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INSULIN-LIKE GROWTH FACTOR-I (IGF-I): ROLE AND APPLICATION IN INTESTINAL DISEASE

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A thesis submitted for the degree of Doctor of Philosophy

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PP1 : Howarth GS, Fraser R, Frisby CL, Schirmer MB, Yeoh EK. Effects of insulin-like growth factor-I administration on radiation enteritis in rats.
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PP5 : Howarth GS, Francis GL, Cool JC, Xu X, Byard RB, Read LC. Milk growth factors enriched from cheese whey ameliorate intestinal disease by methotrexate when administered orally to rats.
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PP6 : *Patent*: Method of preventing or treating alimentary tract damage due to chemotherapy or radiation. *Inventors*: Leanna C Read and Gordon S Howarth.
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SP7. Conlon MA, Tomas FM, Owens PC, Wallace JC, Howarth GS, Ballard FJ. Long R^3-IGF-I infusion stimulates organ growth but reduces plasma IGF-I, IGF-II and IGF binding protein concentrations in the guinea pig. *J Endocrinol* 1995, **146**:247-253.

SP8. Conlon MA, Francis GL, Tomas FM, Wallace JC, Howarth GS, Ballard FJ. Continuous 14 day infusion of Insulin-like growth factor (IGF)-II increases the growth of normal female rats, but exhibits a lower potency than IGF-I. *J Endocrinol* 1995, **144**:91-98.

SP9. Read LC, Lemmey AB, Howarth GS, Martin AA, Tomas FM, Ballard FJ. The gastrointestinal tract is one of the most responsive target tissues for IGF-1 and its potent analogs. In: *Modern Concepts of Insulin-like growth factors*. Ed. E. Martin Spencer. Elsevier Science Publishing Company London. 1991, 225-234.

SP10. Read LC, Howarth GS, Steeb C-B, Lemmey AB. In vivo effects of IGF-I on gut growth and function. In : *The insulin-like growth factors and their regulatory proteins*. Proceedings of the Third International Symposium on Insulin-like Growth Factors. Ed. R.C Baxter, P.D. Gluckman and R.G. Rosenfeld. Elsevier Science Publishing Company London. 1994, 409-416.

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SP13. Read LC, Tomas FM, Howarth GS, Martin AA, Edson K, Owens PC, Ballard FJ. IGF-1 and its N-terminal modified analogs induce marked gut growth in dexamethasone-treated rats. *J Endocrinol* 1992, **133**:421-431.

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ABSTRACT

Insulin-like growth factor-I (IGF-I) : role and application in intestinal disease.

Gordon S Howarth. Child Health Research Institute, CRC for Tissue Growth & Repair and Department of Physiology, University of Adelaide. PhD Thesis. November, 2001.

The principal (**PP**) and supporting (SP) peer-reviewed publications contained within this thesis describe studies which have contributed significantly to the understanding of growth factor functionality in the processes of protection, growth and repair in the diseased or damaged gastrointestinal tract. I am first author on the principal publications and co-author on the supporting publications. My personal contributions to each of these studies [described more comprehensively in Appendices A (**PP**) and B (SP)], ranged from the initial development of hypotheses and the design and execution of experiments, through to the preparation of manuscripts for publication. These studies have extended our understanding of the mechanism of action, and the therapeutic potential, of insulin-like growth factor-I (IGF-I) (**PP1-PP5**) and of several other growth factors, in the normal and damaged gastrointestinal tract. The latter include epidermal growth factor (SP1,SP2), transforming growth factors-α and -β (SP3), betacellulin (SP4), hepatocyte growth factor (SP5) and trefoil peptides (SP6).

Initial observations that IGF-I possessed trophic selectivity for the gut (SP7-SP11) were extended through the use of IGF-I analogues with altered bioavailability which demonstrated IGF binding protein modulation of IGF activities on gut growth and repair. In addition, a number of gastrointestinal conditions for which IGF-I therapy may be effective have been identified from experimental studies utilising animal models of gastrointestinal disease in which IGF-I promotes bowel re-growth. Early indications of IGF-I efficacy in gastrointestinal repair were derived from studies in catabolic states, including bowel resection (SP12) and glucocorticoid-induced villous atrophy (SP13).

Subsequent studies, however, have suggested a primary therapeutic role for IGF-I during the repair phase, immediately following acute gastrointestinal damage, including the recovery phase of radiation enteritis (**PP1**,SP14,SP15), chemotherapy-induced intestinal mucositis (**PP2**) and acute episodes of colitis (**PP3**). As a result of these studies, IGF-I has recently been accorded Orphan Drug status for the treatment of the short bowel syndrome and final planning for a clinical trial is currently in progress. In addition to identifying candidate target conditions in gastrointestinal disease or damage for therapeutic intervention by IGF-I, these studies have also contributed to knowledge on the possible influence of IGF-I treatment on cancer risk. In a study investigating the effects of chronic IGF-I administration in a model of inflammatory bowel disease with neoplastic predisposition (**PP4**), IGF-I did not affect the progression of bowel dysplasia. In more recent studies, therapeutic potential of enterally-administered bioactive formulations comprising many concentrated growth factors including IGF-I has been investigated. One such formulation is the bovine-sourced whey-derived growth factor extract (WGFE), which has demonstrated efficacy in promotion of gut repair in models of chemotherapy-induced intestinal mucositis (**PP5**,SP16) and colitis (SP17). WGFE has since been patented as a potential treatment for oral mucositis following radiotherapy or chemotherapy (**PP6**) and a Phase II clinical trial is currently underway.

The work contained within this thesis has extended our understanding of growth factors, particularly of IGF-I, in gut growth and repair in addition to the physiological role and hence, therapeutic potential, of a range of growth factors in gastrointestinal disease. Future evaluation of the application of growth factors to combat gastrointestinal disorders could focus on functional outcomes such as effects on intestinal absorption. In addition, the efficacy of growth factor analogues with increased bioactivity, possibly incorporated into enterally-administered growth factor formulations, either enriched in, or depleted of, certain specific growth factors, could be investigated as a novel treatment strategy for diseases of the gastrointestinal tract.

STATEMENT OF ORIGINALITY

This thesis contains original material which has been published previously in peer-reviewed scientific journals. I have acted as the principal author of the scientific publications that form the main body of this thesis, and co-author of the supporting publications. This work has not been accepted for the award of any other degree or diploma in any other University or tertiary institution. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to a copy of this thesis being made available for loan and photocopying following its deposition in the University Library.

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Gordon S Howarth

November 5th, 2001.

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After completing an Honours degree in Science at Adelaide University in 1976, and an ensuing productive ten year career in diagnostic medical science, it was not until 1987 that my interests became focussed on the more fundamental aspects of medical research. I would like to thank Drs Tony Clarkson, Andrew Woodroffe, Jane Lomax-Smith and Gerard Hale, then of the Renal Unit at the Royal Adelaide Hospital, for enticing me into a career in medical research. It was a decision I have never regretted. My appreciation must also go to Professor Heddy Zola, Director of the Child Health Research Institute (CHRI), Professor Leanna Read, CEO of the Cooperative Research Centre for Tissue Growth & Repair and now Managing Director of TGR Biosciences, and finally to Professor Caroline McMillen, Head of Adelaide University's Physiology Department for their support, and the support of their departments, in my doctoral studies. My sincere personal appreciation is extended to Professor Leanna Read, who at various times over the past twelve years has acted as my supervisor, adviser, mentor, confidante and friend. I would also gratefully acknowledge my co-investigators and the numerous staff at CHRI who have assisted and supported me over the past twelve years, especially Johanna Cool and Kerry Penning. I am further indebted to Dr Ross Butler of the Women's and Children's Hospital Gastroenterology Department for his valuable input and advice. Similarly, this venture would not have been possible without the support of Associate Professor Julie Owens, my principal supervisor throughout the preparation of this thesis. Finally, my most special thanks must go to my parents, Edna and Stanley Howarth, for providing such a wonderful, warm and loving home environment. For the gifts of balance and perspective I thank my mother, whilst my father can take credit for his gifts of persistence and humour. I thank them both from the bottom of my heart. A very special thankyou must also be extended to my two sons, Christian and Scott, who, hopefully, will understand one day just how important they have been to me throughout the decade's work contained within this thesis. Finally, to Kerry Jolly who kept me laughing, my many supportive friends and colleagues, my brother Gary, and the other members of my extended family who have supported me in so many different ways over the years.

This thesis is dedicated to my parents, Edna and Stanley Howarth, my brother, Gary, and my two wonderful sons, Christian and Scott.

INTRODUCTION

The identification of insulin-like growth factor-I (IGF-I) as a potent trophic factor for the gut in the early 1990s resulted in investigations into its therapeutic potential to induce growth and repair of the gastrointestinal organs following injury. This thesis describes the investigation of IGF-I as a potential treatment for a range of clinically relevant, experimentally-induced, gastrointestinal disease conditions. The thesis also incorporates a review of the relevant scientific literature, including my published contributions to determining the mechanism of action, and therapeutic applicability, of IGF-I and several other related growth factors in the gut. The studies described in this thesis have resulted in five first-authored, and seventeen co-authored, scientific publications which have now culminated in the design of a clinical trial for IGF-I in the treatment of the short bowel syndrome, one of the disease conditions identified from these experimental studies as a candidate target condition for IGF-I therapy. For the purposes of this thesis, the peer-reviewed publications on which I am listed as principal author and which form the main body of the thesis, are identified by the bolded prefix '**PP**' and listed as **PP1 – PP6**. These publications are faithfully reproduced in **Appendix A** with the permission of the respective publishers. Other supporting publications on which I am listed as co-author are prefaced by the identifier '**SP**' and listed in **Appendix B**. The significance of each publication in furthering scientific understanding, together with details of my personal contribution, is described in annotated form in the Appendices. All other reference to the published work of others is cited in the 'References' section.

LITERATURE REVIEW

1. Introduction

Increasingly, the importance of *growth factors,* small molecules responsible for the growth of all tissues, to the processes of tissue homeostasis, development, growth, adaptation, and transformation is being recognized and understood. Although common to all tissues, these processes and their control, are particularly important in the gastrointestinal tract by virtue of its high rate of cell turnover and renewal. These characteristics have driven investigations into the roles that growth factors play in the aetiology and pathogenesis of disorders and diseases affecting the organs of the gastrointestinal system. Understanding the mechanism of action of growth factors, acting either individually or in concert with other factors, in the regulation of bowel protection and repair, provides a powerful tool for the design of new treatment strategies. Defining specific growth factor actions on disease processes in a range of models of gut damage has now made it possible to predict and design potential applications for growth factors at distinct stages of the disease process, for example as agents of gut protection, or alternatively, accelerated gut repair.

The number of known growth factors is large, and the spectrum of known gastrointestinal disorders and disease conditions which afflict man even larger. This review will primarily describe the rationale for investigations into one of these growth factors, insulin-like growth factor-I (IGF-I), as a potential treatment modality for several of these conditions on the basis of their shared disease aetiology or pathogenesis.

