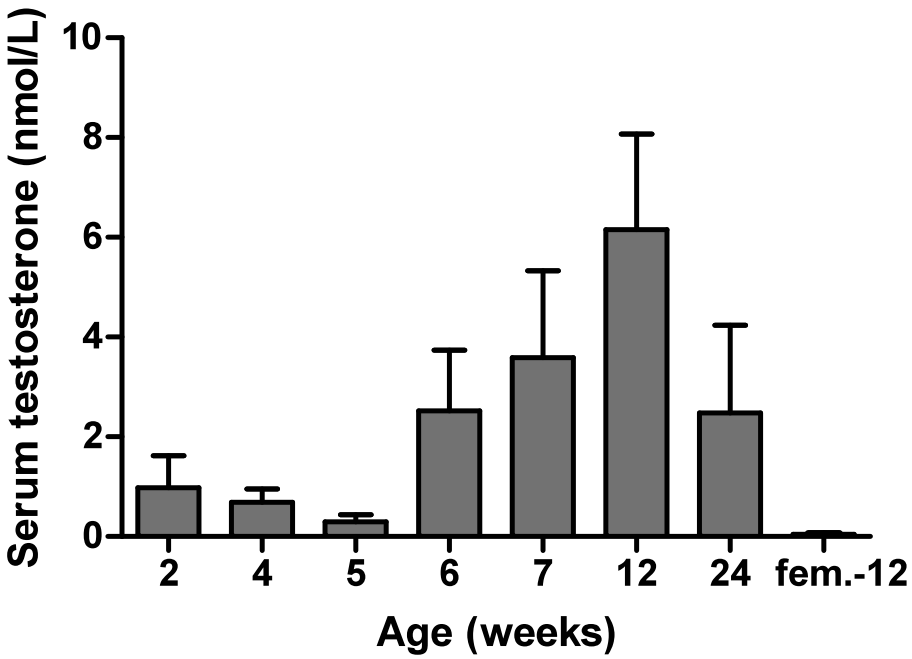
4.5.2 Serum Testosterone Levels in Young and Adult Male DA Rats

Serum was prepared from blood obtained from the male DA rats used above in analysis of liver CD36 expression (ages between 2 and 24 week). Testosterone levels were estimated using a competitive radio-immuno assay, as described in Materials and Methods (section 2.8). A "%B/Bo value", (given by the formula %B/Bo = [standard or sample count] x 100 / zero-standard count) was computed using counts obtained from the gamma-counter. The %B/Bo values were then plotted against standard known concentrations of testosterone (ranging from 0 to 50nmol/L testosterone) in order to generate a standard curve from which to read the concentrations of testosterone in the serum samples. The results are presented graphically in Figure 4.4.

There was considerable variation in testosterone levels between individuals in each group and with a sample size of 5 per group, no significant differences between groups were detected by one-way anova (Tukey's multiple comparison post-test). However, the results the suggest that there is a small decrease in serum levels of the hormone between ages of two weeks to five weeks, followed by a rise at six weeks that appears to continue until 12 weeks of age. There is a good correlation between the rise in testosterone levels at six weeks of age and the slightly delayed trend towards the adult pattern of CD36 expression in the liver at 7 weeks of age (Fig. 4.2D). The highest levels of testosterone were measured in serum from 12 week-old rats and mean levels had decreased by 24 weeks of age to approximately those seen at age six to seven weeks. During this period, expression of CD36 in the liver appeared to be unchanged, suggesting that if testosterone is responsible for suppression of CD36, the levels at puberty are sufficient to induce and maintain maximal suppression.

Figure 4.4: Developmental changes in serum testosterone concentrations in DA rats.

Testosterone levels in serum samples from male rats aged 2 to 24 weeks of age were measured using an indirect radio-immunoassay. A standard curve was used to convert the CPM values obtained into [T], given as nmol/L. The average value obtained from duplicate measurements for each sample was used to generate the data presented in this graph. Bars indicate the mean +/- SE, (n=5 rats) for each age group.


Serum testosterone (nmol/L)
10
8
6
4
2
0
2
4
5
6
7
12
24
fem.-12
Age (weeks)

4.6 Developmental Regulation of SR-BI Expression in the Liver

4.6.1 Background

In Chapter 3, a cross-reactive polyclonal anti-human SR-BI antibody was found to stain hepatocytes in adult liver only weakly and in a diffuse pattern, without clear staining of the cell membranes or the sub-lobular partitioning noted when mAb UA009 was used to detect CD36 (Section 3.3.2). The same antibody demonstrated SR-BI in Western blot analysis of liver proteins and also produced strong staining of SR-BI in tissue sections from adrenal and ovary (Section 3.4). It appeared, therefore, that SR-BI is either expressed by individual hepatocytes at very low levels or that the native form of SR-BI expressed in the liver is recognised poorly by the antibody.

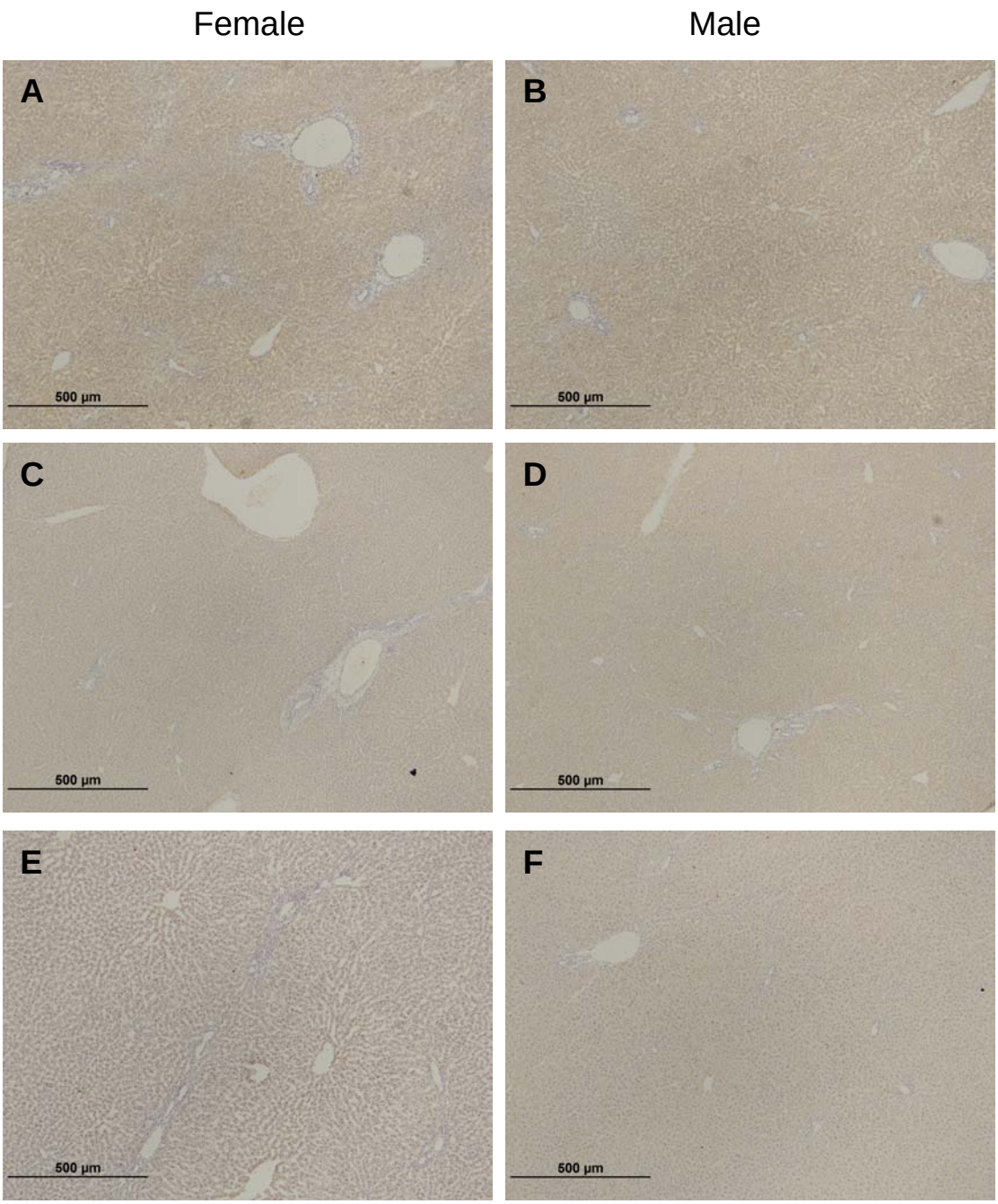
There have been no published studies describing expression of SR-BI in the liver in juvenile or adolescent rats. However, it has been reported that expression of the molecule is regulated by steroid sex hormones (Graf et al., 2001, Landschulz et al., 1996). In particular, expression of SR-BI in the liver is down-regulated by pharmacological doses of estrogen (Landschulz et al., 1996). It is possible, therefore, that levels of SR-BI are suppressed in adult female liver by circulating estrogen or other changes associated with the onset of puberty and that examination of pre-pubertal rats might reveal detectable levels of the molecule. For this reason, frozen sections of liver from young female and male rats were examined by immuno-histochemistry to investigate whether expression of SR-BI can be detected prior to puberty.

4.6.2 Expression of SR-BI in the Liver in Pre-Pubertal DA Rats

Groups of five male or female rats were examined at 2, 4, 5, 6 and 7 weeks of age. As observed in sections of liver from adult male and female DA rats (section 3.3.2), there was only weak staining of hepatocytes in the sections prepared from two week-old pups of either sex (Fig. 4.5). The faint staining did not seem to localise to the hepatocyte cell membranes and was evenly distributed across the liver lobule. Similar staining, which was not present in sections stained with the isotype-matched control mAb, was observed also in liver from the groups of 4-7 week-old male and female rats. It appears therefore

Figure 4.5: Hepatic expression of SR-BI in DA rat during post-natal development.

Liver was obtained at ages two, four and six weeks of age and frozen sections were stained with affinity-purified IgG rabbit polyclonal anti-SR-BI ab using the indirect immunoperoxidase technique. A, C, E; females aged 2, 4 and 6 weeks respectively. B, D and F: males aged 2, 4 and 6 weeks, respectively. Photographed using X10 objective.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Male
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500 µm

that SR-BI is expressed at low levels by hepatocytes, that its expression is not developmentally regulated in the pre-pubertal period and that expression is not affected by hormonal changes associated with puberty.

4.8 Discussion

The experiments described in this chapter examined the relationship between age and expression of CD36 in the liver, with particular attention to the period around puberty. Because there is a marked gender difference in expression of CD36 by hepatocytes in male and female rats, an important hypothesis to be tested was that prior to the onset of puberty the patterns of CD36 expressed would be similar in male and female rats, while the gender difference would become apparent at or soon after puberty. The results supported this hypothesis. In addition, a developmental regulation has been identified that initiates CD36 expression by hepatocytes in male and female rats in the period between 14 and 28 days after birth. What initiates the onset of CD36 expression and exactly when it occurs was not explored further. On the one hand, it may be developmentally programmed and have an endogenous trigger. However, an important event in this period is the change from an entirely milk diet to a partial solid diet, followed by complete weaning at age 21 days by animal maintenance staff.

It is interesting that the hepatocytes do not commence expression of CD36 until approximately the time of weaning and possibly later. During suckling, pups have a very high lipid intake and β-oxidation of FA provides their primary source of metabolic energy. Thus prior to weaning, the liver has a low rate of lipid synthesis and a high rate of lipid degradation. Compared with milk, normal rat chow is higher in carbohydrate and lower in fat. Late in the suckling period, a cluster of genes is switched on whose expression in the liver facilitates adaptation to solid foods. This cluster of genes includes ATP-citrate lyase, pyruvate dehydrogenase, maleate dehydrogenase, fatty acid synthase, glucokinase and pyruvate kinase. Thus the liver switches activity away from lipid degradation and towards synthesis (Bohme et al., 1983). It is not immediately clear why there should be an increase in expression of CD36 by hepatocytes at a time when uptake of exogenous FA should be less important. This might represent a “scavenger” function to collect surplus FA, rather than uptake to meet metabolic demands of the liver per se. This situation contrasts with findings in the heart, where upregulation of

Cd36 mRNA at three weeks of age coincides with increased FA utilization by this tissue (Van Nieuwenhoven et al., 1995). One explanation is that transport of FA is not the primary role of CD36 in the liver, and increased expression occurs to facilitate other metabolic processes *eg* lipoprotein holoparticle uptake or cholesteryl ester selective uptake. Complete investigation of the effect of a solid diet and weaning on hepatic expression of CD36 would require additional age-related sampling and control of access to solid food by the pups. Effects of diet and contributions from changing gut microbiota are intriguing possible triggers of CD36 expression, especially in view of the vital role of the liver in metabolism of nutrients and toxins and its position downstream from the gut in the portal circulation.

With respect to CD36 expression, gender differentiation commences at approximately six weeks in female rats and at approximately seven weeks in male rats. The onset of gender-specific expression coincides approximately with the reported commencement of puberty in female and male SD rats (Odell and Swerdloff, 1976). Furthermore, it is reported that there is a large increase in serum testosterone concentration in male SD rats at age 7 weeks (Dohler and Wuttke, 1975). The present study confirms that an increase in testosterone levels occurs also in male DA rats at this age. However, the bio-availability of plasma testosterone is affected by circulating plasma levels of sex hormone-binding globulin (SHBG). Production of this protein by Sertoli cells is under hormonal control and may therefore vary during development (Munell et al., 2002). The testosterone assay employed in this study does not discriminate between free and bound androgen. Therefore, although the experimental evidence presented does not establish a direct link between regulation of CD36 expression in the liver and puberty, the observations on testosterone levels in male rats are consistent with this possibility.

Unfortunately, the scope of the project did not allow similar correlation between expression of CD36 by hepatocytes in females with serum estradiol levels. Without allowance for the oestrus cycle, random measurements of serum estradiol levels in female rats are of limited value. Unlike male rats, in which pre-pubertal levels of testosterone are relatively low, there are significant amounts of E2 in the serum from juvenile female rats, and concentrations vary during the pre-pubertal period. The peak serum E2 concentrations in adulthood are actually lower than the irregular surges that occur in the first weeks of life (Dohler and Wuttke, 1975). Furthermore, the presence of alpha feto-protein in very young rats reduces the bio-availability of E2. An additional

consideration is that many of the tissues that respond to E2 after puberty (eg those of the hypothalamus/pituitary axis) have not gained full responsiveness to oestrogen during the pre-pubertal period (Ojeda et al., 1986).