2. The gastrointestinal tract

2.1 Structure and function of the gastrointestinal tract

The basic anatomy of the gastrointestinal tract is illustrated in **Figure 1** and described comprehensively in Hamilton WJ et al., 1976. An annotated description of the essential elements of the alimentary system follows. Following oral consumption of nutrients, the stomach provides an initial site for nutrient breakdown through acidic and enzymatic degradation, whilst the completion of their breakdown, subsequent absorption of nutrients occurs in the small intestine. The region of small intestine immediately distal to the stomach is known as the *duodenum*, the site of entry for pancreatic secretions via the bile duct. The duodenum lies adjacent to the *jejunum* (proximal small intestine) which functions as the principal region of nutrient breakdown and uptake. The more distal small intestine is the primary site of bile acid reabsorption and is termed the *ileum*. At the termination of the ileum, the large bowel, or *colon*, the principal site of water reabsorption begins, terminating at the *rectum* and *anus*.

The lining of the gastrointestinal tract is continuous, from mouth to anus, lined by a layer of cells known as the *epithelium* (**Figure 2**). The epithelium, together with the underlying connective tissue of the lamina propria, forms the *mucosa*. The mucosa is separated from the *submucosa* by a thin smooth muscle layer known as the *muscularis mucosae* and the diametrically opposed muscle layers of the *muscularis externa* further underlie the submucosa. Finally, the gastrointestinal organs are enmeshed in the loose connective tissue of the *serosa*. The histology of the alimentary tract is reviewed comprehensively in Whitehead R, (1995) and Jungueira LC et al., 1986).

Small finger-like structures known as villi, lined predominantly by differentiated enterocytes, are responsible for the enzymatic degradation and uptake of nutrients. The villi serve to maximise absorptive surface area so as to optimise digestion and absorption of nutrients. The absorptive surface area of the bowel is in considerable excess, since physiological processes and associated bodily functions are essentially unaffected in humans if up to 50% of the small intestine is resected (Wilmore DW et al., 1999). Villi line the entire small bowel and are renewed continually by cells emanating from the underlying crypts or glands (**Figure 2**). The crypts represent the proliferative compartment of the bowel mucosa, with the epithelium of the small bowel undergoing complete renewal in 48 – 72 hours (Alison MR, 1994). Intestinal homeostasis represents a balance between enterocyte renewal, through proliferation, and cell death, through necrosis or apoptosis (programmed cell death).

Whilst the primary function of the gastrointestinal system relates to the efficient digestion and absorption of orally administered nutrients, the regulation of these processes is complex and influenced by a number of factors. These include nutritional influences and non-nutritional factors such as endogenous secretions, neurovascular components, mesenchymal interactions, peptide hormones and growth factors, whilst the rate of gastric emptying and intestinal motility, each dependent on enteric innervation, also impact on bowel function.

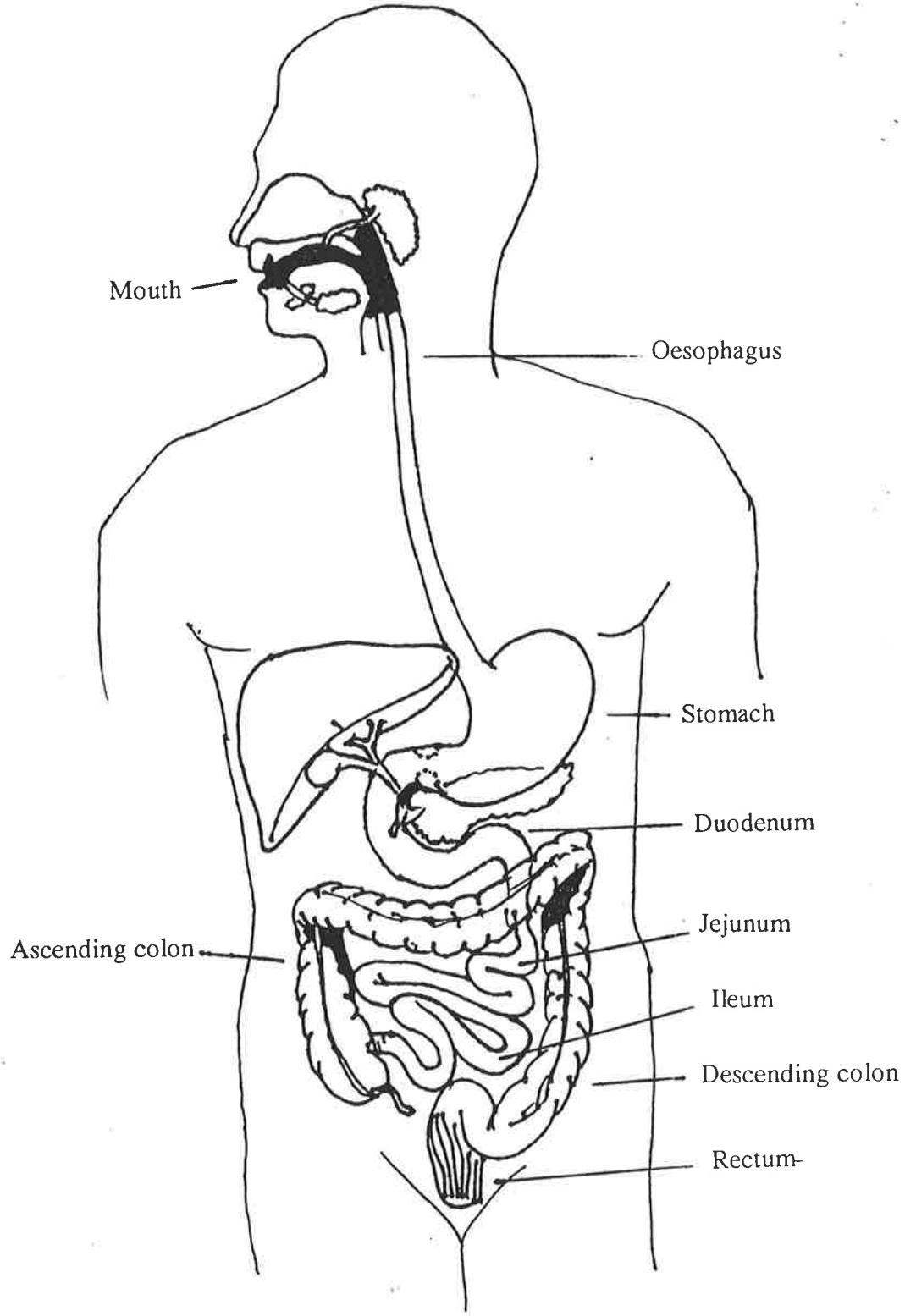


Figure 1

Anatomy of the alimentary tract. Modified from Hamilton WJ et al., 1976.

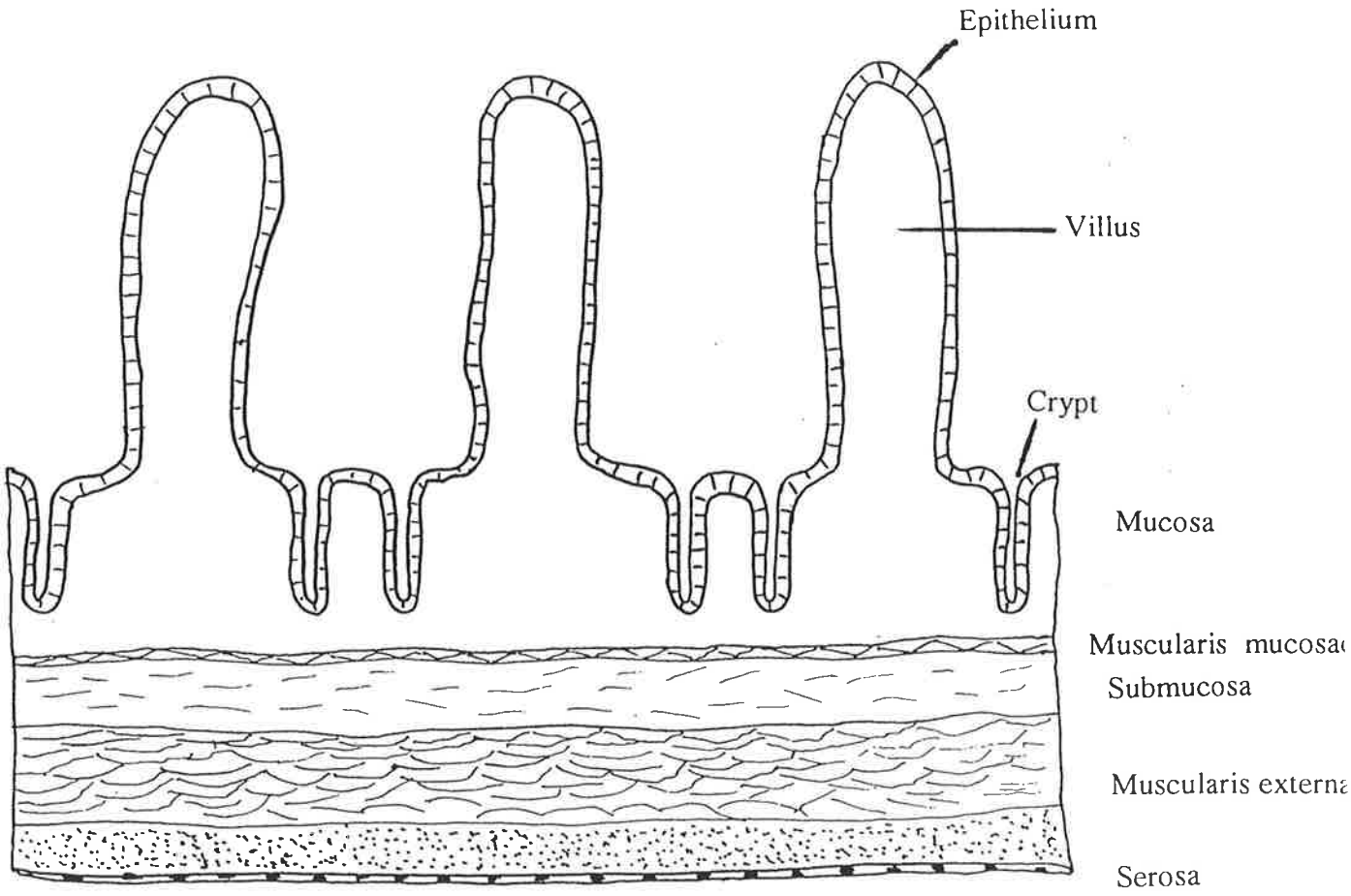


Figure 2

Microscopic appearance of the small intestine. Modified from Hamilton WJ et al., 1976.