Nevertheless, the findings from the present study indicate that expression of CD36 in the liver in female rats increases at around the time of puberty. For the reasons discussed above, this increase is unlikely to be caused by an increase in serum concentration of E2 per se. However, it is possible that other changes occur in the body at adolescence that result in increased sensitivity of the liver to E2. These could include changes in expression of hepatic estrogen receptors (Norstedt et al., 1981), altered metabolism of E2 or increased bioavailability of the hormones. Alternatively, there may be downstream effects resulting from the acquisition of E2 responsiveness by the hypothalamus that are responsible ultimately for the changes that are observed in expression of CD36 by the liver. Clearly, further experiments are required to elucidate the mechanisms that underlie the peri-pubertal regulation of hepatic CD36 expression in both sexes.

The third line of investigation was to examine whether there are age-dependent effects on CD36 expression in the liver in post-pubertal and adult rats. Both male and female rats were found to display the adult pattern of CD36 expression in the liver by eight weeks of age. This pattern was maintained up to 24 weeks of age. This is of interest because testosterone levels have been shown to be in decline by 24 weeks of age (Kaler and Neaves, 1981). There was, however, no corresponding change in hepatic CD36 expression from the young adult pattern. Examination of older male and female rats (twelve to eighteen months) was not possible within the time-frame of this project. Old female rats are reported to undergo menopause (Nass et al., 1984) and it would be interesting to determine whether the increased and subsequently decreased E2 levels are matched by respective changes in CD36 expression in the liver by perimenopausal and post-menopausal females.

Finally, the observation that SR-BI expression in rat liver can be regulated by sex steroid hormones (Landschulz et al., 1996, Graf et al., 2001)(Landschulz et al, Graf et al) led to a consideration as to whether expression of this scavenger receptor may also alter throughout post-natal development. The results presented in Chapter 3 did not provide clear evidence of a gender difference in SR-BI expression by hepatocytes in adult rats.

Extension of these studies to young rats did not reveal any effects of development or puberty on SR-BI expression in either male or female rats. In particular, there was no evidence that the difficulty experienced in detection of SR-BI in hepatocytes in adult rats is due to down-regulation of the molecule by hormonal changes related to puberty.

To provide an overview of the changes in expression of CD36 it is useful to describe two distinct phases that occur during post-natal development. In the first phase, there is induction of a basal level of CD36 expression in hepatic parenchymal cells. At two weeks of age, CD36 is expressed only by hepatic vascular endothelium and Kupffer cells. The earliest time at which CD36 could be detected in hepatocytes was at four weeks of age, (although it is possible that expression may have commenced earlier than this). Three characteristics describe this phase of CD36 expression by hepatocytes. Firstly, expression becomes stronger as the animals mature and secondly, the distribution of the protein within the liver lobules becomes focussed increasingly around the central veins. Finally, expression is independent of gender, as the pattern observed in male and female rats was indistinguishable up to five weeks of age. The second phase in regulation of CD36 expression by hepatocytes is the imposition of gender difference. The present studies show that gender differentiation involves changes in both genders. Compared to their pre-pubertal counterparts, expression of CD36 by hepatocytes in female rats is increased, while expression in male rats is reduced.

Therefore, in relation to the hypotheses enunciated at the beginning of this chapter, the information collected from this series of experiments can be summarised as follows. The characteristic centrilobular distribution of CD36 in the liver is not associated with gender-specific regulatory mechanisms because chronologically, this pattern is established before the advent of gender differentiation. However, the sexually dimorphic pattern of CD36 expression in the liver is only observed in post-pubertal rats. In both sexes, the process(es) leading to gender dimorphism are active, in each case leading to divergence from the non-dimorphic pattern that exists before puberty. The timing of these changes in male rats coincides with an increase in serum testosterone levels, thus implicating the male sex steroid in this process. In female rats, there was also a temporal relationship between changes in CD36 expression and puberty. However, this observation alone does not implicate estrogen in upregulation of CD36 in female rats.

This study provides important information from which to construct hypotheses relating to the role of the endocrine system in controlling expression of CD36 in the liver and for the design of experiments with which to test the role of sex steroids in establishing gender dimorphism in the post-pubertal period.

Chapter 5 THE ROLE OF SEX STEROIDS IN REGULATING GENDER-SPECIFIC PATTERNS OF CD36 EXPRESSION IN THE LIVER.

5.1 Introduction

The work presented in Chapter 4 demonstrated that the sexually dimorphic pattern of CD36 expression in the liver occurs only in post-pubertal rats. The difference in expression between the genders was a consequence of post-pubertal up-regulation of expression in females and down-regulation of expression in males. Because one of the most significant changes that occurs in puberty is an increase in the serum levels of gender-specific sex steroids (as well as acquisition of responsiveness to these hormones), the next stage was to test the hypothesis that gonadectomy or *in vivo* manipulation of E2, progesterone and/or testosterone concentrations will result in changes in the expression of CD36 in the liver in young adult rats.

In male rats, testosterone is produced mainly in the testes, while in females the main source of circulating E2 and progesterone is the ovaries. Therefore, surgical gonadectomy (Gx) is an effective method for assessing the effects of sex hormone withdrawal. Although small quantities of sex steroids can be produced by the adrenal glands in some species, this is unlikely to affect gene expression in the liver due to the efficiency of the organ in metabolizing steroid hormones (Eisenfeld and Aten, 1987). In Gx animals, the effects of administering particular sex hormones can be examined in the absence of the corresponding endogenous hormones, thus providing clear evidence of their effects on the liver. This approach has been used to study the expression of numerous hepatic genes that exhibit gender-specific regulation, including SR-BI/II (Stangl et al., 2002), PPARalpha (Jalouli et al., 2003), cytosolic alcohol dehydrogenase (Harada et al., 1998), neutral cholesterol ester hydrolase (Gandarias et al., 1984), glutathione s-transferase subunit A5 (Staffas et al., 1998), the LDL receptor (Staels et al., 1990), plus many cytochrome P450 isoenzymes (Kalsotra et al., 2002, Lax et al., 1979). The mechanisms underlying the gender-biased expression of some of these genes

have been shown subsequently to involve other hormones (e.g. ACTH or GH) as well as the gonadal steroids. Nevertheless, the response (or lack thereof) of a gene to gonadectomy and/or sex steroid reinstatement provides an obvious starting point from which to investigate the complexities of gender-specific gene regulation.

Important additional information on regulation of CD36 expression can be obtained by examining transcriptional activity of *Cd36*, using quantitative RT-PCR (qRT-PCR) to measure relative levels of *Cd36* transcripts. If either E2 or testosterone acts directly or indirectly on regulatory elements in the *Cd36* promoter, via engagement of nuclear hormone receptors or other transcription factors, then change in levels of *Cd36* transcripts should be detected. Indeed, work described in Chapter 3 shows that in three inbred mouse strains, there are gender differences in levels of *Cd36* mRNA in the liver. The work in this chapter includes, therefore, analysis of both CD36 and *Cd36* transcripts during various manipulations of endocrine status.

The general hypothesis examined in this chapter is:

Gonadectomy, or manipulation of E2, progesterone and/or testosterone concentrations, will result in changes in expression of CD36 by hepatocytes.

The work described tests the following sub-hypotheses:

Levels of *Cd36* mRNA are higher in the liver in adult female rats than in aged matched males.
The liver contains less *Cd36* mRNA/CD36 protein in Gx female rats compared with age- and gender-matched sham-operated controls.
The liver contains more *Cd36* mRNA/CD36 protein in Gx male rats compared with age- and gender-matched sham-operated controls
Administration of E2 to Gx female rats and testosterone to gonadectomised male rats will restore the gender-bias to expression of CD36 in the liver.
In hepatocytes cultured *in vitro*, expression of *Cd36* mRNA/CD36 protein increases in the presence of E2 and decreases in the presence of testosterone.

5.2 Strategies For Measuring Cd36 mRNA And CD36 Protein In Rat Liver

A number of assays were assessed for their suitability for measuring changes in expression of CD36 and transcripts encoding CD36. A real-time PCR assay was established to measure *Cd36* RNA transcripts and this is described in Section 5.2.1. Although readily detectable in tissues by immuno-histochemical techniques, measurement of CD36 levels in protein extracts was not possible at the time that this work was conducted. Neither mAb UA009 nor commercially available polyclonal antibody L-17 (Santa Cruz biotechnology), were successful in Western blot. However, towards the end of the study, a mouse anti-human CD36 mAb that cross-reacts with rat CD36 (Tandon et al., 1989) was provided by Dr Narendra Tandon. This allowed semi-quantitative comparison of the levels of CD36 in normal male and female liver samples by Western blot (section 5.2.2). Unfortunately, this antibody was not available early enough to estimate relative levels of CD36 in the samples of liver from Gx rats or rats that had been treated with sex steroid hormones. Stored frozen liver samples from these experiments were lost during a freezer break-down. Quantitative video-image analysis (VIA) of immuno-histo-chemistry was used for this purpose, allowing measurement of the relative areas of the liver stained by mAb UA009 and thus an indirect estimate of the relative proportion of CD36-positive hepatocytes. This technique is described in section 5.2.2.

5.2.1 Measurement Of Relative Levels Of mRNAs Encoding CD36 And SR-BI By Reverse Transcription And Real-Time PCR (RT-PCR)

5.2.1.1 Background

A SYBR-green based real-time PCR assay was established to measure the relative amounts of *Cd36* mRNA in livers from the various experimental groups. In addition, primers were also designed to amplify *Sr-bI/II* and the housekeeping gene *36b4*. The gene *36b4* encodes a ribosomal phosphoprotein that is expressed ubiquitously. It was chosen as an appropriate control because its expression is not influenced by estrogen (Laborda, 1991).

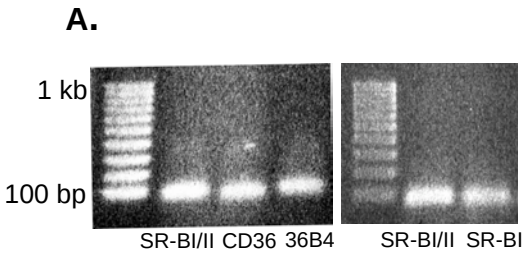
When SYBR-green technology is used to compare levels of transcripts encoding a nominated gene, relative to the levels encoding a control housekeeping gene, it is necessary to understand the reaction characteristics of each of the primer pairs. To be meaningful in estimating relative amounts of the transcripts, the efficiency of amplification of the target gene must be comparable to that of the housekeeping gene. In the following section, evidence is presented to validate each of the primer pairs used to estimate relative levels of *Cd36* and *Sr-b1* transcripts in samples by qRT-PCR. The technique is then used to examine levels of *Cd36* and *Sr-BI/II* mRNA transcripts in liver from adult male and female rats.

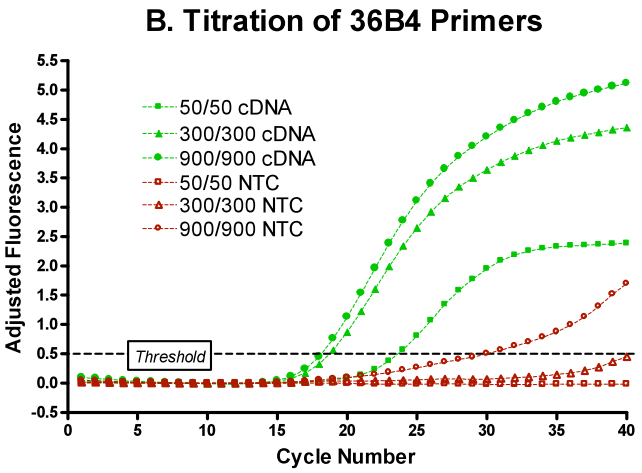
5.2.1.2 Validation of the qRT-PCR Assay Used To Measure Relative Levels of *Cd36* and *Sr-bI/II* mRNA

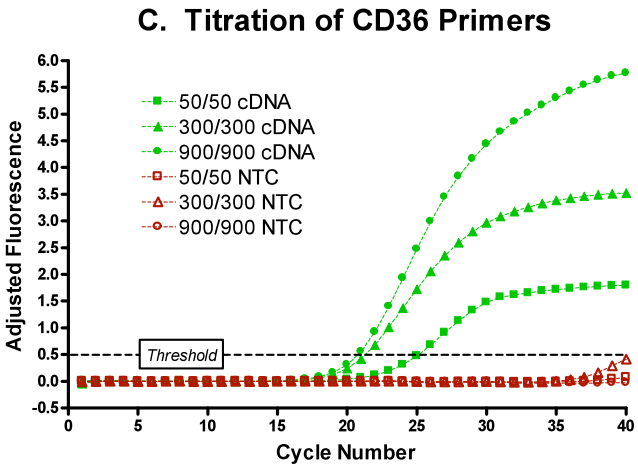
A number of conditional assumptions are inherent in the calculations used when the comparative C_T method is employed to measure the amounts of a target cDNA relative to an endogenous reference cDNA. These assumptions must be tested experimentally for each primer pair. The first assumption is that the relative efficiencies of amplification of the target and the reference cDNAs are comparable, with a ratio close to 1. To this end, the variables in the reaction (such as the affinity of the primers for template, primer concentration, template concentration, etc) are optimized to ensure high efficiency amplification. The Primer Express software program (Applied Biosystems) allows generation of high efficiency primer sets that are constrained with respect to melting temperature, GC content, primer length and amplicon length. Primers for *Cd36*, *Sr-bI*, *Sr-bI/II* and *36B4* were assessed for suitability in this assay. The details for each of these primers sets are listed in Table 2.3. Initially, each primer was tested using liver cDNA to ensure production of a single amplicon. This is crucial, as the fluorescent reporter dye SYBR-green detects all double-stranded DNA that is present, and the presence of non-target amplification products may result in false positives or over-estimation of the amounts of specific cDNA. PCR amplification of cDNA from adult female liver was performed under standard real-time amplification conditions, using each of the primer sets listed above. The reaction products were then resolved on 1.5% agarose gel by electrophoresis, post-stained with ethidium bromide and visualized using an UV illuminator. Each of the four primer sets produced a single amplicon that was less than 100 bp in size (Fig. 5.1 A).