2.2 Gastrointestinal dysfunction

A wide range of bowel disorders afflict man, both in the western and third world, and the concomitant morbidity and mortality associated with bowel dysfunction results in a significant health burden (**Table 1**) with an estimated total cost in excess of $40 billion ($US) in the United States for the year 2000 (Source: American Gastroenterological Association report on Burden of Gastrointestinal Disease, AGA, Bethesda, MD, USA, 2001). The prevalence of digestive disease in the United States is estimated at 60 to 70 million people with an annual mortality of 191,000 (1985). (Source: National Digestive Diseases Information Clearinghouse, National Institute of Health, Bethesda, MD, USA, 2001). For the purposes of this review, many of the gastrointestinal conditions contributing to these figures have been grouped and discussed on the basis of their shared pathogenesis and aetiology.

2.2.1 Reduced bowel mass

Reduced bowel mass, either congenital in origin, or as a consequence of surgical or pathological intervention, reduces absorptive surface area and diminishes bowel function. The short bowel condition is therefore multi-factorial in its aetiology, resulting in its collective description as the SBS (short bowel syndrome). Although absorptive surface area of the intestine may be reduced following atrophic or ulcerative insult, by far, the principal cause of impaired absorptive function in SBS, is the loss of substantial (greater than 75%) bowel length, usually resulting in a remnant bowel length of less than 150 cm (Reviewed in Wilmore DP et al., 1997).

Disease	1998				2000*
	Prevalence (in thousands)	Direct Cost ($ in millions)	Indirect Cost*** ($ in millions)	Total ($ in millions)	Total ($ in millions)
GERD	18,600	9,325	479	9,804	10,070
Gallbladder Disease	20,500	5,755	294	6,049	6,467
Colorectal Cancer	422.3	4,846	106	4,952	5,294
Peptic Ulcer Disease	6,730	3,059	201	3,260	3,441
Diverticular Disease	2,254	2,357	141	2,498	2,667
Diseases of Pancreas**	1,151	2,123	212	2,335	2,492
Non-foodborne Gastroenteritis and Other Intestinal Infections	135,000	1,598	505	2,103	2,238
Chronic Liver Disease and Cirrhosis	5,490	1,421	222	1,642	1,752
Irritable Bowel Syndrome	15,396	1,353	205	1,558	1,658
Liver Cancer	10	1,266	78	1,344	1,518
Pancreatic Cancer	18	1,225	30	1,255	1,370
Foodborne Illness	76,000	887	233	1,119	1,192
Crohn's Disease	359	707	75	781	826
Chronic Hepatitis C	2,530	693	51	744	758
Chronic Diarrhea	3,080	493	129	622	661
Ulcerative Colitis	619	388	36	423	443
Barrett's Esophagus	808	351	22	372	389
TOTAL****	---	36,310	2,817	39,197	41,816

*Inflated to year 2000 dollars using Medical CPI

** Excludes diabetes mellitus

***Indirect cost estimates include only lost days of work due to consumption of health care. Estimates based on other studies in the literature are mentioned throughout the study.

****Total cost estimate does not include costs of Barrett's esophagus, chronic hepatitis C, and chronic diarrhea due to double counting (Barrett's esophagus is included in GERD, chronic liver disease and cirrhosis are often caused by hepatitis C, and chronic diarrhea is often a symptom of irritable bowel disease). These diseases represent only a portion of all GI diseases, hence this estimate does not represent the cost of GI diseases overall, but rather this group of diseases only.

Table 1

Prevalence and health burden of selected gastrointestinal diseases. Source : American Gastroenterological Association report on Burden of Gastrointestinal Disease (2001).

Short-bowel syndrome is characterised by maldigestion, malabsorption, dehydration, electrolyte abnormalities, and both macronutrient and micronutrient deficiencies (Bernard et al., 1993). SBS in its most severe form may restrict sufferers to a life-time reliance on total parenteral nutrition (TPN).

The incidence of SBS in newborns has been estimated at between 0.3 and 0.5 per 10,000 births (Wallander L et al., 1992). Based on current data, the total incidence of small intestinal resection resulting in long term TPN treatment is approximately 9000 per year in the United States (Howard L et al., 1996). TPN is associated with a significant health burden estimated at 3 billion dollars ($US) per year in the United States alone (Warner BW, 2001). The development of new agents capable of increasing bowel mass is therefore, highly desirable. An increase in intestinal mass and hence, in absorptive surface area, could be achieved through either an increase in mucosal thickness by increasing villus length, or alternatively, an increase in linear length of the bowel. An optimal therapeutic strategy for the treatment of SBS would therefore be the capacity to increase both two- and three-dimensional growth of the bowel.

2.2.2 Atrophy

The general term for dysfunction of the bowel is enteropathy, and the infective enteropathies generally account for the greatest morbidity and mortality on a world-wide basis. For example, **Table 1** illustrates that the infective enteropathies (including food-borne infections) accounted for an estimated total health burden of more than seven billion dollars ($US) in the United States in 2000, with a prevalence in excess of 200

million affected individuals. These enteropathies encompass those induced by viral-, bacterial- and protozoal-agents. Although there are many differences between the mechanistic pathways of these differing initiating agents, infection with these agents often results in a common bowel pathology. The malabsorption which results from bowel infection is generally a consequence of an infection-induced flattening of the villi which line the bowel mucosa resulting in a condition known as *atrophy* (Whitehead R, 1995). Villous atrophy is frequently the end-result of many conditions differing in their aetiology. Thus, in addition to the infective enteropathies, villous atrophy can result from the hypo-proliferative effects of chemotherapy and abdominal radiotherapy and also uncontrolled coeliac disease due to a sensitivity to wheat gluten.

2.2.3 Ulceration

Focal erosion and superficial or penetrating ulceration of the epithelial barrier and deeper mucosa can result from several idiopathic or known causative initiators. Perhaps the best characterised are the anti-proliferative and prostaglandin-inhibitory effects of non-steroidal anti-inflammatory drugs (NSAIDs) (Chan TA, 2002), such as aspirin in inducing focal ulcerations of the jejuno-ileum (Aabakken L, 1999). Idiopathic inflammatory bowel disease (IBD), particularly the Crohn's disease variant, manifests as deep focal erosions and ulcerations which may become transmural resulting in bowel perforation (Jewell DP et al., 1992). Since the denuded epithelium provides a readily accessible route of entry for pathogens, secondary sepsis may be a serious consequence of this mucosal ulceration (Gottlieb JE et al., 1986).

2.2.4 Dysmotility

Bowel function is a product of efficient digestive and absorptive processes combined with appropriate time of transit through the bowel. Accordingly, agents which influence the peristaltic motion, or motility of the bowel, influence the rate of passage of nutrients through the intestine and the extent to which they are digested and absorbed. Dysregulation of intestinal motility is observed following abdominal irradiation, presumably through a direct toxic effect on neuronal innervation of the gut (Fraser R et al., 1997). Indeed, the retrograde passage of gastric contents through dysregulated alimentary transit is known as gastroesophageal reflux disease (GERD) and its estimated annual health burden cost of 10 billion dollars ($US) makes it the single greatest contributor to the health burden associated with gastrointestinal disease in the United States (**Table 1**).

2.3 Conventional treatment strategies

A wide range of treatments are available to combat the variety of disorders which affect the digestive system, although a detailed discussion of these treatment strategies is outside the confines of this thesis. Historically, however, the development of many of these treatments has been somewhat empirical and targeted toward disease symptomatology as opposed to disease aetiology. Clinical symptomatology in the upper alimentary tract may vary from mild dyspepsia through to the severe 'heart-burn' that typifies oesophageal reflux. Similarly, the spectrum of conditions and associated symptomatology affecting the lower bowel is just as diverse, varying from mild cramping through to severe pain with concomitant bowel dysfunction.

For the purposes of this review, discussion will be restricted to disorders of the small and large intestine, with clinical disease manifestations mediated by the mechanisms described in section (2.2). Based on these, the principal criteria for effective prevention or treatment of gastrointestinal dysfunction should encompass the prophylactic protection of the bowel, the promotion of repair and re-epithelialisation of mucosal breaches and finally, the normalisation of dysregulated motility.

Whilst current therapeutic strategies for many disorders of the lower bowel are effective, there are a number of conditions which remain relatively refractory to conventional treatments. Examples of these conditions include the mucositis that commonly accompanies chemotherapy and radiotherapy (Wilkes JD, 1998), ulcerative conditions such as the idiopathic inflammatory bowel diseases (Jewell DP et al., 1992), and other conditions in which bowel mass has been severely reduced, such as the short bowel syndrome (Wilmore DW et al., 1997). In each of these disease categories, there is a clear need to develop new therapeutic approaches to protect the intestinal epithelium from luminal or systemic insult, to promote the repair process, and finally, to stimulate regrowth of the damaged bowel. Our current understanding of the mechanisms and actions of several known growth factors implicates these as potential candidates in achieving these aims. Growth factors and their known actions in the gut are described in the following sections.

3. Growth factors and the gastrointestinal tract

Bowel growth and regeneration is influenced and regulated by a range of nutritional and non-nutritional factors (Bakseev L et al., 2000). Non-nutritional factors include endogenous secretions, neurovascular components, mesenchymal interactions, peptide hormones and growth factors, within the gastrointestinal system itself as well as endocrine influences. In general terms, growth factors are low molecular weight peptides which exert their effects via binding to specific cell-surface receptors. There are a number of excellent reviews available on the role and actions of growth factors in the gut (Beck PL et al., 1999; Herndon DN et al., 1993; Burgess AW, 1990; Podolsky DK, 1994).

Constitutive growth of the gastrointestinal tract is an integral feature of the digestive system. Enterocytes lining the intestinal epithelium migrate from the basal proliferative zone of the crypts of Lieberkuhn to the tips of the villi. In the proximal small intestine of the rodent this may take 2–3 days (Clarke RM, 1970) resulting in complete renewal of the entire small bowel epithelium every 48–72 hours. In the human, however, crypt cells may take 5-6 days to transit the villus (Eastwood GL, 1977), whilst gastric epithelial cells migrate toward the lumen in a journey which may take one to two weeks (Lee ER, 1985). Moreover, enterocyte transit in the colon is slower still, since crypt cell production rate in this region is less than half that of the small intestine (Goodlad RA, 1992). In addition to the maintenance of functional bowel integrity, this dynamic cell turnover is vital to the reconstitution of the mucosa through growth and cellular replacement in response to a variety of different forms of injury in the intestine.

In addition to growth factors, two peptide hormones, gastrin (Boel E et al., 1983) and enteroglucagon (Bell GJ et al., 1983), have been identified as trophic regulators in the gastrointestinal tract. However, the evidence for a role for enteroglucagon in the control of colonic mucosal proliferation is purely correlative and is derived from serum levels under different pathological or experimental conditions (Gornacz GE et al., 1984). However, whilst there is compelling evidence that gastrin stimulates the production of stomach cells (Ryberg B et al., 1990) the reports on its effects on cell production in the small intestine and colon have been less definitive.

3.1 Endogenous growth factors

Growth factors, produced locally or at distant, non-gastrointestinal sites (Podolsky DK, 1994), play an important role in the processes of bowel growth, protection, repair and homeostasis, though the associated kinetics of epithelial proliferation and apoptosis are complex (Reviewed in Alison MR et al., 1994). Although growth factors are associated with the growth of all tissues, the term 'growth factor' may sometimes be a misnomer since some growth factors, for example transforming growth factor–β (Barnard III JA et al., 1994) are associated with the *inhibition* of cell proliferation. A wide array of known endogenous growth factors is therefore required to maintain bowel homeostasis (**Figure 3**). Epidermal growth factor (EGF) has been the most well-characterised growth factor influencing maintenance, protection, adaptation and regeneration of the intestine. EGF is a member of a broader family of peptides with shared physico-chemical features. These are described in Section 3.2.

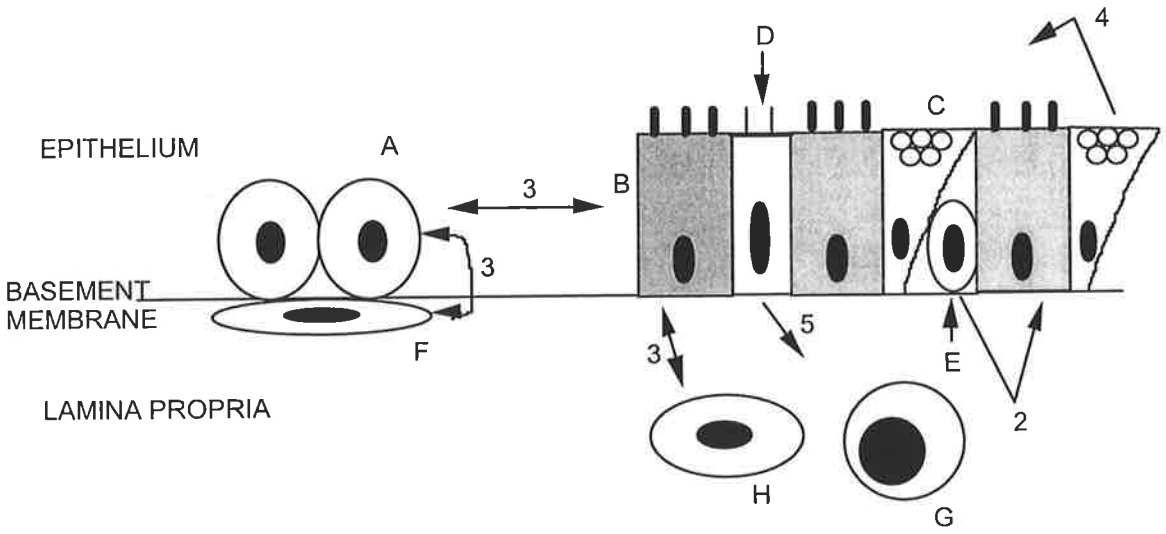


Figure 3. Overview of peptide growth factors and growth regulation in the intestine. Crypt (A) and differentiated columnar (B) epithelial cells produce a variety of peptide growth factors that can modulate proliferation of themselves, adjacent cells, and nearby cells, including lamina propria cell populations. The latter include pericryptal fibroblasts (F), lymphocytes (G) and macrophages (H). These effects are mediated by mechanisms designated autocrine, juxtacrine and paracrine, respectively [indicated by arrows 1, 2 and 3]. Both secretion and response to these factors by the columnar epithelial cells occurs at basolateral surfaces. Goblet cells (C) produce growth-modulatory proteins (trefoil peptides) in addition to their predominant secretory mucin glycoprotein product. These factors appear to be secreted through the apical pole onto the luminal surface where they may interact with the apical surfaces of other epithelial cells (4). Enteroendocrine cells (D) may secrete growth modulatory protein products (eg. GLP-2) through their basolateral pole in response to luminal factors bound at their apical surface (5). Luminal factors include nutrients and other dietary constituents. Intraepithelial lymphocytes (E) could also contribute to this network. Modified from Podolsky DK (1994).

3.2 Epidermal growth factor (EGF) family [SP1, SP2, SP3, SP4]

Although the EGF family of growth factors is relatively large, incorporating cripto, amphiregulin, heregulin, heparin-binding EGF, epiregulin and betacellulin, the archetype parent molecule, EGF, and to a lesser extent, the related peptide, transforming growth factor-α (TGF-α) have been studied most extensively with respect to the gastrointestinal tract (Karnes WE, 1994).

3.2.1 EGF [SP1, SP2]

EGF is a 53 amino acid molecule localised to submandibular glands, duodenal Brunner's glands and Paneth cells of the small intestine. The EGF peptide family binds to cell-surface receptors known as erb receptors. There are four known erb receptors, erb B1 (the EGF receptor), erb B2, erb B3 and erb B4. The EGF receptor has been localised to the baso-lateral surface of intestinal enterocytes (Playford et al., 1996). Indeed, recently a role has been assigned to the erb B1 receptor in the regulation of paracellular permeability in gastric epithelial cells (Chen MC et al., 2001), and up-regulated expression of its receptor has been described following bowel resection (Avissar NE et al., 2001).

EGF is resistant to degradation by gastric proteases and acids and has been demonstrated to increase mucosal hyperplasia and proliferation, intestinal weight, crypt depth, villus height, digestive enzyme activity and transport mechanisms (Karnes WE, 1994). Wright et al., (1990) reported that EGF was secreted by a novel intestinal cell lineage, termed the ulcer-associated cell lineage adjacent to intestinal ulcerations, suggesting therapeutic potential for EGF in conditions in which re-epithelialisation was desirable.

Prior to the availability of transgenic animals over-expressing EGF (Erwin CR et al., 1999), the therapeutic potential of EGF in bowel disease was investigated by the administration of recombinant EGF to laboratory animals, primarily in experimental studies of bowel adaptation following resection (Chaet MS et al., 1994; Schwartz MZ et al., 2000). In these studies, EGF restored mucosal protein, DNA and brush border enzyme activity in the small intestine. Similarly, in our own studies in normal animals (**SP1**: *Subcutaneous but not intraluminal epidermal growth factor stimulates colonic growth in normal adult rats*. Ribbons KA, **Howarth GS**, Davey KB, George-Nascimento C, Read LC. [1994]) crypt depth and hence, mucosal thickness, were increased in the proximal and distal colon following administration of EGF by the systemic route, although luminally-administered EGF was ineffective. These effects are consistent with the proposed baso-lateral localisation of EGF receptors, supported by similar colonic growth effects of EGF in bowel conditions associated with ulceration (**SP2**: *Effects of epidermal growth factor administration on repair of acetic acid-induced colonic ulcerations in rats*. Ribbons KA, **Howarth GS**, Ford WDA, George-Nascimento C, Bourne AJ, Read LC. [1997]).

3.2.2 Transforming growth factor-α (TGF-α) [SP3]

TGF-α is highly homologous to EGF (Karnes Jr. WE, 1994) and signals through the erb B1 EGF receptor. Although its expression is greatest in the colon, TGF-α is expressed abundantly on crypt enterocytes throughout the gastrointestinal tract (Jaeger LA, 1996). Evidence of an active role for TGF-α in colonic protection has been derived from reports of reduced (Egger B et al., 1998) or increased (Egger B et al., 1997) susceptibility to

experimental colitis in mice either over- or under-expressing, TGF-α, respectively. Although studies of TGF-α administration in vivo have been rare, Blikslager et al., (1999) described an enhanced recovery of villous surface area when TGF-α, acting synergistically in combination with glutamine, was administered to pigs with experimentally-induced ischaemic damage to the intestine. In summary, both EGF and TGF-α play an important role in protection and repair of the bowel.

Further insight into the role of TGF-α in vivo was gained from the findings of **SP3**: *Site-specific changes in transforming growth factor-α and -β expression in colonic mucosa of adolescents with inflammatory bowel disease.* Xian CJ, Mardell CE, **Howarth GS**, Byard RW, Moore DJ, Miettinen P, Read LC. [1999]. In this study, TGF-α immunostaining and mRNA expression were unchanged in the epithelium of specimens sourced from adolescents with active IBD compared to normal subjects. This study suggested that TGF-α itself may not have an important role in the pathogenesis of IBD, further implying a degree of functional redundancy within members of the EGF peptide family. This conclusion has since been further supported in as yet unpublished studies of experimentally-induced intestinal damage in mice with targeted deletion of the TGF-α gene (*Effects of TGF-α gene knockout on epithelial cell proliferation, migration, apoptosis, and repair of acute damage in the small intestine.* Xian CJ, Cool JC, **Howarth GS,** Read LC. : Submitted to Cellular Physiology : 2001). This study found that TGF-α deficiency had minimal impact on the processes involved in small bowel protection and repair, supporting a degree of redundancy within members of the EGF peptide family since other EGF ligands capable of signalling through the erb B1 receptor would

presumably be capable of maintaining bioactivity in response to mucosal injury.

3.2.3 **Betacellulin** [SP4]

There have been few reports of studies examining the relative importance and contribution of the other EGF family members to the process of intestinal growth. Recently, we have identified betacellulin as a trophic agent for the gastrointestinal tract (**SP4**: *Systemic administration of betacellulin to rats promotes growth of the gastrointestinal organs*. **Howarth GS,** Bastian SEP, Dunbar AJ, Kallincos NC, Goddard C. [2000]). Betacellulin was initially identified in the conditioned medium of pancreatic beta-cell tumours in mice (Shing Y et al., 1993). Human betacellulin is a 32 kDa glycoprotein, with three glycosylation sites, processed from a large transmembrane precursor by proteolytic cleavage, subsequently described as a potent mitogen for retinal epithelial cells and vascular smooth muscle cells (Dunbar AJ et al., 2000).

Although betacellulin is capable of activating erb B4 homodimerisation, to date, no physiological function has been assigned to this interaction. Preliminary studies (**SP4**) have prescribed a selectivity of betacellulin action to the distal bowel, consistent with the intestinal distribution of erb B1 receptors. Betacellulin could therefore hold clinical potential in conditions associated with ileal dysfunction, such as IBD disease or the distal enteropathy associated with consumption of non-steroidal anti-inflammatory drugs.

3.3 Transforming growth factor-β (TGF-β) [SP3]

TGF-β is a multipotent cytokine capable of promoting tissue restitution through fibroblast migration and inhibition of epithelial cell proliferation in the bowel mucosa (Barnard III JA et al., 1994). Several major conceptual problems regarding specific in vivo functions of the TGF-beta family members remain the key focus of many researchers studying the biology of these secreted signalling molecules. More than 45 members of this family of growth factors have been identified and partially characterized for their molecular roles in numerous processes such as cell proliferation and differentiation, embryonic development, carcinogenesis, immune dysfunction, inflammation and wound healing. The high degree of similarity that exists at the structural level among the isoforms of these growth factors is accompanied by a significant overlap in function, as defined by many *in vitro* model systems and *in vivo* systems involving administration of exogenous ligand or of ligand-specific blocking antibodies. The ability to discern the critical functions of these molecules based on patterns of expression has also often been quite difficult. The evolution of more sophisticated functional genomics approaches has been recently instrumental in generating unique perspectives into the mechanisms governing the activity of the members of the TGF-beta family, reviewed comprehensively in Kulkarni AB et al., (2002).