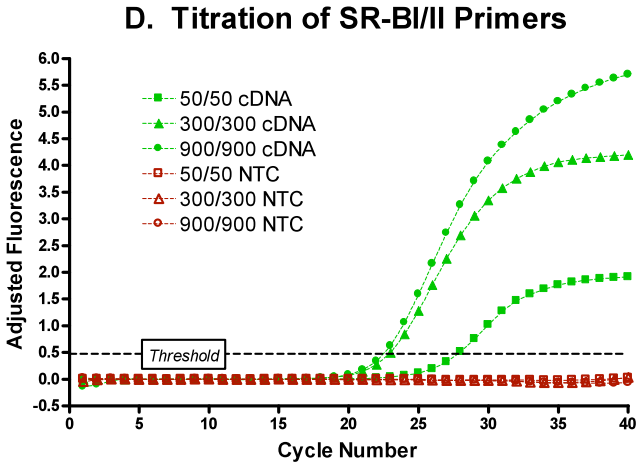
Figure 5.1: Validation of qPCR assay to measure levels of *Cd36* and *Sr-bI* mRNA trancripts.

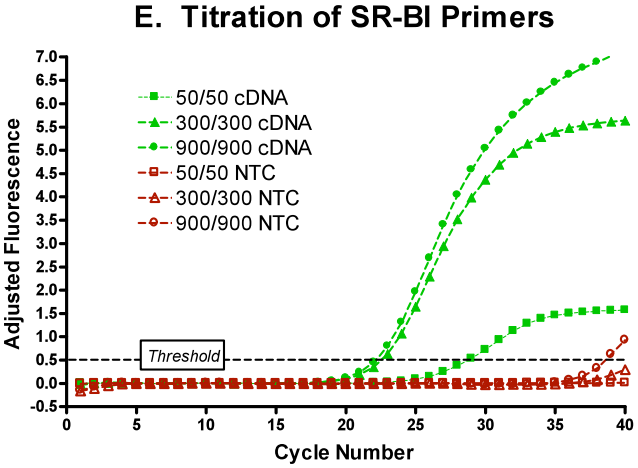
Photograph showing Cd36 (68 bp), Sr-bI (70 bp), Sr-BI/II (80 bp) and 36B4 (81 bp) PCR products resolved by agarose gel electrophoresis (A). Amplification of liver cDNA by conventional PCR using the respective real-time primer sets (sequences provided in Table 2.3) produced a single amplicon in each case. Titration of each of the primer sets was performed using either liver cDNA template, or an equal volume of H_2O (no template control). Primer concentrations were tested at 50, 300 and 900 nM and the adjusted fluorescence values obtained were plotted against the cycle number. Reactions performed using a 50 nM concentration of 36B4 (B), CD36 (C), SR-BI/II (D) or SR-BI (E) primers resulted in a reduced efficiency of amplification, whilst increasing the primer concentration to 900nM resulted in detection of fluorescence in the no template control reactions. Therefore, subsequent experiments were performed using primer sets at a concentration of 300nM.


A.
1 kb
100 bp
SR-BI/II CD36 36B4
SR-BI/II SR-BI


B. Titration of 36B4 Primers
50/50 cDNA
300/300 cDNA
900/900 cDNA
50/50 NTC
300/300 NTC
900/900 NTC
Threshold
Adjusted Fluorescence
Cycle Number


C. Titration of CD36 Primers
50/50 cDNA
300/300 cDNA
900/900 cDNA
50/50 NTC
300/300 NTC
900/900 NTC
Threshold
Adjusted Fluorescence
Cycle Number


D. Titration of SR-BI/II Primers
50/50 cDNA
300/300 cDNA
900/900 cDNA
50/50 NTC
300/300 NTC
900/900 NTC
Threshold
Adjusted Fluorescence
Cycle Number


E. Titration of SR-BI Primers
50/50 cDNA
300/300 cDNA
900/900 cDNA
50/50 NTC
300/300 NTC
900/900 NTC
Threshold
Adjusted Fluorescence
Cycle Number

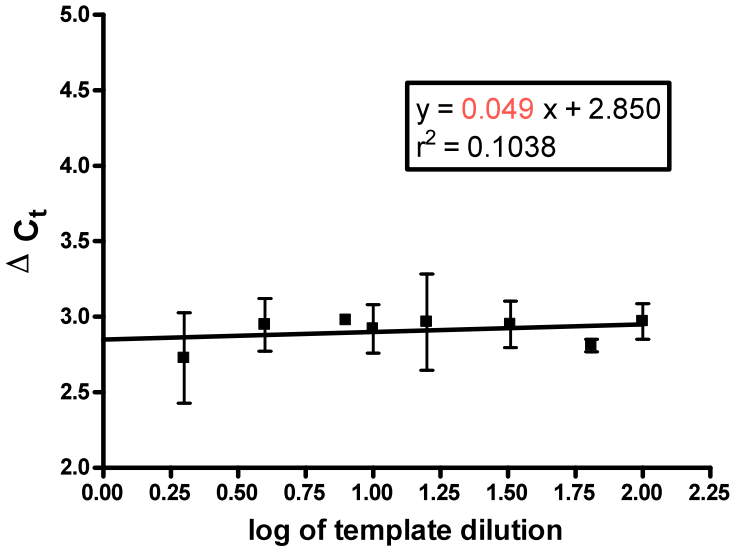
The second condition for optimisation was primer concentration, in order to determine the minimum primer concentration that resulted in maximum yield and minimum non-specific amplification. Real-time PCR reactions were performed, using cDNA from female rat liver as a template and with forward and reverse primers each at a concentration of 50, 300 or 900nM. The amplification curves for each of the primers pairs are shown in Figure 5.1 B-E. Each of the four primer sets performed well at concentrations of forward and reverse primers of 300nM. When the concentration of one or both of the primers was 50nM, the cycle threshold (C_T) of the reaction increased. If the concentration of one or both primers was increased to 900nM, the cycle threshold remained comparable but non-specific amplification was detected in the no template control reactions. Therefore, primers were used at a concentration of 300nM in all subsequent experiments.

The third condition tested was the relative efficiency of target cDNA (*Cd36, Sr-bI, Sr-bI/II*) and reference cDNA (*36B4*) amplification, assessing the effect of diluting the concentration of cDNA template on the ΔC_T values of the reaction (defined by $C_{T(target)} - C_{T(reference)}$). Validation was performed using standard real-time PCR amplification conditions, according to the guidelines outlined in the Applied Biosystems User Bulletin #2: *Relative Quantitation of Gene Expression*, in which the log of the template cDNA dilution factor is plotted against the ΔC_T value obtained at each dilution. If the relative efficiencies of target and reference cDNAs are equal, the slope of this graph will be zero (the efficiencies can be considered satisfactory if the absolute value of the slope is < 0.1). The relative efficiency plots for *Cd3*6, *Sr-bI/II* and *Sr-bI* cDNAs are shown in Figure 5.2 A, B and C respectively. The value of the slopes for *Cd36/36B4* and *Sr-bI/II/36B4* were found to be 0.049 and 0.019 respectively, indicating that the amplification efficiencies of both of these target cDNAs are approximately equal to that of the reference *36B4* cDNA. These primer sets are, therefore, suitable for estimating relative amounts of *Cd36* and *Sr-bI/II* cDNAs using the ΔC_T method. However the value of the slope obtained using the primer pair that binds exclusively to cDNA encoding the SR-BI splice variant was 0.426, indicating that the efficiency of amplification differed from *36B4* cDNA, thus excluding this primer set for use in the assay. For this reason, the measurements performed in this chapter actually describe the levels of transcripts encoding both splice variants (*Sr-bI* plus *Sr-bII*).

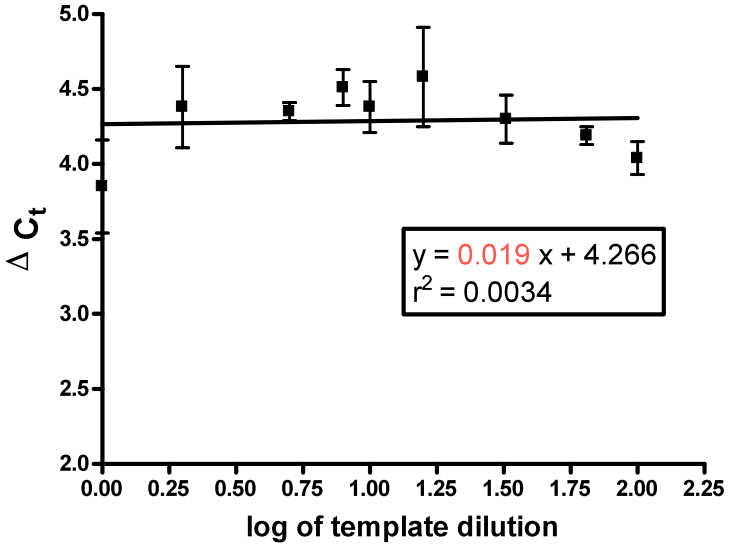
Figure 5.2: Relative Efficiencies of the *Cd36*, *Sr-bI* and *Sr-bI/II* Real-time PCR primer sets.

The amplification efficiencies of the primer sets designed to target *Cd36*, *Sr-bI* and *Sr-bI/II* cDNAs (relative to the 36b4 reference primers) were determined empirically by performing real-time PCR using a series of template cDNA dilutions. The slope of the line produced when the ΔC_T ($C_{T\ (target)}$- $C_{T\ (reference)}$) is plotted against the log of the template cDNA dilution was calculated and is shown inset (red) in graphs for *Cd36* (A), *Sr-bI/II* (B) and *Sr-bI* (C).

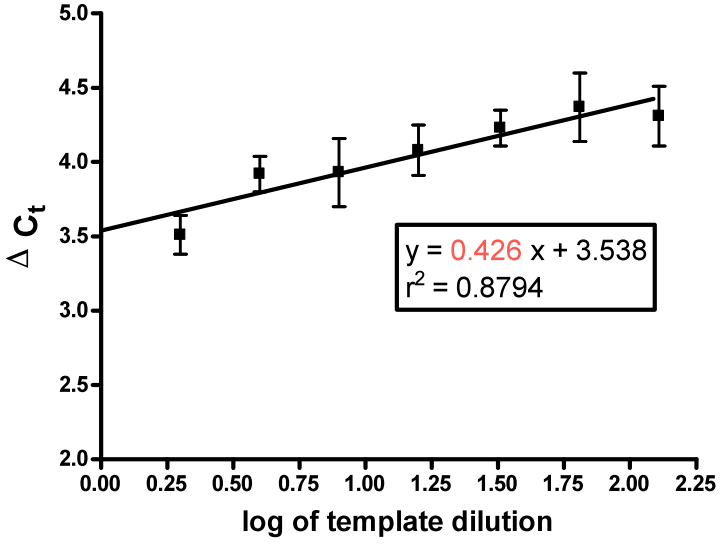
A. Efficiency Plot: CD36 & 36B4



B. Efficiency Plot:SR-BI/II &36B4



C. Efficiency Plot:SR-BI &36B4



For the purposes of quantitation and to monitor inter-assay variation, a reference cDNA sample was run on every plate. Because the levels of *Cd36* transcripts were likely to be highest in cDNA prepared from adult female livers, a pool of cDNA prepared from five male adult livers was used as the reference. Levels of transcripts in experimental groups are, therefore, expressed relative to the levels present in the reference cDNA preparation (*i.e.* relative to levels in adult normal males).

5.2.1.3 Differences In Transcription Of *Cd36* In The Liver In Female And Male Rats

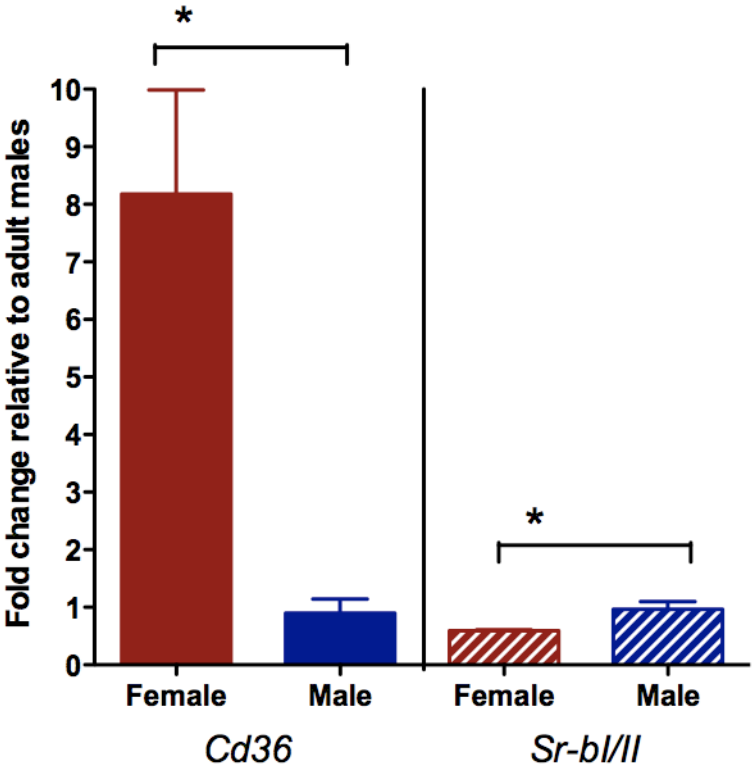
For qRT-PCR, RNA was extracted from samples of liver from twelve-week old male and female DA rats (n=3/group) that had been stored in RNAlater® at -80°C. Reverse transcription was performed as described in Methods (sections 2.17 and 2.18). Real-time PCR was performed on these samples (in duplicate) to detect *Cd36*, *Sr-BI/II* and *36b4*, according to the protocol described in section 2.22. The adjusted fluorescence measurements collected during amplification were then plotted against cycle number, generating an amplification curve for each reaction. A threshold was set at the point where fluorescence was detected above baseline levels and the cycle number at which threshold was reached was denoted as the cycle threshold (or C_T). To calculate the relative quantities of *Cd36* or *Sr-BI/II* cDNAs, the duplicate C_T values obtained from each sample were averaged. From the mean C_T was subtracted the average C_T value of *36b4* obtained from the same sample, thus providing the ΔC_T value for the respective cDNA. This value was then normalized against the C_T obtained from the reference cDNA sample with the corresponding set of primers (giving $\Delta\Delta C_T$). The relative fold change in expression was then calculated using the formula $2^{-\Delta\Delta C_T}$.

Figure 5.3 shows the mean fold-differences in levels of *Cd36* and *Sr-bI/II* transcripts in liver from male and female rats. There was an approximately eight-fold higher expression of *Cd36* transcripts in liver cDNA samples from females compared with males ($p<0.016$). In the case of *Sr-bI/II*, the difference in levels of transcripts was small (less than two-fold) but significant, with lower expression in females than in males ($p<0.049$). As expected, absolute levels of *36b4* transcripts were similar in liver cDNA samples from males and females.

Figure 5.3: Real-time PCR estimation of levels of mRNA transcripts encoding CD36 and SR-BI/II in cDNA prepared from adult female and male DA rat liver.

Real-time PCR was performed on liver cDNA samples to analyse expression of *Cd36* and *Sr-bI/II* in male and female DA rats (relative to a calibrator sample of normal adult male liver cDNA). Data is presented as mean + SEM (n=3/group). Female liver contained eight fold higher levels of *Cd36* mRNA compared to normal males. Conversely, male liver contained approximately two-fold higher levels of *Sr-bI/II* transcripts.

* $p<0.05$


*
10
9
8
7
6
5
4
3
2
1
0
Fold change relative to adult males
Female
Male
Female
Male
Cd36
Sr-bI/II
*

5.2.2 Measurement of CD36 in Liver from DA Rats

5.2.2.1 Measurement of CD36 by Western Blot

Total proteins were extracted from samples of snap-frozen liver and 50µg of protein from each sample was subjected to reduced SDS-PAGE and transferred to PVDF membrane. The membrane was cut horizontally at the 47kDa marker. The upper portion was probed with anti-CD36 mAb (MΦ25, diluted 1:2000) and the lower portion was probed with anti-β-actin mAb (diluted 1:15000), which served as a loading control. Both halves were then incubated with anti-mouse HRP-conjugate and bound antibody was detected using reagents from the FemtoWest ECL detection kit (Pierce Biochemicals), as described in Methods (section 2.16). Samples of liver proteins from three male and three female rats were tested individually.

A representative Western blot is shown in Fig. 5.4A. A single band was observed at approximately 88 kDa in samples from both male and female rats, corresponding to the reported size of glycosylated CD36 (Barnwell et al., 1985, Greenwalt et al., 1990). This band was considerably more intense in the liver samples from females compared with the males. These bands, were subjected to semi-quantitative analysis by densitometry. Liver samples from females contained 4.5 fold more CD36 than those obtained from males (Fig. 5.4B). The bands detected by the anti-β-actin mAb were too dark to perform densitometry on, even when the film was exposed for only 2 seconds. However, on visual inspection of the film, there was no apparent difference in the intensity of the female samples compared to the male samples. A gender difference in CD36 expression was also observed in liver membrane proteins samples (N=3 per group) obtained from male and female Sprague-Dawley (SD) rats (Fig. 5.4C and D). However, in contrast to the results of immuno-histochemical studies in DA rats (described in section 3.4.2), Western blot analysis of pooled adrenal gland samples from SD rats (three rats per pool) did not reveal any difference in levels of CD36 between males and females.

5.2.2.2 Estimation of CD36 Expression By Hepatocytes, Using Immuno-Histochemistry And Video-Image Analysis

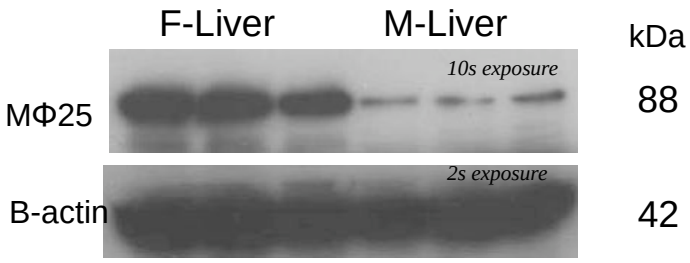
As discussed earlier, staining of liver sections from DA rats with mAb UA009 revealed that CD36 had a centrilobular pattern of expression (Chapter 3, section 3.2.2). The distribution of CD36 included most of the lobule in female rats but only a rim of

Figure 5.4: Detection and measurement of CD36 in liver tissue from female and male DA and SD rats by Western analysis.

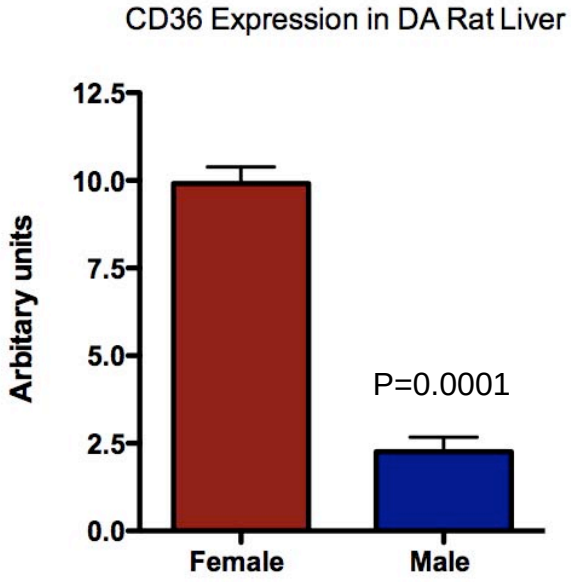
Equal amounts of total proteins (50µg) from liver samples obtained from three male and three female adult DA rats were subjected to reduced SDS-PAGE (12%) and electro-blotted to PVDF membrane. Membrane was cut at the 47 kDA marker. The upper protion of the membrane was probed with mAb MΦ25 antibody and staining with ECL showed a band of approx. 88kD in all samples. The lower portion of the membrane was probed with anti-β-actin mAb as a loading control and showed bands of approx 42 kDa of equivalent intensity in all samples. A representative blot is shown in (A). The intensities of the 88kD bands obtained from all liver samples were measured by densitometry (mean +/- SD, n = 3 rats per group). The results are expressed in arbitary units and statistical analysis showed that there was significantly higher expression of CD36 in females compared with males (B). Western blot of liver membrane proteins from female and male SD rats. 25 µg of liver membrane proteins were loaded from each sample individually and 10 µg of pooled adrenal samples were loaded into each well. PVDF membrane was probed with mAb MΦ25 antibody and staining with ECL resulted in an 88k Da band in each sample (C). The intensities of the 88kD bands obtained from all liver samples were measured by densitometry (mean +/- SD, n = 3 rats per group). Like DA rats, liver membrane proteins from female SD rats had significantly more CD36 compared to males (D).

Abbreviations: FH – female adrenal, MH- male adrenal, M-Liver – male liver, F-Liver – female liver.

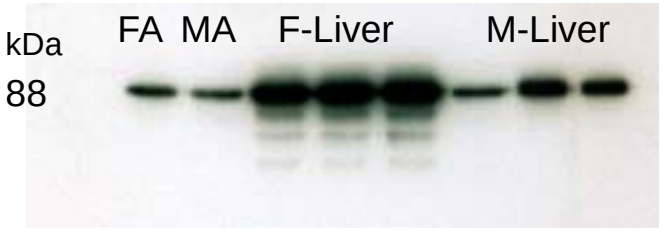
A


F-Liver
M-Liver
kDa
10s exposure
MΦ25
88
2s exposure
B-actin
42

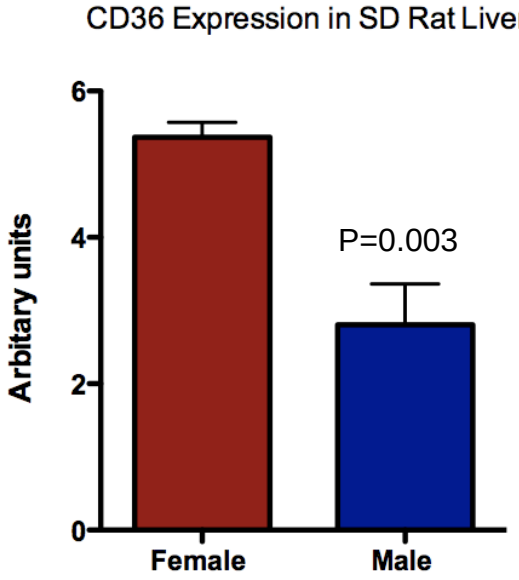
B


CD36 Expression in DA Rat Liver
Arbitary units
12.5
10.0
7.5
5.0
2.5
0.0
P=0.0001
Female
Male

C


kDa
88
FA
MA
F-Liver
M-Liver

D


CD36 Expression in SD Rat Liver
Arbitary units
6
4
2
0
P=0.003
Female
Male

hepatocytes around the central vein in males. Video-image analysis (VIA) of liver sections labeled with mAb UA009 by the indirect immuno-peroxidase technique (as described in Methods section 2.11) was used to obtain an indirect estimate of the proportion of hepatocytes that express CD36. Following image capture, vascular structures were excluded from further analysis as illustrated in Figure 5.5A. The stained area was then measured as a percentage of the total area of parenchyma within the field. The mean percentage of parenchymal area that was stained was calculated (obtained from 4-6 fields per section), allowing an estimate of the fraction of hepatocytes that express CD36. In liver sections from normal 12 week-old female DA rats (n=3/group), the ratio of stained to unstained parenchyma was 3.9-fold greater than in sections of male liver (Fig. 5.5B).

5.3 Effects Of Oophorectomy And Administration Of Sex Steroid Hormones On CD36 Levels In Female Rat Liver

5.3.1 Background

As shown in Chapter 4, expression of CD36 in the liver increases in female rats at approximately the time of puberty. Furthermore, qRT-PCR indicates that levels of *Cd36* mRNA transcript are higher in the liver in adult female rats than in males. To obtain direct evidence that the pattern of expression of CD36 in female liver is due to the effects of ovarian hormones, observations were made on Oophor-X and sham-operated eight week-old female DA rats. The effects of Oophor-X, which ablates production of estrogen and progesterone, were monitored by VIA of CD36 expression in liver sections and by qRT-PCR measurements of *Cd36* transcripts on liver RNA.

5.3.2 Effects Of Oophor-X and Steroid Hormone Replacement on CD36 Expression in the Liver

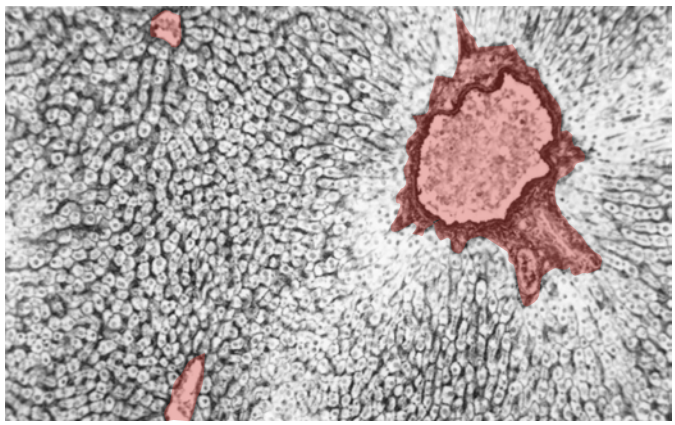
Oophorectomy (Oophor-X), with removal of the associated ovarian adipose tissue, was performed on four groups of eight week-old female DA rats (n = five per group). An additional group underwent a sham-operation, in which only the associated ovarian adipose tissue was excised. Following a three-week recovery period, each group of Oophor-X animals received a seven day course of daily sub-cutaneous doses of either

Figure 5.5: Estimation of CD36 expression by hepatocytes, using immuno-histochemistry and video-image analysis.

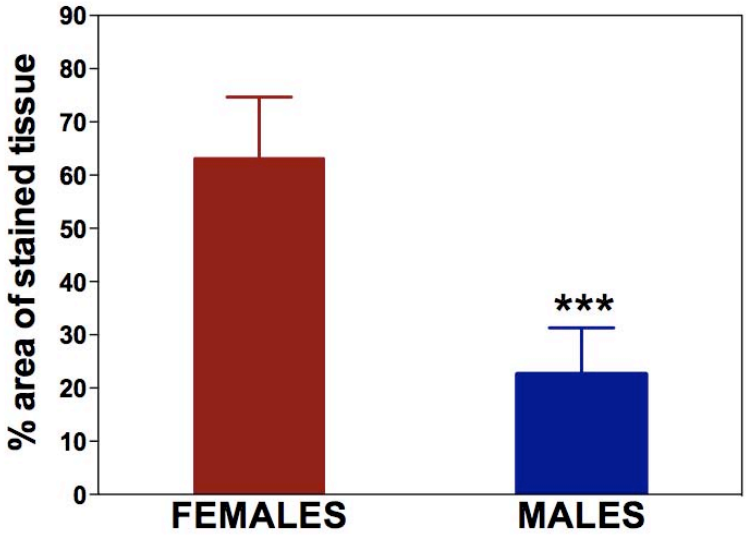
High power view of a representative section of female liver, stained with mAb UA009 by the indirect immunoperoxidase technique. The areas outlined in red demonstrate how vascular structures were excluded for video-image analysis (A). Mean percentage of the area of parenchyma stained by mAb UA009 in sections of male and female DA liver was calculated using video-image analysis. A significantly higher proportion ($p = 0.0001$) of hepatocytes express CD36 in females compared to males (B).

X20 objective

A



B


% area of stained tissue
90
80
70
60
50
40
30
20
10
0
***
FEMALES
MALES

E2, progesterone, testosterone or vehicle (peanut oil), as described in Section 2.4. At the conclusion of the treatment period, the animals were killed and liver was obtained to analyse hepatic expression of CD36 in frozen sections. Additional samples were stored in RNAlater® for RNA extraction. Samples were also snap-frozen in liquid nitrogen for protein analysis but these were thawed in a –80°C freezer failure.

Immuno-histochemical examination revealed a reduction in the area of parenchymal staining by mAb UA009 in sections of liver from Oophor-X rats (Fig. 5.6A) compared with sham-operated control rats (Fig. 5.6B). Furthermore, administration of E2 appeared to reinstate the normal adult female pattern of CD36 expression (Fig. 5.6C).

VIA confirmed these subjective impressions (Fig. 5.6D). The percentage of liver parenchyma stained by mAb UA009 was significantly lower in the Oophor-X group than in the sham-operated controls (43% vs 61%, $p<0.001$). Administration of peanut oil alone (vehicle control), testosterone or progesterone to Oophor-X females did not change in the areas of parenchymal staining significantly. However, there was a greater area of stained parenchyma in E2-treated Oophor-X rats (59%) compared with either the Oophor-X group (43%) or the Oophor-X plus peanut oil treatment group (37%) and these differences were significant ($p<0.01$). There was no significant difference in the areas of stained parenchyma between sham-operated control group and the Oophor-X plus E2 treated group.