The two variants of inflammatory bowel disease, Crohn's disease and ulcerative colitis, are characterised by type 1 (Th1) and type 2 (Th2) immune responses, respectively (Monteleone I et al., 2002). TGF–β is also associated with down-regulation of type 1 (Th1) immune responses and its capacity to suppress type 1 helper cell responses has

been exploited as a potential application in the treatment of IBD. The latter is a condition characterised by an increased density of TGF-β-positive immune cells in the lamina propria (**SP3**) and an uncontrolled activation of the Th1-type immune response (Neurath MF et al., 1996). At least three distinct types of TGF-β receptor have been identifies on a variety of cell types. These receptors appear to include conventional transmembrane proteins, which serve as high, and low, affinity receptors (Cheifetz S et al., 1990). In addition, a third receptor with relatively low affinity has been found to consist of a complex cell-bound proteoglycan designated betaglycan (Wang X-F et al., 1991; Lopez-Casillas F et al., 1991). This receptor, in concert with proteoglycans within the extracellular matrix, may provide a further level of regulation in the complex control of TGF-β bioactivity.

Intestinal and colonic epithelial cells both produce significant amounts of TGF-βand are markedly responsive to this peptide (Suemori S et al., 1991a). In addition to its immunomodulatory properties, TGF-β directly, or indirectly, promotes acquisition of some features of differentiation and reconstitution of mucosal integrity has been described following injury in an in vitro model of epithelial wounding (Feil W et al., 1989). In summary, TGF-β is an important growth factor associated with protection and repair of the damaged bowel. It is capable of inhibiting proliferation whilst stimulating migration and restitution of epithelial cell populations and has also been demonstrated to be a potent chemotactic cytokine for neutrophils (Feil W et al., 1989).

3.4 Hepatocyte growth factor (HGF) [SP5]

HGF is a multifunctional growth factor with mitogenic, motogenic and morphogenic properties which was originally identified through its enhancement of hepatocyte DNA synthesis (Weidner KM et al., 1993). Enhanced gut function following HGF administration has been described in massive small-bowel resection (Schwartz MZ et al., 2000). Moreover, expression of HGF and its receptor (c-met) have recently been localised in the intestinal tract, and up-regulated expression has been reported in the bowel epithelium following damage induced by methotrexate (**SP5**: *Increased expression of HGF and its receptor c-met in rat small intestine during recovery from methotrexate-induced mucositis.* Xian CJ, Couper RT, **Howarth GS**, Mardell CE, Read LC, Kallincos NC. [2000]). HGF therefore joins the growing list of growth factors with therapeutic potential for the management of the short bowel syndrome.

3.5 Keratinocyte growth factor (KGF)

KGF is a heparin-binding member of the fibroblast growth factor (FGF) family in which it is termed FGF-7 (Herndon DN et al., 1993). KGF acts as a paracrine mitogen throughout the gastrointestinal tract and its mRNA and associated receptor have been detected in human adult and rodent gut tissue (Chailler P et al., 2000). Pre-treatment with KGF has been demonstrated to protect mice from chemotherapy- and radiation-induced gastrointestinal damage (Farrell CL et al., 1998) and oral mucositis (Dorr W et al., 2001). Playford RJ et al., (1998), utilising a model of indomethacin-induced enteropathy, concluded that KGF was involved in the control of proliferation of the small intestine, although there was no evidence for a role in the early reparative process.

Enteral nutrition has been described as a potent inducer of intestinal KGF and KGF receptor expression indicating nutrition as a primary regulator of KGF abundance (Estivariz CF et al., 2000). Moreover, precocious gut adaptation has been reported following KGF administration in a rat model of the short bowel syndrome (Johnson WF et al., 2000) and evidence of reduced mucosal damage has been described in an experimental model of Crohn's disease (Zeeh JM et al., 1996). The data accumulated thus far would suggest a role for KGF-1 in protection and repair of the bowel epithelium, further substantiated by recent reports of efficacy with KGF-2 (FGF-10), a cloned homologue of KGF, in indomethacin-induced bowel ulceration (Han DS et al., 2000).

3.6 Trefoil peptides [SP6]

Trefoil factors (TFFs) are small protease-resistant peptides produced by mucus-secreting cells, including goblet cells, in the gastrointestinal tract (Suemori S et al., 1991b). Three members of the trefoil peptide family have been described to date, *p*S2, spasmolytic peptide (TFF2) and intestinal trefoil factor (TFF3) which is specifically expressed in the intestine. Trefoil peptides play an important role in maintenance and protection of the intestinal mucosal barrier through promotion of wound healing, stimulation of epithelial cell migration and/or differentiation of epithelial cells (Podolsky DK et al., 1993). The growth factor properties of trefoil peptides are believed to be associated with reduced apoptosis but not by increased proliferation. Nevertheless, accelerated intestinal repair has been demonstrated following TFF2 administration in a rat model of colitis (Tran CP et al., 1999). In addition, temporal fluctuations in expression of the anti-apoptotic peptide, TFF3 have been described during the phases of damage and repair associated with acute

intestinal injury (**SP6**: *Temporal changes in TFF3 expression and jejunal morphology during methotrexate-induced damage and repair.* Xian CJ, **Howarth GS**, Mardell CE, Cool JC, Familari M, Read LC, Giraud AS. [1999]).

3.7 Glucagon-like peptide-2 (GLP-2)

Proglucagon is synthesized and differentially processed in pancreatic alpha cells and intestinal L-cells (Cordier-Bussat M et al., 1998). In 1961, Unger et al., (1961) first identified glucagon-like immunoreactivity in dogs and humans which was subsequently termed enteroglucagon. Serum enteroglucagon levels have since been associated with bowel growth (Frame CL, 1977). In recent years, the greatest interest in the role of growth factors in intestinal growth and function, and by association, in their clinical application, has been focused on glucagon-like peptide 2 (GLP-2), a member of the glucagon-related peptide superfamily.

For a comprehensive review of GLP-2, the review by Drucker DJ (2002) should be consulted. GLP-2 is a 33 amino acid peptide secreted by L-type enteroendocrine cells of the distal small intestine and colon (Drucker DJ et al., 2000). The primary stimulus for GLP-2 secretion is believed to be nutrient intake via direct luminal stimulation of L-cells (Burrin DG et al., 2001). Since enteroglucagon and GLP-2 are derived from a common precursor, L-cells simultaneously secrete these two peptides. Enteroendocrine L-cells are also responsible for the synthesis of other bioactive products such as peptide-YY (PYY), an effector of smooth muscle contraction and proliferation.

GLP-2 acts via specific binding to a single G protein-coupled GLP-2 receptor (GLP-2R) in epithelial cells of the small intestine (Drucker DJ et al., 2000) and Yusta B et al., (2000) using immunocytochemical techniques, have localised GLP-2R to the enteroendocrine cells of the stomach, distal small intestine and colon in rats and humans, consistent with the sites of GLP-2 synthesis. Further to this, Lovshin J et al., (2000) have demonstrated differential expression of GLP-2R in foetal and neonatal gastrointestinal tissue, suggesting a role for the GLP-2/GLP-2R axis in gastrointestinal development. GLP-2 has now been identified as a potent intestinotrophic factor involved in both the stimulation of epithelial proliferation and inhibition of apoptosis, and inhibits gastric acid secretion, emptying and up-regulates intestinal hexose transport (Drucker DJ et al., 2000).

The epithelial selectivity of GLP-2 has emerged as a powerful therapeutic strategy in a variety of clinical and experimental settings of epithelial insufficiency. For example, Thulsen J et al., (2001) reported a more pronounced adaptive response in the small bowel following resection of the proximal portion compared to the distal portion. The differences in the adaptive response were coupled to changes in circulating GLP-2 levels, identifying plasma levels of GLP-2 as an indicator of adaptive growth of the intestine.

Evidence of a potential role for GLP-2 in the treatment of IBD has been described by Alavi K et al., (2000) using a rodent model of spontaneous IBD induced by the micro-injection of human HLA-B27 and β2-microglobulin genes. In this study a marked reduction in gross and histologically-assessed bowel lesions following treatment with GLP-2 was observed. Although GLP-2 has also been proposed as a potential therapy for

chemotherapy-induced enteritis (Tavakkolizadeh A et al., 2000), its intestinotrophic properties would suggest application in the treatment of short bowel syndrome. Indeed, Sigalet DL et al., (2000) have reported an improvement in short-term weight gain following GLP-2 administration after massive bowel resection in a rat model. More recently, in a small group of patients, GLP-2 has been demonstrated to improve nutritional status in colon-deficient SBS sufferers (Jeppesen PB et al., 2001). This preliminary clinical study suggests that GLP-2 action on the damaged gut could have a substantial and favourable impact on nutrient digestion and absorption.

In summary, a wealth of information has accumulated on the role of the EGF family of peptides, and increasingly for that of other growth factors, in the intestine. Nevertheless, historically, perhaps the most important discovery in the past decade with respect to growth factor activity in the gut, was the identification of insulin-like growth factor-I (IGF-I) as an agent with trophic properties selectively targeted toward the bowel.

4. IGF-I and the gastrointestinal tract

Since the main body of this thesis relates to my contribution to the initial identification of IGF-I as a trophic factor for the gut, and its subsequent development as a potential treatment modality for a range of bowel disorders utilising various animal model systems, it is important that the key features of the IGF system and its regulation be reviewed.

4.1 The IGF system

There are a number of excellent reviews which describe aspects of the IGF system including the essential elements, regulation and proposed mechanism of action of IGF-I and –II in post-natal tissue growth, including the gastrointestinal organs (Jones JI and Clemmons DR, 1995; Clemmons DR, 1998; Noguchi T, 2000; Ghigo E et al., 2000; Ross RJ, 2000; Holly JM et al., 2000; Fryburg DA et al., 1999; Le Roith D et al., 1999). Current understanding of the IGF system is outlined in the following sections and refers to these reviews as well as other studies where most relevant (4.1.1, 4.1.2 and 4.1.3).

4.1.1 IGF ligands and receptors

There are two distinct types of IGFs, IGF-I and IGF-II, each single chain polypeptides of 7.5 kDa with 70% amino acid sequence homology (Blundell TL et al., 1978; Hill DJ, 1990). IGF-I and IGF-II are comprised of 70- and 67 amino acids, respectively (Daughaday WH, 1982). In many mammalian species, including humans, IGF-II is more abundant prenatally, but gene deletion experiments in mice suggest both IGFs are important for growth, differentiation and development before birth (Butler AA et al., 2001). IGF-I is structurally related to proinsulin, highly conserved across species and is a potent mitogen for many cell types.

The signalling pathways that culminate in IGF-I bioactivity are initiated by the interaction between IGF-I and the cell-surface located type I IGF-I receptor. IGF-II is additionally able to bind to a mannose-6-phosphate receptor known as the type II or mannose-6-phosphate IGF-II receptor. The IGF-I receptor is a disulphide-linked dimer which

belongs to the tyrosine kinase family of receptors and resembles the insulin receptor. The IGF-I and insulin receptors are closely related proteins that share an $\alpha 2\beta 2$ heterotetrameric structure and possess tyrosine kinase activity. The subsequent phosphorylation by receptor tyrosine kinase is the first step in the intracellular signalling process.

Receptor autophosphorylation amplifies the tyrosine kinase activity of the receptor and creates binding motifs for downstream signalling molecules. The IGF-I receptor, and the closely-related insulin receptor, utilize a family of large docking proteins (insulin receptor substrate, IRS) which bind to phosphotyrosine motifs on the receptor and, in turn, are phosphorylated on multiple tyrosine residues, creating binding motifs for the regulatory subunit of phosphoinositide 3-kinase (p85), the adapter protein Grb-2 and Nck, and the tyrosine phosphatase, Syp (Dey BR et al., 1998). The adapter protein Shc also binds directly to the IGF-I receptor, providing an alternative pathway for the activation of Ras via Grb-2 and SOS (Porras A et al., 1992).

STAT (signal transducer and activator of transcription) proteins including STAT1-4, -5a, -5b and –6 have been shown to play an important role in cytokine signalling (Ihle JN, 1996; Darnell JM Jr, 1997). Zong CS et al., (2000) investigated the activation of STAT proteins by the IGF-I receptor *in vivo* and *in vitro* concluding that IGF-I-induced STAT3 activation could be inhibited by interaction with a family of recently described proteins known as SOCS (Suppressor of cytokine signalling), which includes SOCS1-7 and CIS (Starr, R et al., 1999) which have been isolated and shown to act as negative regulators of

cytokine-induced signalling. SOCS-2 has been cloned by a yeast two-hybrid screen of a human fetal brain library, using the IGF-I receptor as bait, and demonstrated to bind to the IGF-I receptor from mouse fibroblasts (Dey BR et al., 1998). These results raise the possibility that SOCS proteins may play an important role in IGF-I receptor signalling.

4.1.2 Regulation of IGF bioactivity

The regulation of IGF bioactivity and bioavailability is not restricted to its receptor interaction but is also modulated by a series of up to six specific, high affinity binding proteins (IGFBPs) designated IGFBP-1 to -6, which, in concert with IGFBP proteases, play an important role in the regulation of IGF action through non-covalent associations with the IGFs. Postnatally, IGF-I in the circulation exists predominantly as a ternary complex comprising the IGF-I moiety, binding protein species and a growth hormone-dependent acid labile subunit (ALS).

More recently, a series of low affinity IGFBPs have been identified. Mac25 is a recently discovered member of the IGFBP family, assigned the name IGFBP-7, which is hypothesized to have growth-suppressing activity (Swisshelm K et al., 1995). In 1997, Kim HS et al., described a second low affinity IGFBP (IGFBP-8) also known as connective tissue growth factor (CTGF), further speculating on the existence of an IGFBP superfamily (**Figure 4**).

Although IGFBP-3 was the first IGFBP to be identified in the ternary complex, recent data suggest that IGFBP-5 can complex with IGF and ALS (Twigg SM et al., 2000). The

release of IGFs from these large complexes leads to the formation of smaller complexes with other IGFBPs, complexes that are believed to transport the IGFs out of the circulation. In addition to their roles in the circulation, most target tissues also express IGFBPs, which regulate the local action of IGFs (Rechler MM, 1993).

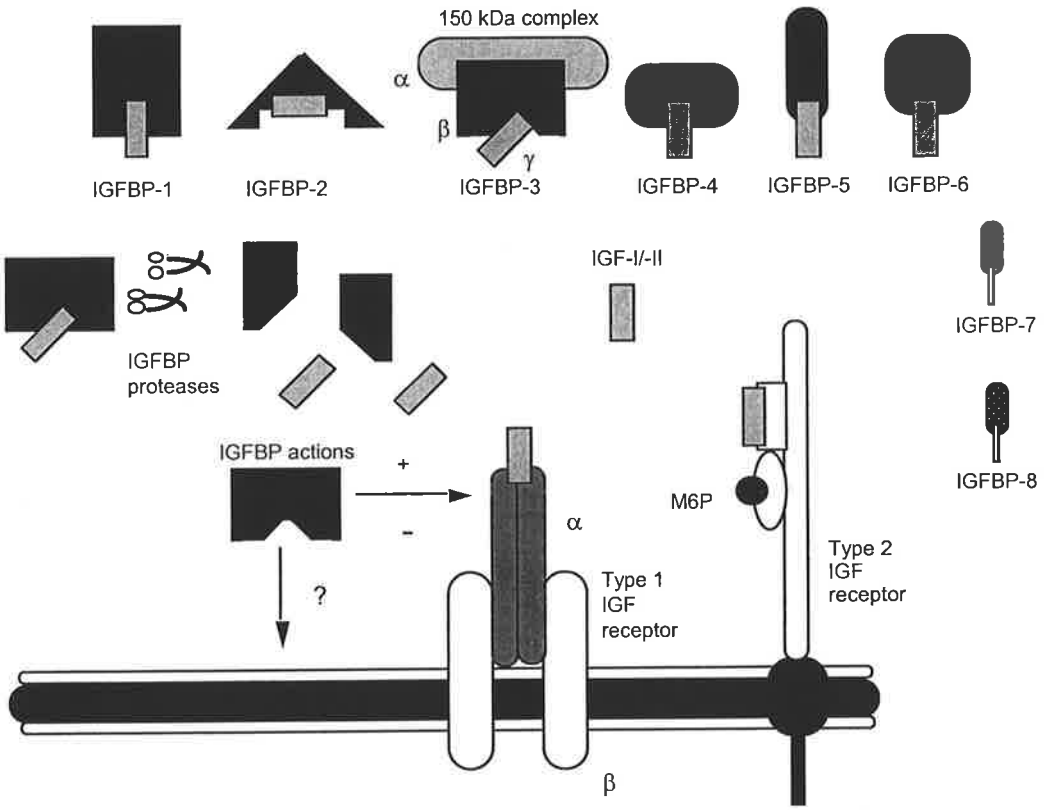


Figure 4. The IGF axis. The regulation of IGF-I and -II bioactivity and bioavailability is not restricted to the interaction with the type 1 IGF or type 2 (mannose-6-phosphate) IGF receptor, but is also modulated by a series of up to six, non-covalently bound, high affinity binding proteins (IGFBPs) designated IGFBP-1 to -6, which, in concert with IGFBP proteases, play an important role in the regulation of IGF action. Recently, low affinity IGFBP species (IGFBP-7 and –8) have been identified suggesting the existence of an IGFBP superfamily. Postnatally, in the circulation, IGF-I exists predominantly as a 150 kDa ternary complex comprising the IGF-I moiety, binding protein species and a growth hormone-dependent acid labile subunit (ALS). Modified from Rosenfeld RG et al., (1994).

4.1.3 The GH/IGF-I axis

The original hypothesis for IGF-I-actions on tissue growth was known as the somatomedin hypothesis (Daughaday WH 2000). This hypothesis suggested that the effects of growth hormone (GH) on post-natal growth were mediated by somatomedins (now known as IGF-I and -II), thought to be derived from the liver and secreted into the circulation. The hypothesis was later revised to include evidence that IGF-I was produced by most, if not all tissues, and that GH affected circulating levels of liver-derived IGF-I, as well as the stability of circulating IGF-I via the IGFBP-3/ALS complex. In addition, GH was capable of influencing local production of IGF-I, thereby maintaining its control on post-natal growth. However, recent studies in mice bearing tissue-specific deletions of the IGF-I or IGF-IR genes (reviewed in Butler AA et al., 2001), have suggested that liver-derived IGF-I is not essential for post-natal growth of tissues such as muscle and bone, and the importance of circulating levels of IGF-I remains controversial (Le Roith D et al., 2001; Rosen CJ et al., 1999; Hall K et al., 1999). These suggested mechanisms of IGF-I action in post-natal growth are illustrated diagrammatically in **Figure 5**.

Previous studies in hypophysectomized rats have indicated that GH is most effective in promoting bone growth and that IGF-I is more effective in promoting kidney and spleen growth (Guler HP et al., 1988). However, pre-natally, GH is not essential for intra-uterine growth and development, as demonstrated by the existence of normal-sized infants with either congenital absence of the pituitary or deletions of the genes encoding GH or the GH receptor (Laron Z, 1993; Takahashi Y et al., 1996). Moreover, foetal

tissues such as brain and skeletal muscle are differentially responsive to IGFs through a mechanism attributed to differential expression of IGFBPs in these tissues (Funk B et al., 1992).