It is clear, therefore, that removal of ovarian hormones by oophorectomy reduces CD36 expression in the liver. Expression was re-instated by treatment with a pharmacological dose of E2 but not by treatment with progesterone. Treatment with testosterone did not cause a further reduction in levels of CD36 expression.

5.3.3 Effects of Oophor-X and E2 Reinstatement on Levels of *Cd36* and *Sr-bI/II* mRNA in the Liver

Levels of *Cd36* transcripts were estimated by qRT-PCR, using total liver RNA extracted from the liver samples that were obtained from the same Oophor-X, sham-Oophor-X, and Oophor-X plus oestrogen-treated groups of rats that were described in 5.3.2. The groups of Oophor-X rats that received either progesterone or peanut oil alone were not included, as these treatments had not affected expression of CD36.

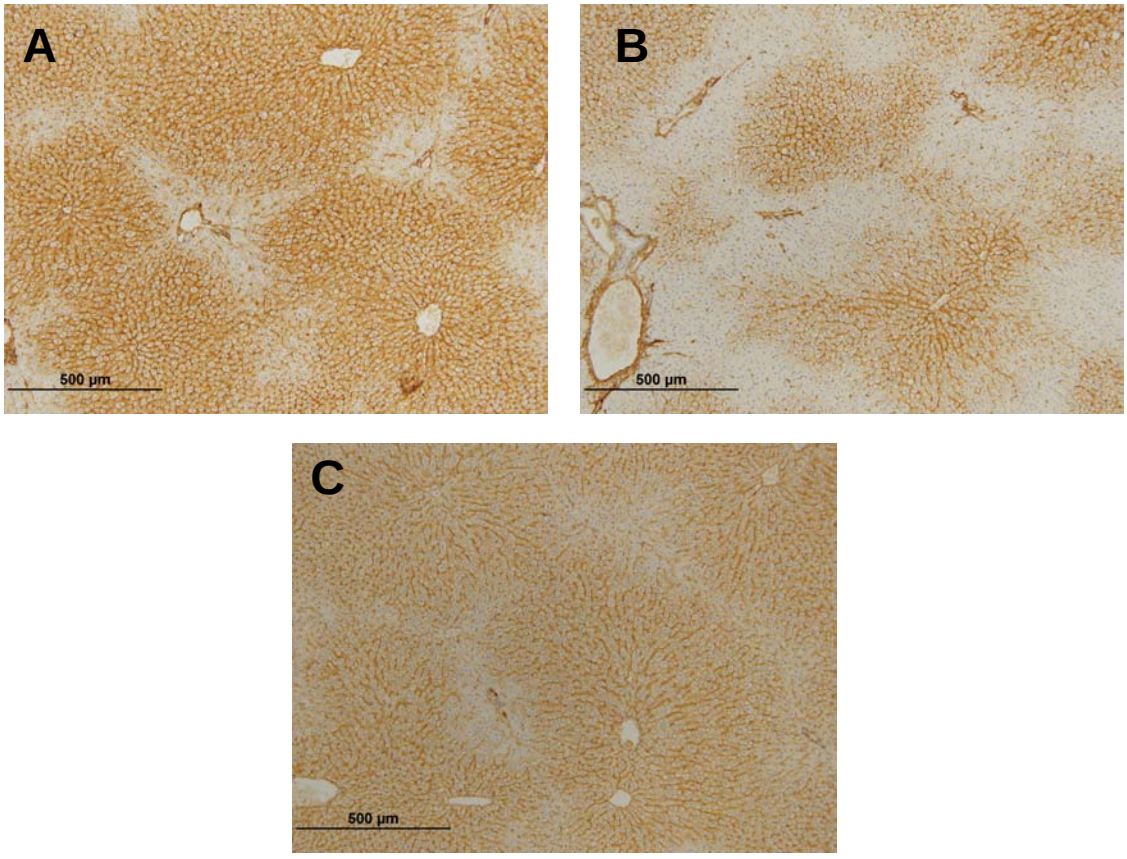
Figure 5.6: Effect of oophorectomy and steroid hormone replacement on expression of CD36 in the liver of female rats

Oophor-x was performed at age eight weeks and where applicable, hormone treatment commenced at age eleven weeks. Liver samples were obtained at age 12 weeks. Frozen sections of liver were stained using mAb UA009, using the indirect immunoperoxidase technique. Compared to Sham-operated control rats (A), Oophor-x females (B) displayed decreased area of parenchymal cell staining. This was reversed by the administration of E2 (C). Video-image analysis was performed on sections of liver from the groups of rats (D). Areas around vascular structures were excluded from image analysis, as shown in Fig. 5.5A. The results are expressed as percentage of the area of parenchyma analysed that was stained by mAb UA009 (mean +/- SD, n = 5 per group).

**$p<0.01$, *** $p<0.001$

Abbreviations: Sham, sham oophorectomy; Oophor-X, oophorectomy; E2, 17-β-estradiol treatment; P, progesterone treatment T, testosterone (Sustenon) treatment.

Photographed using x10 objective


A
500 µm
B
500 µm
C
500 µm
D
% Area of Stained Tissue
70
60
50
40
30
20
10
0
***
**
**
Sham
Oophor-X
Oophor-X + vehicle
Oophor-X + E2
Oophor-X + P
Oophor-X + T

The mean levels of *Cd36* and *Sr-bI/II* mRNA transcripts (relative to the male liver reference cDNA) in each of the experimental groups are shown in Figure 5.7. Liver from the sham-operated group contained approximately 8.1 fold more *Cd36* mRNA than the reference standard and this value was comparable to that obtained in an analysis of liver from intact normal adult female rats (Fig. 5.3). As was observed in VIA of CD36 expression in the liver (Fig. 5.6D), Oophor-X resulted also in a decrease in *Cd36* mRNA levels. When Oophor-X rats were treated with E2, *Cd36* mRNA in the liver increased dramatically to levels 15.2 fold higher than in normal males and exceeding the levels in sham-operated female controls by approximately 2 fold and Oophor-X females by over 6 fold. Intriguingly, levels of *Cd36* transcripts did appear slightly higher in Oophor-X rats that were treated with testosterone although this was not statistically significant. Furthermore, administration of testosterone to Oophor-X rats did not appear to affect the proportion of hepatocytes expressing CD36 protein (Fig. 5.6D).

In comparison with the substantial effects on levels of *Cd36* transcripts that were observed after Oophor-X, or treatment of Oophor-X rats with E2, changes in the levels of *Sr-BI/II* transcripts were rather modest. As displayed in Fig 5.7, there was a 1.6 fold increase in *Sr-BI/II* transcripts in the Oophor-X group ($p > 0.05$) and a 1.7 fold increase in the testosterone-treated Oophor-X group ($p > 0.05$) compared to control females. Both the Oophor-X and testosterone-treated Oophor-X females displayed levels of *Sr-BI/II* transcripts that were comparable to the normal male reference samples (1.02 fold and 1.07 fold respectively). However, levels of *Sr-BI/II* transcripts in Oophor-X rats that were treated with E2 were not statistically different from any other groups.

5.4 Effects of Castration and Administration of Sex Steroid Hormones on CD36 Levels in Male Rat Liver

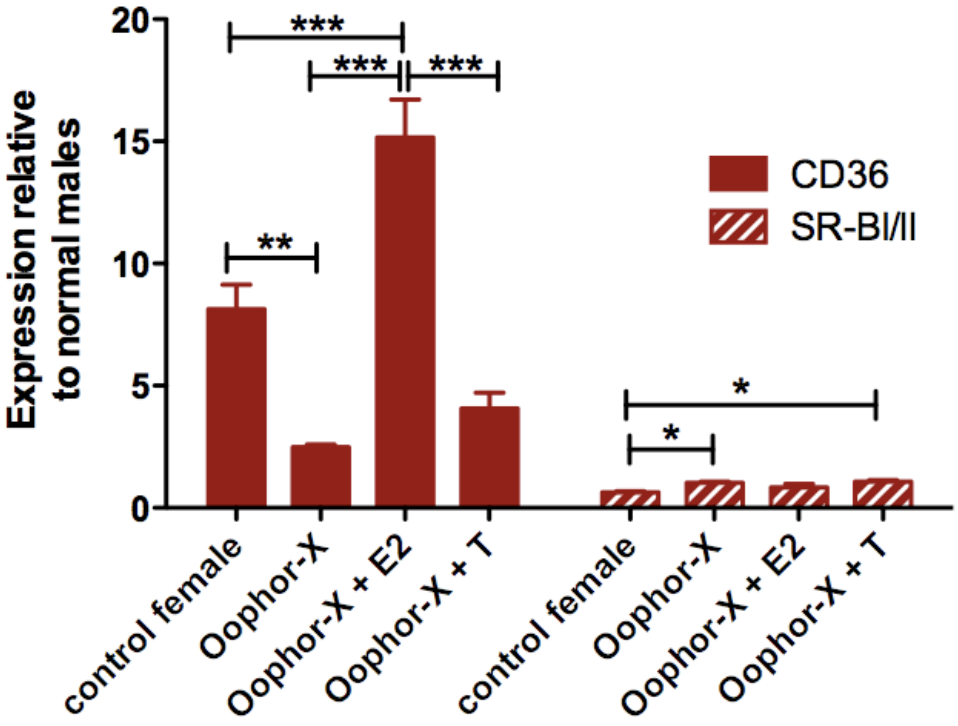
5.4.1 Background

While the results of Oophor-X and E2 reinstatement in female rats suggested that this hormone exerts a positive regulatory effect on expression of CD36 in the liver, the effects of testosterone in Oophor-X females were less clear (Fig. 5.6D and 5.7). To investigate further the role of testosterone in regulation of CD36 expression in the liver

Figure 5.7: Real-time PCR analysis of *Cd36* and *Sr-BI/II* mRNA levels in liver from female DA rats after Oophorectomy, and effects of treatment with steroid sex hormones.

Real-time PCR was performed on cDNA samples prepared from liver of animals in each group shown in Fig. 5.6. The cycle threshold (C_T) value for transcripts encoding each scavenger receptor was normalised against the C_T value for transcripts encoding the 36B4 ribosomal protein in the corresponding sample. The results are expressed as the fold difference in the normalised C_T value for each transcript, relative to the C_T value for the transcript in a reference sample of cDNA prepared from normal DA male liver (mean +/- SD, n = 5 rats per group).

* $p<0.05$, **$p<0.01$, *** $p<0.001$


Expression relative to normal males
20
15
10
5
0
***
***
***
**
*
*
CD36
SR-BI/II
control female
Oophor-X
Oophor-X + E2
Oophor-X + T
control female
Oophor-X
Oophor-X + E2
Oophor-X + T

and to determine whether the effects of E2 are exerted only on a female background, an experimental design similar to that described for female rats was performed using groups of male rats. Briefly, four groups of eight-week old DA rats underwent castration (removal of the testes plus the testicular fat pads), while another group received a sham-operation (removal of the fat pads only). Three weeks post-operatively, the castrated rats received either; no treatment, daily injections of peanut oil alone, or E2 or testosterone on alternate days subcutaneously for a period of one week as described in detail in section 2.4. At the conclusion of the treatment period, the animals were killed and tissues (liver, heart, adrenal gland) were collected to analyse expression of CD36 by immuno-histochemistry and *Cd36* and *Sr-bI* transcripts by qRT-PCR. As noted above, samples for analysis of proteins were lost in a freezer break-down.

5.4.2 Effects of Castration and Steroid Hormone Relacement on CD36 Expression in the Liver

A greater area of parenchyma was stained by mAb UA009 in liver sections from castrated rats than in sections from the sham-operated controls (Fig. 5.8A-B). When measured by video-image analysis (Fig 5.8E) the area of stained parenchyma rose from 22.8% in the sham-operated group to 40.8% in the castrated group ($n=6$, $p<0.01$). Although the proportion of stained liver parenchyma was higher in castrated rats that received daily injections of peanut oil (vehicle control) than in the untreated castrated group (52.4%), this difference was not significant. In castrated rats that received testosterone supplementation, the area of stained parenchyma (27.9%) was similar to that in liver sections from sham-operated controls (n.s.) and significantly less than in sections from the castrated rats that received vehicle alone ($p<0.01$).

It appears, therefore, that castration leads to increased expression of CD36 in the liver and that in the background of a castrated male, testosterone suppresses expression of CD36 in the liver. To examine whether longer treatment with testosterone would further suppress the proportion of hepatocytes that express CD36, the treatment period was extended to two weeks in an additional group of castrated rats. The pattern of CD36 expression in this group was indistinguishable from that observed after the single week of treatment (data not shown).