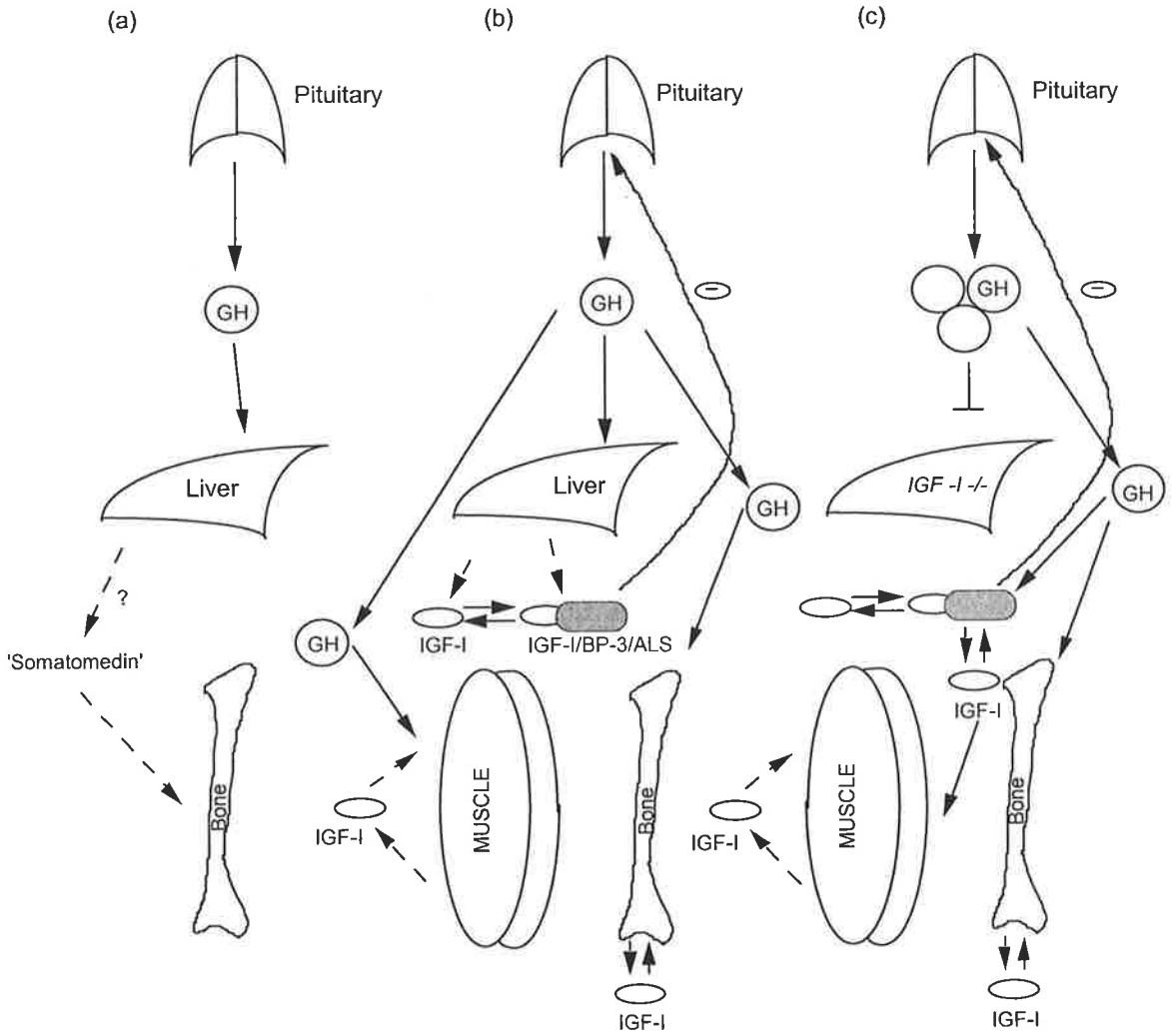


Figure 5. GH/IGF-I axis. The original hypothesis for IGF-I-mediated tissue growth was known as the somatomedin hypothesis (Daughaday WH, 2000). This hypothesis (a) suggested that the effects of growth hormone (GH) on post-natal growth of tissues such as bone and muscle, were mediated by somatomedins (now known as IGF-I), thought to be derived from the liver and secreted into the circulation under GH control. The hypothesis was later revised (b) to include evidence that IGF-I was produced by most, if not all tissues, and that GH affected circulating levels of liver-derived IGF-I, as well as the stability of circulating IGF-I via the IGFBP-3/ALS complex. In addition, GH was capable of influencing local production of IGF-I. However, the most recent studies (c) have suggested that, at least in mice, liver-derived IGF-I is not essential for post-natal growth whilst the importance of circulating levels of IGF-I remains controversial. Modified from Le Roith D et al., 2001.

4.1.4 IGF-I and cell kinetics

The role of IGF-I as a mitogen was deduced largely from in vitro studies of growth-arrested fibroblast cell lines (Pardee AB et al., 1989). Peptides such as platelet-derived growth factor, fibroblast growth factor, and epidermal growth factor were termed competence factors, as they stimulated quiescent cells to enter the G1 phase of the cell cycle (**Figure 6**).

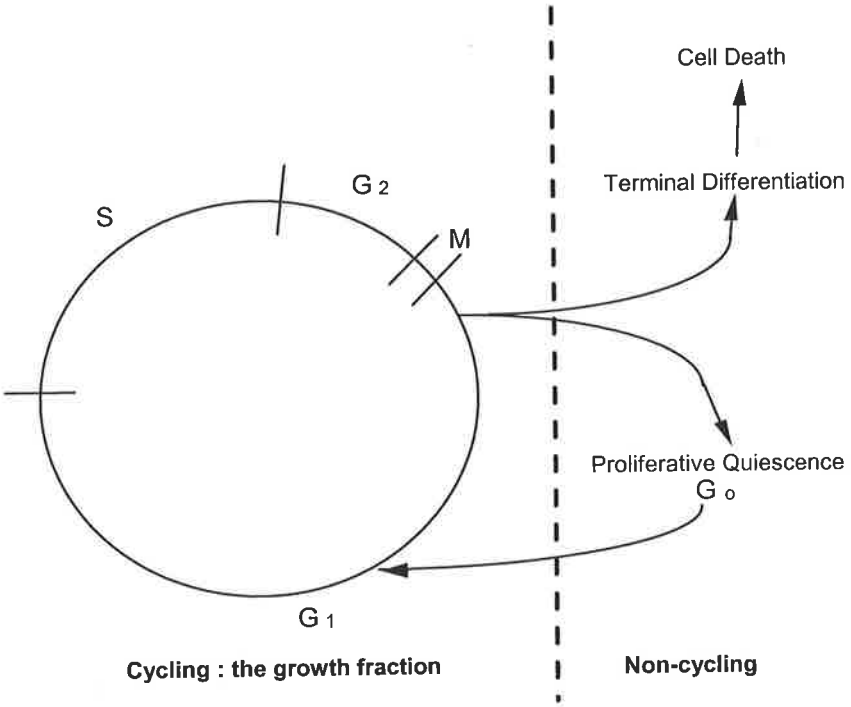


Figure 6. Schematic diagram of the cell cycle. Gap 1 (G_1) is the most variant phase in the cell cycle. G_1 can be almost non-existent in exponentially growing cultured cells, but can be 5-6 days in basal cells in stratified squamous epithelia. The synthetic phase (S-phase) is commonly complete in 6-8 hours, the second gap phase (G_2) in 1-4 hours and mitosis (M-phase) in 1-2 hours. Cells not actively cycling are in G_0, but can be triggered back into the cell cycle. Adapted from Alison MR (1995).

IGF-I spurs progression through G1 to S phase and was designated a G1-progression factor (Stiles CD et al., 1979). (**Figure 7**) IGF-I is a potent stimulator of proliferation in the crypts of the small and large intestine. In a study, Egger et al., (2001) investigated the effects of IGF-I on cell proliferation, as assessed by incorporation of the thymidine analogue, 5'-bromo-2'-deoxyuridine (BrdU), following partial colonic resection and anastomosis. IGF-I (1 mg/kg ip) almost doubled the BrdU labelling index (BrdU positive cells per crypt) two days following resection. Similarly, utilising tritiated thymidine incorporation as a marker of epithelial cell proliferation, Steeb, C-B et al., (1997) described increased proliferation in the jejunum of suckling rats following infusion of IGF-I or long-R^3-IGF-I, which was correlated exactly with crypt growth in the region. Moreover, Ohneda K et al., (1997) have described increased numbers of cell mitoses in the crypts of the small and large bowel epithelium in transgenic mice over-expressing human IGF-I. Studies using cells derived from knockout models of the IGF-I and IGF-I receptor suggest that IGF-I is not indispensable for progression through G1. Rubin R et al., (1995) reported that fibroblasts derived from IGF-I receptor null embryos had a fourfold longer duration G2/M phase in the cell cycle than comparable wild-type cells. In this model, G1 was not blocked but was longer in duration. Moreover, in intact IGF-I null mice, progression through G1 and S phases is normal, indicating that IGF-1 has a minor role as a G1 progression factor in vivo as evidenced by its requirement for EGF-mediated cell cycle progression in mammary epithelial cells (Stull MA et al., 2002) and for G2 progression in the estradiol-induced mitotic cycle from studies in IGF-I null mice (Adesanya OO et al., 1999). IGF-I, therefore, appears to be required for normal progression through the latter phases of the cell cycle

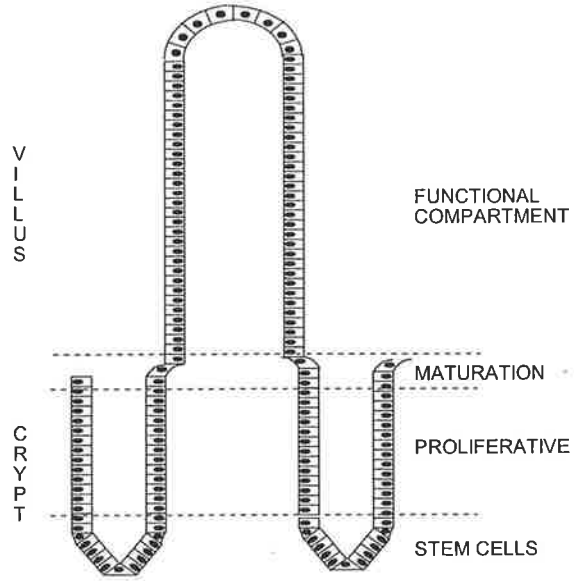
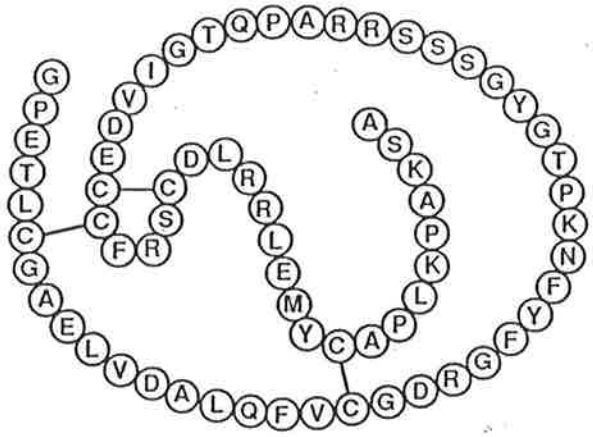


Figure 7. Diagrammatic representation of the kinetic compartments in the small intestine. IGF-I is a potent stimulator of enterocyte proliferation and is additionally capable of promoting differentiation whilst inhibiting apoptosis. Modified from Alison MR (1995).

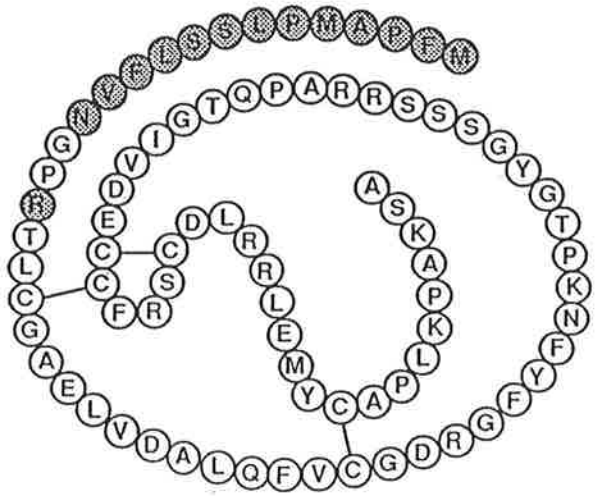
4.1.5 Analogues of IGF-I

Initial studies utilising IGF-I revealed that native IGF-I was difficult to produce recombinantly. Consequently, a number of analogues of IGF-I were generated for in vitro and in vivo applications (Ballard FJ et al., 1989). Examples of these more potent analogue peptides include des-(1-3)-IGF-I, which bears a three amino acid truncation at the N-terminus, and long-R^3-IGF-I with a 13 amino acid N-terminal extension peptide and an arginine substitution for glutamate at amino acid position three (Francis GL et al., 1992) (**Figure 8**). It was subsequently demonstrated that these analogue peptides had a reduced affinity for IGF binding proteins, resulting in an increased bioavailability and hence, biological potency both in vivo and in vitro. Comparative studies between IGF-I and analogue peptides with reduced affinity for IGFBPs has generated important information on the facilitatory or inhibitory influences of IGFBP species on IGF action.