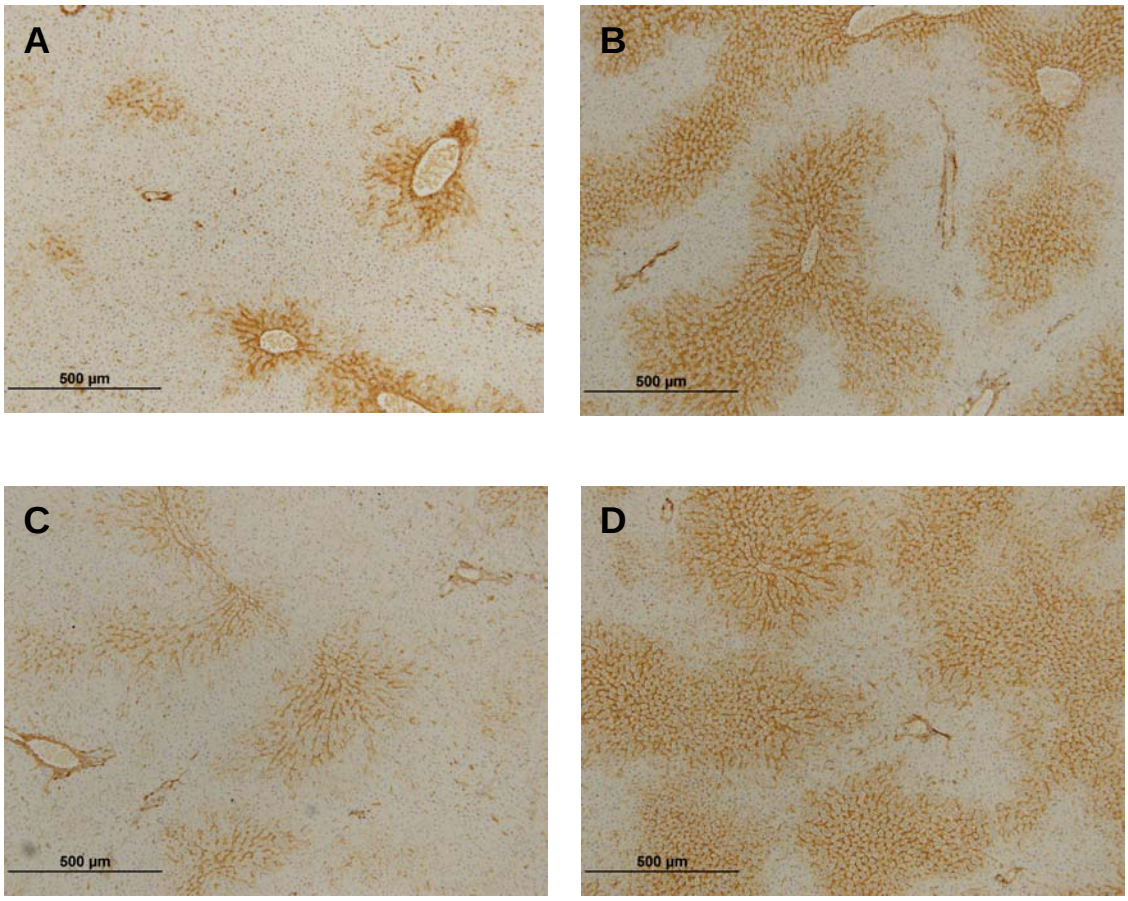
Figure 5.8: Effect of castration and steroid hormone replacement on expression of CD36 in the liver of male DA rats

Castration was performed at age eight weeks and where applicable, hormone treatment commenced at age eleven weeks. Liver samples were obtained at age 12 weeks. Frozen sections were stained using mAb UA009, using the indirect immunoperoxidase technique. Compared to Sham-operated control rats (A), Castrated males (B) displayed an increased area of parenchymal cell staining. This was reversed by the administration of testosterone (C) and re-instated by treatment with E2 (D). Video-image analysis was performed on sections of liver from all rats in each of the groups. Areas around vascular structures were excluded from image analysis (E). The results are expressed as area of parenchyma stained by mAb UA009, as a percentage of the total area included in the analysis (mean +/- SD, n = 5 per group).

**$p<0.01$, *** $p<0.001$

Abbreviations: Sham, sham castration; Cast, castration; VC, vehicle control; T, testosterone (Sustenon) treatment; E2, 17-β-estradiol treatment.

Photographed using x10 objective


A
500 µm
B
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C
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D
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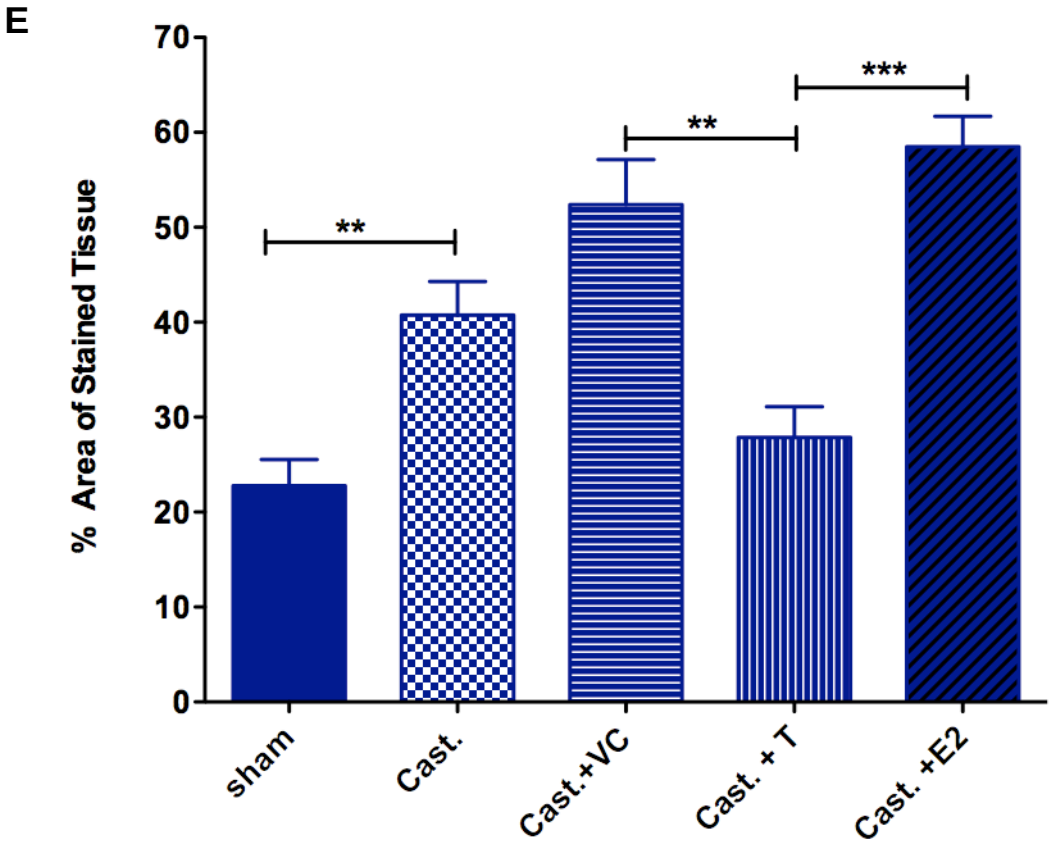

E
% Area of Stained Tissue
70
60
50
40
30
20
10
0
**
**
***
sham
Cast.
Cast.+VC
Cast. + T
Cast. +E2

Conversely, treatment of castrated males with E2 increased the proportion of the parenchyma that was stained by mAb UA009 relative to sections from the castrated group (Fig 5.8D). However, the difference did not reach statistical significance when measured by VIA. Nevertheless, the proportions of stained parenchyma in sections from E2-treated castrated males (58.5 $^{+}$/- 7.2%) and E2-treated Oophor-X females (58.7 $^{+}$/- 5.0%) was similar. It appears, therefore, that expression of CD36 in the liver is sensitive to the effects of E2 when administered in the background of a castrated male.

5.4.3. Effects of Castration and Testosterone Reinstatement on Levels of *Cd36* and *Sr-b1/II* mRNA in the Liver

Liver RNA samples prepared from each of the experimental groups described in Section 5.4.1 (n=5 per group) were analyzed by qRT-PCR. In Figure 5.9, levels of *Cd36* mRNA are presented as means (+/-SD) for each condition, relative to the reference sample of cDNA from normal male liver. No significant differences were observed between the sham-operated, castrated, or castrated plus testosterone-treatment groups. Thus although castration resulted in an increase in CD36 expression in the liver, and administration of testosterone restored the adult male pattern of expression (Fig 5.8E), these changes were not mirrored at the level of *Cd36* transcripts. In contrast, when castrated males were treated with E2, levels of *Cd36* transcripts were 3.8 fold higher than in either their castrated counterparts ($p<0.001$) or in intact males. Therefore, the effects of E2 on transcription were similar in Oophor-X females and castrated males.

In contrast to levels of *Cd36* transcripts, castration led to a small but significant increase (2.0 fold) in levels of *SR-bI/II* mRNA transcripts in the liver compared with the sham-operated controls (Fig 5.9). Levels of *Sr-bI/II* transcripts were returned to control levels when pharmacological doses of testosterone were administered to the castrated animals. Paradoxically, treatment with E2 produced a similar reduction in *Sr-bI/II* transcripts.

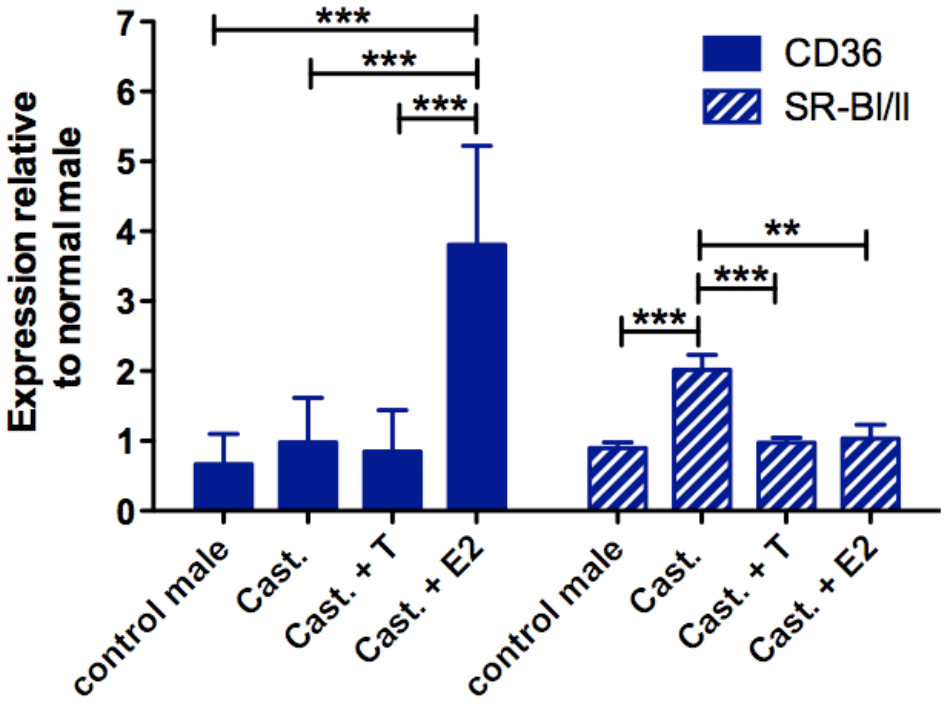
In summary, surgical gonadectomy resulted in significant changes in CD36 and *Cd36* mRNA levels in the liver in both male and female rats. The removal of the principle sex steroid-producing organs decreased expression of CD36 in females and increased expression of the protein in males. Thus, the net effect of gonadectomy was to reduce considerably the gender difference in expression of CD36 in the liver. Parallel changes in *Cd36* mRNA levels were observed following Oophor-X but not castration. Treatment

Figure 5.9: Real-time PCR analysis of *Cd36* and *Sr-BI/II* mRNA levels in liver from male DA rats after castration, and effects of treatment with steroid sex hormones.

Real-time PCR was performed on cDNA samples prepared from liver of animals in each group shown in Fig. 5.8. The cycle threshold (C_T) value for transcripts encoding each scavenger receptor was normalised against the C_T value for transcripts encoding the 36B4 ribosomal protein in the corresponding sample. The results are expressed as the fold difference in the normalised C_T value for each transcript, relative to the C_T value for the transcript in a reference sample of cDNA prepared from normal DA male liver (mean +/- SD, n = 5 rats per group).

**p<0.01, *** p<0.001

Abbreviations: Cast, castration; T, testosterone (Sustenon) treatment; E2, 17-β-estradiol treatment.


Expression relative to normal male
7
6
5
4
3
2
1
0
***
***
***
**
***
***
CD36
SR-BI/II
control male
Cast.
Cast. + T
Cast. + E2
control male
Cast.
Cast. + T
Cast. + E2

with E2 increased expression of CD36 and *Cd36* mRNA levels in the liver in gonadectomised female rats, and interestingly the liver in male rats also responded to estrogen. However, the effects of testosterone were dependent on gender-background. In males, there was a decrease in the percentage of liver parenchyma that expressed CD36 but this was not paralleled by a decrease in levels of *Cd36* mRNA. Paradoxically, treatment with testosterone appeared to increase the levels of *Cd36* mRNA in the liver of female rats (although this did not obtain statistical significance in this experiment). It is possible that the dose of testosterone used was insufficient to replace physiological levels of the hormone in male rats. However, this seems unlikely, because the dose of testosterone was chosen to be supra-physiological. In the case of Oophor-X females, some of the administered testosterone esters could have been converted to E2, thus providing an explanation for the increased levels of *Cd36* transcripts in the liver of these animals. To resolve these issues, serum levels of the respective hormones were measured in the gonadectomised and hormone treated rats.

5.5 Serum Hormones Levels in Rats Following Gonadectomy and Hormone Administration

5.5.1 Background

The levels of circulating hormones achieved in gonadectomised rats by the treatment regimens were determined by RIA assays for E2 and testosterone. Serum was prepared from blood samples obtained from male and female rat in the groups that had undergone either sham-operation, gonadectomy (Oophor-X or castration), or gonadectomy followed by one week of hormone treatment as described in sections 5.3.2 and 5.4.2. The samples were stored at -20^{o}C until used to measure either total testosterone or E2 concentrations.

5.5.2 Serum Testosterone And E2 Concentrations, Measured by Radioimmunoassay

Serum testosterone concentrations in 12 week old male and female sham-operated DA rats were 4.2 ± 1.705 nmol/L and 0.05 ± 0.009 nmol/L (mean ± SEM) respectively (the

levels in females were below the manufacturer's recommended 0.1nmol/L sensitivity of the assay). The mean testosterone concentrations in serum from gonadectomised rats of both genders were undetectable by this assay. Sera collected from male and female rats on the last day of treatment with testosterone ester injections contained 29.35 ± 3.083 and 29.16 ± 1.59 nmol/L of testosterone respectively. The results indicate that castration was effective in reducing serum concentrations of testosterone to levels comparable with those observed in both intact and Oophor-X females. Furthermore, subcutaneous injection of testosterone esters produced supra-physiological levels of testosterone that were similar in the sera of both genders.

E2 concentrations were measured in sera from female sham-operated, Oophor-X and E2 treated animals. Unfortunately, available resources did not allow measurements in the other groups. The mean E2 concentration in sera from sham-operated females was 0.037 ± 0.009 nmol/L (mean ± SEM), whereas concentrations of E2 in sera from Oophor-X rats were below the linear range of the assay (0.019nmol/L). Treatment with E2 produced serum concentrations of 0.092 ± 0.015 nmol/L (mean ± SEM) by the end of the treatment period, indicating that the treatment regimen had achieved serum levels of E2 that were above the normal physiological range.

5.6 Timecourse Of Changes In Hepatic Expression Of CD36 Following Oophorectomy

5.6.1 Background

It was of interest to investigate in more detail the changes in CD36 expression that occur in the liver following Oophor-X and to determine whether these changes coincide with changes in serum E2 levels. Female rats were chosen, because the changes in expression of CD36 and levels of *Cd36* mRNA following Oophor-X are less complicated than those observed in males after castration.

5.6.2 Temporal Course Of Hepatic CD36 Expression In Female Rats After Oophorectomy

Oophor-X was performed on four groups of 11 week old female rats (3/group). The rats were killed one, two, three or seven days later to collect liver and blood samples. To control for effects of surgery, two additional groups underwent a sham-operation and they were killed at either day one or day seven after surgery. At 1 day post-surgery, the concentration of E2 in serum from Oophor-X rats was already below the limits of detection by the RIA (0.019nmol/L), whereas the mean concentration in serum from the sham-operated group was 0.062 ± 0.036 nmol/L (mean ± SEM). Therefore, serum E2 levels decrease very rapidly following removal of the ovaries and remain at undetectable levels for the following two weeks (Section 5.5.2).

Frozen sections of liver from the groups of rats were stained with mAb UA009, using the indirect immuno-peroxidase technique. Photomicrographs from representative sections are shown in Figure 5.10. The distribution of CD36 was similar in sham-operated controls at days one and seven after surgery (Fig. 5.10A and E) and in normal female DA rats (Fig. 4.3). No difference was observed in patterns of CD36 expression between Oophor-X and control (day one or day seven after sham-operation) rats in the first three days after removal of the ovaries (Fig. 5.10B, C, D and Fig. 5.10A and B, respectively). Only seven days after Oophor-X was there a reduction in the area of centrilobular staining (Fig. 5.10F), although at this time the area and intensity still appeared to be greater than in Oophor-X rats killed 21 days after surgery (Fig. 5.6B). The results indicate that there is a delay of at least 7 days between removal of the ovaries and reduction in CD36 expression in the liver, and that this delay is not due to persistence of E2 in the circulation. It appears, therefore, that the effects of withdrawl of E2 are delayed by several days, possibly related to the time necessary for turnover of existing mRNA and/or protein.

5.7 Studies on Hepatocytes In Vitro

5.7.1 Background

The effects of steroid sex hormones on expression of CD36 by hepatocytes could be either directly on the cells, or via the hypothalamo-pituitary system and involving other

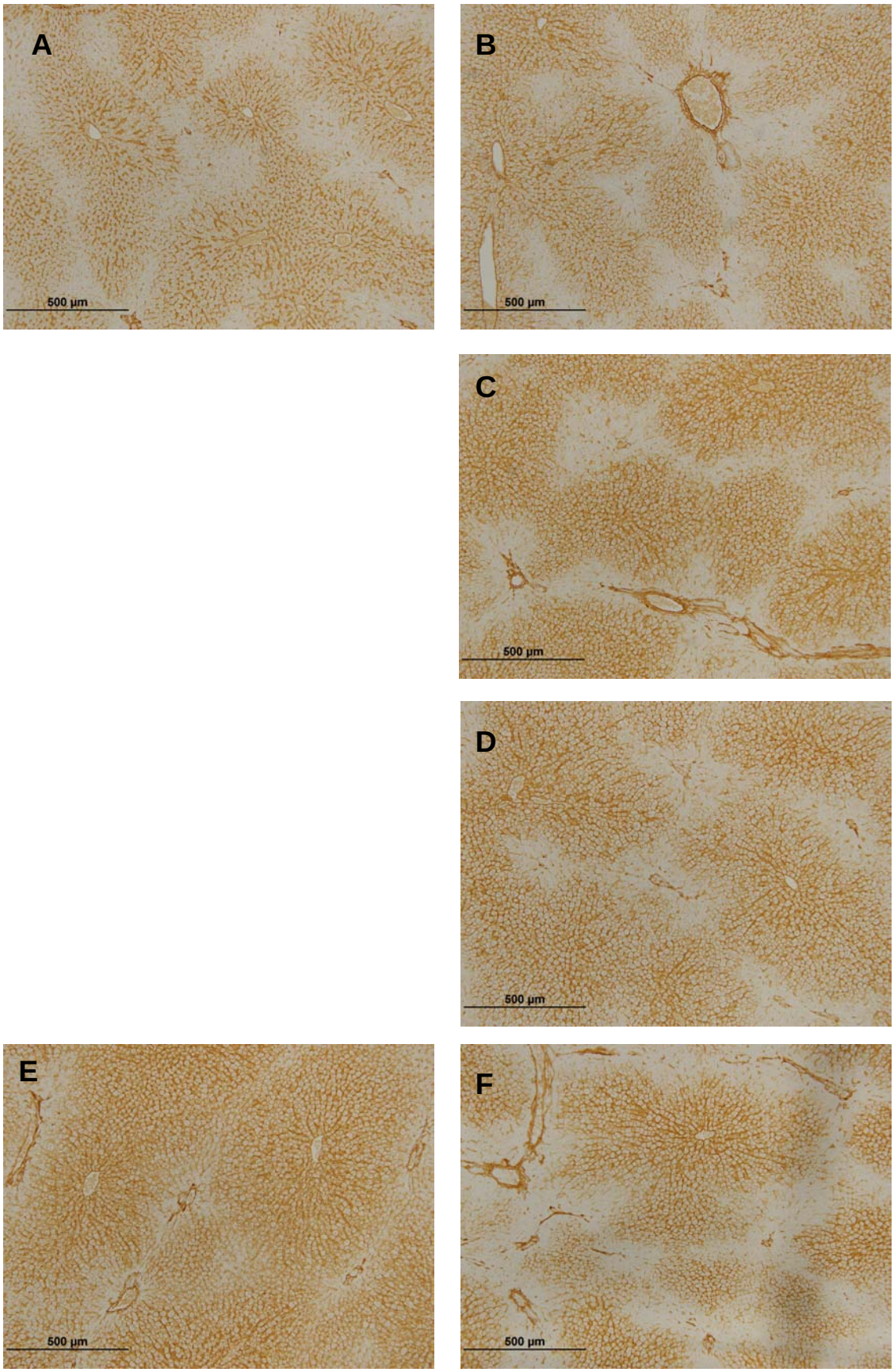
Figure 5.10: Timecourse of expression of CD36 in the liver of female rats following Oophorectomy

Oophorectomy (Oophor-x) was performed at age eleven weeks and samples of liver were obtained on post-operative days 1 (A-B), 2 (C), 3 (D) and 7 (E-F). Frozen sections were stained using monoclonal antibody UA009, using the indirect immunoperoxidase technique. Compared to Sham-operated control rats (A), Oophor-X females displayed no changes in the area of parenchymal cell staining at days 1, 2 or 3. A moderate decrease in the area of stained cells was observed at day 7 (F), compared to the sham-operated controls.

Photographed using x10 objective

Sham-Operated Females

Oophor-X Females



hormones. The aim of the following set of experiments was to determine whether a direct link could be established between sex steroids and expression of CD36 by primary rat hepatocytes *in vitro*. The cells were cultured initially in the absence of steroid hormone and then following addition of the relevant hormone to the culture medium. Expression of CD36 was monitored by flow cytometry. Liver perfusion to produce primary rat hepatocytes was performed by Dr Peter Coyle (Institute of Medical and Veterinary Science, Adelaide).

5.7.2 Expression of CD36 by Primary Hepatocytes *In Vitro*

Freshly isolated primary hepatocytes from adult female DA rats were allowed to adhere to collagen coated plates and were then cultured in the absence of added oestrogen. Cells were harvested either immediately following attachment (T=0) or at 24 hour intervals for up to 72 hours. The harvested cells were labelled by indirect immuno-fluorescence with either mAb UA009 or the isotype-matched control mAb 1B5 and examined by flow cytometry. In preparations from female rats, ~ 44% of the freshly isolated liver cells expressed CD36 (T=0). After culture for 24 hours, the proportion of hepatocytes that expressed CD36 fell to 9.74% (Fig. 5.11A) and remained at ≤5% after 48 and 72 hours in culture. Addition of 0.1nM E2 to the culture medium did not prevent this rapid *in vitro* loss of CD36 expression (not shown).

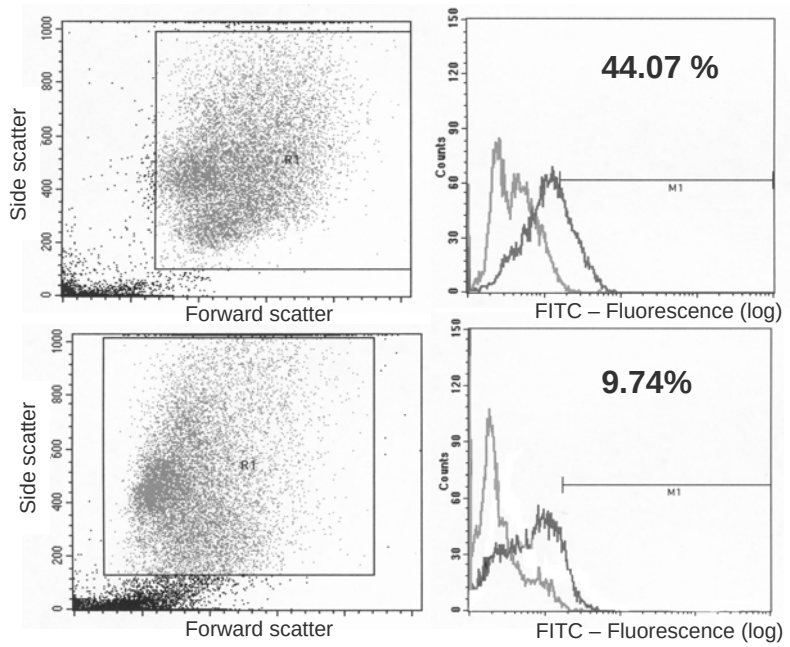
In similar experiments using primary hepatocytes isolated from adult DA male rats, ~22.7% of the freshly isolated cells expressed CD36 (T=0). This proportion was not increased by lack of exposure to testosterone (T=24, Fig. 5.11B). As in female hepatocytes, following culture (in the absence of testosterone) for 48-72 hours, <4% of the cells were found to express CD36.

It was noted that the density of adherent cells decreased after 24 hours and that fewer of the events detected by flow cytometry had the forward and side scatter properties of viable cells. To test the viability of the cells, lactate dehydrogenase (LDH) leakage into the culture medium was measured at each of the time points, as described by Berry et al (1991). As shown in Figure 5.11C, approximately 70% of the total LDH was contained in hepatocytes cultured for 4 hours. However, there was marked leakage of LDH in the later cultures and by 72 hours, most of the LDH was extra-cellular. These observations indicate that the majority of the cells in the later cultures were non-viable.

Figure 5.11: Expression of CD36 by primary rat hepatocytes *in vitro*

Primary hepatocytes were obtained from female (A) and male (B) adult rats by perfusion with collagenase (see Methods 2.3). Freshly isolated hepatocytes, or hepatocytes harvested after 24 hours of monolayer culture, were labelled using either mAb UA009 or the negative control antibody, mAb 1B5. Bound antibody was detected using a FITC-conjugated sheep anti-mouse Ig and the cells were then analysed by flow cytometry. Representative plots of forward and side scatter of hepatocytes from a female rat show the events (R1 gate) that were analyed for fluorescence. Dark and light histograms show the fluorescence of freshly isolated hepatocyes, and hepatocytes after culture for 24 hours, stained with mAb UA009 or mAb 1B5 respectively (A). Histograms of fluorescence of hepatocytes (gated in R1) from a male rat (freshly isolated or after 24 hours culture), after labelling with either mAb 1B5 or mAb UA009 (B). CD36 expression by hepatocytes was diminished after 24 hours of *in vitro* culture in preparations from both female and male rats. Release of lactate dehydrogenase (LDH) into the culture medium was measured using an Cobas Bio clinical analyzer (C). Efflux of LDH into the supernatant provides an indication of membrane integrity. The proportion of the total LDH (cells + medium) that was present in the cell-free supernatant after each period of culture is expressed as a percentage.

A


0 HR
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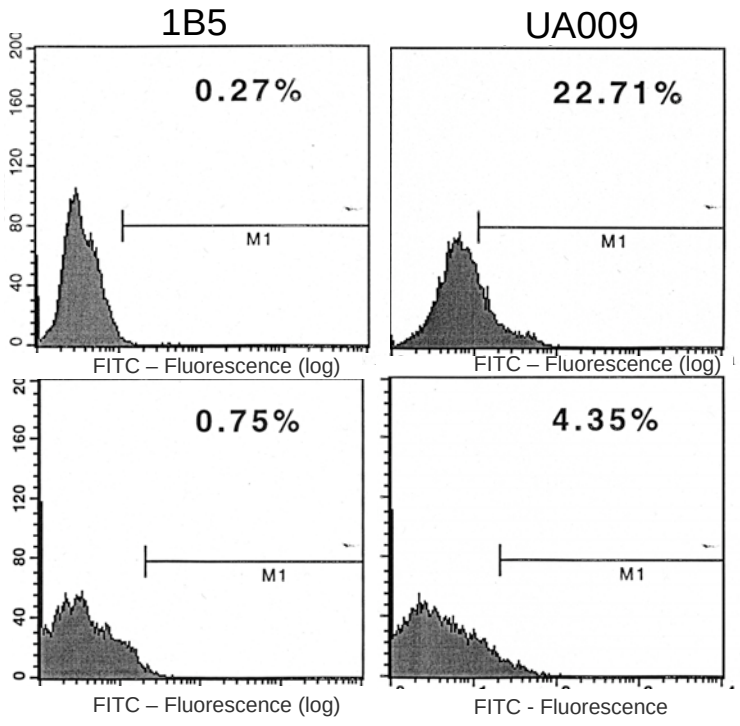
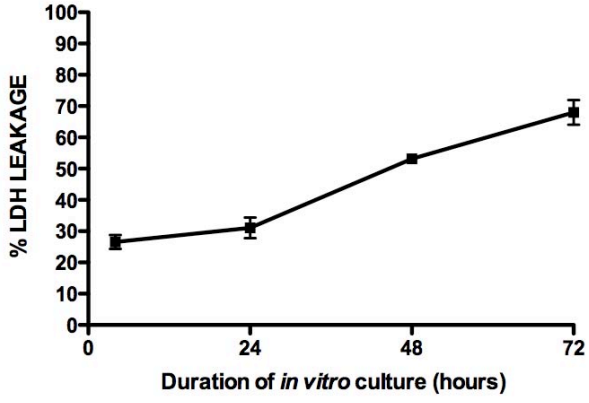
Side scatter
Forward scatter
R1
44.07 %
9.74%
M1
Counts
FITC – Fluorescence (log)

B


1B5
UA009
0 HR
24 HRS
0.27%
22.71%
0.75%
4.35%
M1
FITC – Fluorescence (log)
FITC - Fluorescence

C


% LDH LEAKAGE
Duration of *in vitro* culture (hours)
0
10
20
30
40
50
60
70
80
90
100
24
48
72

The fact that primary hepatocytes appeared to lose CD36 expression rapidly and were only healthy for approximately 24 hours under these culture conditions made them unattractive for further studies on hormonal regulation of CD36. Nevertheless, neither E2 nor testosterone appeared to affect CD36 expression during the first 24 hours in culture, when viability of the cells was satisfactory. As with a number of characteristics that have been studied in primary hepatocytes, the cells appear to lose differentiation markers rapidly in simple monolayer cultures.

In summary, no direct effects of either E2 or testosterone were observed on CD36 expression by primary rat hepatocytes. Expression of CD36 by primary hepatocytes (from both genders) was lost rapidly in culture and this loss was not affected by the presence of either hormone.

5.8 Discussion

The experiments described in this chapter used quantitative immuno-histochemistry and Western blot to measure differences in CD36 expression by hepatocytes in male and female rats, and also examined level of transcripts encoding the protein. These methods were then applied to investigating the role of sex steroid hormones in regulation of CD36 expression by hepatocytes. In general, the sexually dimorphic expression of CD36 protein and levels of *Cd36* transcripts were concordant. Liver from female rats contained approximately eight-fold more *Cd36* transcripts than male liver, a difference that is consistent with the differences in CD36 measured by immunoblot densitometry. Furthermore, the total amount of CD36 protein present in a liver sample correlated approximately with the proportion of liver parenchyma (percentage of hepatocytes) stained by mAb UA009 in immuno-histochemically stained sections, as measured by video-image analysis. However, simple video-image analysis does not discriminate variations in the intensity of staining or differences in the amounts of protein produced by individual cells. While it inherently underestimates differences in total levels of CD36 protein, the technique has the advantage of allowing exclusion of vascular tissues, which probably contribute a significant proportion of the CD36 protein in male liver. With these qualifications, quantitative immuno-histochemistry was the method used to measure CD36 protein expression in relation to steroid hormone status, because immunoblot densitometry was not possible until late in the project.

The *in vivo* experiments clearly indicate a relationship between sex hormones and the regulation of hepatic CD36 protein expression in adult rats. Gender-specific levels of CD36 protein in the liver were only observed when the relevant sex-specific steroid hormones were present *i.e.* in intact adult animals, or when the appropriate exogenous hormone was administered after Gx. In the liver of Gx male and female rats, there was no gender difference in the percentage of hepatocytes that expressed CD36. Furthermore, re-instatement of the appropriate sex steroid restored the gender bias in CD36 expression in both male and female Gx animals. These results support the hypotheses that estrogen regulates hepatic expression of CD36 positively while testosterone has negative regulatory effects. This conclusion is consistent with the findings described in Chapter 3, which show that the emergence of gender-biased expression of CD36 in the liver coincides with the onset of puberty. However, analysis of *Cd36* mRNA levels in liver from Gx and hormone-treated rats showed that the regulation of CD36 in the liver is complex and there may be multiple levels of control.

In male rats, CD36 appears to be regulated at more than one level. Consistent with differences at the protein level, levels of *Cd36* mRNA are considerably lower in male rats than in females. Furthermore, Gx and hormone replacement experiments showed a relationship between androgen status and expression of CD36 protein. Nevertheless, the characteristically low levels of *Cd36* transcripts persisted after castration. This was not attributable to technical error, because serum analysis indicated that the castration and hormone replacement protocols employed had been effective. Two pathways have been described to account for the actions of testosterone on gene expression. The well-defined “classical” mechanism of androgen action is mediated via a direct interaction between cis-acting response elements in the promoters of target genes with an androgen/androgen receptor complex (reviewed in Roy and Chatterjee, 1995). This usually but not always results in induction of the target gene, rather than repression. It is noteworthy, therefore, that *in silico* analysis of the published upstream regulatory sequence of the rat *Cd36* gene (PMID: AF317787), using the Swiss-Prot software program, did not indicate the presence of putative AR-specific responsive elements (Truss and Beato, 1993). An alternative mechanism of action of the androgen/androgen receptor is by “indirect” modulation of either expression or activity of secondary transcription factors, which in turn regulate the transcriptional activity of target genes. The latter mechanism can enable tissue or cell-type specific effects of androgens,

depending on the availability of particular transcription factors, co-activators and/or co-repressors (reviewed in Verhoeven and Swinnen, 1999). However, irrespective of 'direct' or 'indirect' modes of action, if testosterone was exerting an androgen-receptor dependent effect on expression of the *Cd36* gene, then the removal of ligand should release the repression and this would result in increased levels of *Cd36* transcripts. Clearly, the evidence herein suggests that neither of these mechanisms can explain the experimental findings in castrated rats.

An alternative mechanism by which *Cd36* could be regulated in males is via the "masculinising" effects of the male-specific pattern of growth hormone secretion, which becomes established during the pre-pubertal period. The sexually dimorphic pattern of growth hormone secretion is responsible for maintaining gender-specific regulation of a number of genes in the liver (Legraverend et al., 1992). Both androgens and estrogens influence the secretion of growth hormone through the endocrine regulatory network known as the gonadal-hypothalamic-pituitary axis (Gatford et al., 1998). Interestingly, genes that are controlled in this manner often do not display sexually dimorphic expression until puberty, and this follows a latent pre-pubertal period in which there is no gender difference (Denef and De Moor, 1972). This pattern is consistent with that observed for CD36 expression in pre-pubertal and juvenile rats (described in Chapter 4). The possible role of the gonadal-hypothalamic-pituitary axis in the regulation of CD36 is explored in detail in Chapter Six. It is clear, however, from the data obtained on *Cd36* mRNA levels that any indirect effects of testosterone via the gonadal-hypothalamic-pituitary axis do not act at the level of gene regulation.

Thus the mechanism responsible for the changes in liver CD36 protein expression following Gx and T administration to male rats remains unsolved. A number of mechanisms have been suggested for "non-genomic" regulation of specific physiological changes by androgens. For example, testosterone has been reported to trigger; rapid Ca2+ influx by cardiac myocytes (Vicencio et al., 2006) and neural cells (Estrada et al., 2006), Ca2+ influx and insulin secretion by pancreatic islet cells (Grillo et al., 2005), and vasodilation of vascular endothelial cells (Yildiz and Seyrek, 2007). All of these actions occur in a rapid, androgen-receptor independent manner. To date, however, there are no reports of this type of direct, non-genomic effects of testosterone in the rat liver. In addition, these effects tend to be rapid and transient and do not fit well with the active, long-term suppressive effect that testosterone has on expression of

CD36 protein in male rat liver, or the failure of isolated hepatocytes to rapidly upregulate CD36 when cultured *in vitro* in the absence of testosterone.

A more likely explanation is that hepatic CD36 protein levels are regulated at a post-transcriptional level, possibly in response to down-stream physiological changes that result directly or indirectly (via GH) from removing or re-instating testosterone. It is becoming apparent that like E2, testosterone may play numerous roles in regulating energy homeostasis in important metabolic tissues such as liver, skeletal muscle and adipose tissue, acting directly via androgen receptor (AR) activity. For example, administration of testosterone plus a specific aromatase inhibitor to male-Gx mice resulted in changes to the fat mass and serum triglyceride and HDL levels (Moverare-Skrtic et al., 2006). Likewise, compared to wild type mice, male AR knockout mice exhibited greater adiposity, increases in both serum triglyceride and glucose levels, as well as insulin resistance in skeletal muscle and the liver. This phenotype was not reversed by administration of DHT, suggesting the direct involvement of androgen-AR activity in metabolic processes involving both lipid and carbohydrate metabolism (Lin et al., 2005a). In human macrophages, glucose concentrations have been shown to have post-transcriptional effects on the rate of translation of *Cd36* mRNA by altering the proportion that is present in the translationally active polysomal pool (Griffin et al., 2001). Hence, it is speculated that increased translational efficiency of hepatic *Cd36* mRNA (resulting from metabolic perturbations caused by the removal or reconstitution of testosterone) could explain the changes in CD36 protein expression observed in castrated rats and castrated rats treated with testosterone. However, effects via changes in stability of mRNA, or in the rate of CD36 turnover, cannot be excluded.

In contrast to male rats, in which hepatic levels of *Cd36* transcripts were unaffected by Gx or T treatment, levels of hepatic *Cd36* transcripts changed dramatically in female rats according to their E2 status. Gonadectomy of female rats markedly reduced the overall gender difference from males in the levels of *Cd36* transcripts in the liver. Furthermore *Cd36* mRNA levels increased Gx rats that were treated with E2, irrespective of gender, although the effects were more pronounced in females than males (6 fold and 3.9 fold respectively). Interestingly, treatment of Gx females with T did not suppress hepatic levels of *Cd36* mRNA. In contrast, there was a trend towards increased (1.6 fold) expression of *Cd36* transcripts, although this was not statistically significant. A possible explanation the latter observation is that small amounts of T can

be converted to E2 by aromatase in cells such as adipocytes, although typically estrogen produced in this way tends to have autocrine rather than paracrine effects. Although an "ultra-sensitive" RIA was used to measure serum E2 levels, the concentrations in the female rat serum bordered on the sensitivity threshold of the kit and the presence of trace amounts of E2 would not be detected using this method. Future studies using a non-aromatizable metabolite of T, such as DHT, might resolve this issue.

Taken together, the results from these experiments suggested that *Cd36* is regulated by E2 at a transcriptional level in the liver. E2 regulation of *Cd36* has been demonstrated previously in a number of human breast cancer cell lines, although in that case E2 treatment resulted in ER-mediated transcriptional repression (Uray et al., 2004). Subsequent experiments were designed, therefore, to investigate E2 regulation of *Cd36* in more detail. Examination of the kinetics of E2 regulation after gonadectomy of female rats revealed that although serum E2 levels fell rapidly (undetectable by 24 hours), levels of CD36 protein did not change significantly until at least seven days after gonadectomy. There are a number of potential explanations for this delay. Firstly it is possible that the turnover of CD36 protein occurs very slowly in the liver. Evidence from myocytes and macrophages suggests that these cells contain intracellular stores of CD36 and that rapid translocation between the cytoplasm and the cell membrane can occur in response to metabolic stimuli (Bonen et al., 2000). If similar intra-cellular traffic can occur in the liver, a change in the level of *Cd36* transcription, it might take some time to be reflected by a reduction in surface expression of CD36 by hepatocytes. On the other hand, E2 might not act directly on the *Cd36* promoter, but instead regulate transcription indirectly - either by influencing the activity of transcriptional regulatory factors, or via other physiological effects (such as endocrine or metabolic changes). In the latter case, the time required to establish these other changes could account for the delay in responsiveness of hepatic *Cd36* transcript levels to withdrawal of E2.

Direct *in vitro* experiments, undertaken to eliminate extra-hepatic effects of Gx or sex hormone treatment on metabolism or the gonadal-hypothalamic-pituitary axis, failed to establish a direct link between steroid hormone treatment and CD36 expression by hepatocytes. The presence of E2 was not sufficient to maintain *Cd36* expression in primary rat hepatocytes, and neither did treatment of HepG2 cells with E2 induce increased expression of CD36 (not shown). It is recognized, however, that the problems encountered in maintaining cell viability of primary hepatocytes *in vitro* make it

difficult to draw solid conclusions. Factors other than (or in addition to) E2, such as cell anchorage, might be required to maintain expression of CD36 or steroid hormone receptors in primary hepatocytes. It has also been shown that both T and E2 are very rapidly depleted from the media by primary hepatocytes *in vitro* (Coecke et al., 1998). Furthermore, although nuclear translocation of the estrogen receptor has been studied in freshly isolated primary rat hepatocytes (Dickson and Eisenfeld, 1979), it appears that the ER content of hepatocytes also diminishes rapidly in culture (Crabb and Roepke, 1987). In addition, it has been shown that ER expression in hepatocytes is under multi-hormonal control (Norstedt et al., 1981) and primary cultures may require supplementation with growth hormone, dexamethasone or estrogen in order to maintain expression *in vitro* (Stavreus-Evers et al., 1997). All of these issues would need to be resolved before it could be concluded decisively that E2 and T do not act directly on hepatocytes to modulate CD36 expression. The effect of adding E2 to cultured HepG2 cells was also tested in pilot studies, as this has been shown previously to modulate SR-BI expression by these cells (Graf et al., 2001). No induction of CD36 expression was observed following up to 48 hours exposure to E2 (not shown). However, when investigating regulation of the LDL-R in HepG2 cells, Croston et al (1997) reported that transcription of an LDL-R reporter construct was dependent on co-transfection with ER. Clearly, an extensive series of promoter-reporter studies would be required to gain definitive insight into the mechanisms that underlie the *in vivo* observations described above. Nevertheless, the pilot studies described in section 5.7.2 suggest that E2 and T are not solely responsible for the *in vivo* gender difference in CD36 expression by hepatocytes in adult rats, or for the changes that were observed after modulating sex hormone status. These difficulties focused the project towards determining *in vivo* whether other constituents of the endocrine milieu, in particular GH, were involved in CD36 regulation. That is the focus of the following chapter.

In summary, with respect to the listed sub-hypotheses, the following conclusions can be drawn. The levels of *Cd36* mRNA transcripts present in adult female and male rat liver reflect the gender difference in hepatic CD36 protein. Gonadectomy of adult rats resulted in comparable levels of CD36 protein in male and female liver, and reduced the overall difference in *Cd36* transcript expression. However, the latter observation was due to a decrease in transcript levels in Gx-females (although these animals still expressed more *Cd36* mRNA than their Gx-male counterparts, whose transcript levels did not change). Treatment of Gx animals with E2 increased expression of both *Cd36*

mRNA and protein in female and male rats, whereas testosterone treatment repressed CD36 protein expression in Gx-males only and did not affect *Cd36* transcript levels at all. Hence, although these studies demonstrated that the sex steroids E2 and testosterone are important regulators of CD36 in the liver (via different mechanisms), the sexually dimorphic pattern of expression is only partially ablated in their absence.