Since the IGFBPs are predominantly, but not exclusively, inhibitory in action, these IGF-I analogues have proved important in studies associated with therapeutic applications of IGF-I, since they have the potential to be more effective, therapeutically, as a result of their increased bioavailability. This application will be discussed in more detail in the ensuing sections.



IGF-I



LONG R3 IGF-I

Figure 8. Human IGF-I and its analogue, long-R^3-IGF-I. The analogue peptide bears a 13 amino acid extension peptide at the N-terminus and an arginine substitution for glutamate at position three.

4.2 Endogenous IGF-I and the gastrointestinal tract

There is now a considerable body of evidence implicating IGF-I as an important endogenous factor for cell renewal and growth during intestinal development. Receptors for IGF-I and -II have been described throughout the gastrointestinal tract, localised to both the muscle and mucosal layers of the intestine, with their greatest concentration occurring in the crypt enterocytes (Heinz-Erian P et al., 1991). Utilising immunocytochemical and immunoblotting techniques, these authors reported that expression of the type 2 (IGF-II/mannose-6-phosphate) receptor was two to three times greater than that of the type 1 IGF-I receptor. Furthermore, although expression of both receptors was greatest in the colon and ileum, compared to other gastrointestinal regions, expression of the IGF-II/mannose-6-phosphate receptor was greatest in the mucosa, whereas type 1 IGF-I receptor expression was maximal in the submucosa and muscularis externa. Utilising radiolabelled ligand binding methods, IGF-I receptors have been localised predominantly to the baso-lateral membrane of crypt enterocytes (MacDonald RS, 1999) with isolated reports of brush border membrane localisation (Sullivan TA et al., 1995). Expression of IGF-I, -II and all IGFBP species has been reported in the bowel (Reviewed in Ziegler TR et al., 1999; Chen K et al., 1999; Lund PK, 1998), suggesting autocrine, paracrine and endocrine modes of IGF-I action in tissue homeostasis.

4.3 Exogenous IGF-I and the gastrointestinal tract

The first reports of IGF-I as a trophic factor with gastrointestinal selectivity were generated from our laboratory in the early 1990s. Steeb C-B et al., (1994) administered IGF-I continuously to adult rats for 14 days resulting in preferential growth of the

gastrointestinal organs, compared to anabolic effects on the whole animal, since IGF-I treatment increased gut weight as a fraction of body weight by up to 32%. Since transgenic animals over- or under-expressing IGF-I were not available at that time, our early investigations into the actions of IGF-I in vivo, manipulated IGF-I abundance by systemic administration of exogenous IGF-I in rodents. This early work formed the foundation for subsequent investigation of IGF-I as a factor with potential applications for conditions in which bowel growth was compromised. IGF-I has also been demonstrated to induce an increase in cell size (hypertrophy) although this phenomenon has not been fully characterised in the intestine. For example, Semsarian C et al., (1999) have reported skeletal muscle hypertrophy mediated by a Ca2+-dependent calcineurin signalling pathway, following administration of recombinant human IGF-I.

4.3.1 Exogenous IGF-I and the adult gut [SP7, SP8, SP9, SP10, SP11]

Early indications of anabolic potential for IGF-I were reported by Ballard FJ et al., (1989), describing a dual anabolic effect of IGF-I on protein metabolism in cultured cells. Subsequent development and understanding of IGF-I actions in vivo was gained by infusion of IGF-I into normal adult animals. Systemic infusion of IGF-I or more potent IGF-I analogue peptides into normal young adult rats resulted in the anabolic effects predicted from the in vitro studies (Ballard FJ et al., 1989), with improvements in body weight gain, nitrogen retention and food conversion efficacy (Tomas FM et al., 1993).

Significant increases in epithelial proliferation occur in the small intestinal epithelium after only three days administration of IGF-I or more potent IGF peptides with reduced

affinity for IGFBPs (Steeb C-B et al., 1995). Infusion of IGF-I peptides for longer periods (14 days) markedly increased total gut weight, small intestinal weight, and importantly, small intestinal length (Steeb CB et al., 1994). These weight increases were accompanied by increases in crypt cell population (+33%), cells per villus column (+34%), and villus cell density (+20%). IGF-I treatment resulted in the most profound effects on villus height and crypt depth in the more proximal bowel regions where the mucosal and muscularis layers were affected proportionally. The trophic properties of IGF-I for intestinal tissue have been confirmed by Ohneda K et al., (1997) in over expression of IGF-I which enhanced growth of the small bowel, evidenced by increased jejunal villus height, crypt depth, and crypt cell mitoses. The intestinotrophic properties of IGF-I have since been confirmed in other species as disparate as the guinea pig (**SP7**: *Long R^3-IGF-I infusion stimulates organ growth but reduces plasma IGF-I, IGF-II and IGF binding protein concentrations in the guinea pig.* Conlon MA, Tomas FM, Owens PC, Wallace JC, **Howarth GS**, Ballard FJ. [1995], pig and marmoset (Tomas et al., 1997).

There have been fewer studies investigating the effects of IGF-II on gastrointestinal growth compared to IGF-I (**SP8**: *Continuous 14 day infusion of Insulin-like growth factor (IGF)-II increases the growth of normal female rats, but exhibits a lower potency than IGF-I.* Conlon MA, Francis GL, Tomas FM, Wallace JC, **Howarth GS,** Ballard FJ. [1995]) though their findings are consistent with the current view that IGF-II is less potent than IGF-I in postnatal life. In support of this view, while transgenic mice over-expressing IGF-II in liver, kidney and intestine resulted in circulating IGF-II levels 2-3-

fold higher than in wild-type controls, no major changes in body weight, skeletal growth or cell proliferation were evident (Wolf E et al., 1995).

In summary, exogenous IGF-I administration to normal adult mice, rats and guinea pigs induces a selective, three-dimensional growth of the gastrointestinal organs, affecting the mucosal and muscularis layers proportionally.

4.3.2 Exogenous IGF-I and the neonatal gut

The studies conducted in the early 1990s describing the responsiveness of the gut to IGF-I and its more potent analogues were mainly restricted to studies in adult animals in which gastrointestinal development was complete. However, gastrointestinal dysfunction is a common consequence of prematurity in human infants and necrotizing enterocolitis (NEC) is a potentially fatal result of bowel dysfunction with approximately 1500-2000 deaths recorded annually in the United States (Neu J et al., 1999) in the first year of life. Since surgical resection of the necrotic intestinal segment is unavoidable in infants affected by NEC, the stimulation of gastrointestinal mass and length by IGF-I peptides, described earlier in the adult gut, would be highly desirable, if translatable to the neonatal, developing gut. Subsequent studies of IGF-I peptide infusion into neonatal rats (Steeb C-B et al., 1998) revealed that systemically, but not oro-gastrically, delivered IGF-I stimulated intestinal disaccharidase activity in two age groups of suckling rats, a response that was consistent with the localisation of IGF-I receptors to the baso-lateral enterocyte surface. Further studies in neonatal rats revealed that treatment with IGF-I peptides stimulated gut weight and length by up to 60 and 32%, respectively, with the

long-R^3-IGF-I variant more potent for all growth parameters (Steeb et al., 1997). Thus, the neonatal gut appeared more responsive to IGF-I administration compared to adults, providing further support for its potential therapeutic application in short bowel conditions affecting neonates.

Studies into the responsiveness of the immature (foetal) gut to IGF-I administration have been less definitive, with Kimble RM et al., (1999) describing enhanced gut development in oesophageal-ligated foetal sheep and Tarantal A et al., (1997) describing increased intestinal length in foetal rhesus monkeys, whilst Lok F et al., (1996) reported no significant effects of IGF-I administration on gut weight or length in foetal sheep in late gestation. Highly significant with respect to NEC were the findings associated with the administration of IGF-I or more potent IGF-I analogues to neonatal rats described by Steeb C-B et al., (1998) in which the length of the small intestine increased by up to 32% by infusion of IGF-I peptides. This finding identified the surgical resection of significant lengths of bowel in the neonate, for example, following an episode of NEC in perinatal life, as a candidate target condition for subsequent IGF-I treatment. The importance of IGF-I and bowel resection will be re-visited later, in sections 5.1 and 7.2.

5. IGF-I and bowel disease

The initial studies on administration of IGF-I and IGF-I analogues to normal adult and neonatal rodents clearly identified their anabolic and intestinotrophic properties indicating therapeutic potential for conditions associated with bowel dysfunction. These have been

reviewed in SP9, SP10 and SP11. Historically, the initial studies of IGF-I applicability to gastrointestinal disorder were targeted at the bowel dysfunction associated with states of catabolism, whilst subsequent experimental studies were focussed on the potential application of IGF-I to more specific disease entities such as radiation enteritis, chemotherapy-induced mucositis and inflammatory bowel disease. These are described in sections 5.1, 5.2, 5.3, 5.4.

5.1 IGF-I and catabolic conditions associated with bowel dysfunction [SP12, SP13]

There are a number of excellent reviews available describing the applicability of IGF-I to hyper-catabolic states (Gore DC, 1998; Welle S, 1998; Gelato MC, 2000). Early realisation of the intestinotrophic effects of IGF-I resulted in investigations into its application in models of catabolism associated with a degree of intestinal dysfunction. These include the respective rat models of small intestinal resection (**SP12**: *Treatment with IGF-I peptides improves absorption of the remnant gut following small bowel resection in rats*. Lemmey AB, Ballard FJ, Martin AA, Tomas FM, **Howarth GS**, Read LC. [1994]) and glucocorticoid-induced villous atrophy (**SP13**: *IGF-1 and its N-terminal modified analogs induce marked gut growth in dexamethasone-treated rats.* Read LC, Tomas FM, **Howarth GS**, Martin AA, Edson K, Owens PC, Ballard FJ. [1992]).

IGF-I and its analogues have been demonstrated to be beneficial to indices of gut function and mucosal absorptive area in the catabolic state induced by massive bowel resection (**SP12**). In this study, IGF-I, long-R^3-IGF-I or des-(1-3)-IGF-I, administered following 70% jejuno-ileal resection, significantly attenuated malabsorption of fat and nitrogen.

Total gut weight was increased by up to 21%, due predominantly to increased weight of the stomach and proximal small bowel, with the latter effect attributable, at least in part, to an increased bowel length. Long-R^3-IGF-I was more potent than IGF-I at stimulating body weight gain and food conversion efficiency in the bowel-resected rat, but its potency advantage on gut absorptive function and small intestinal re-growth was less marked. This outcome, confirmed by similar findings of Vanderhoof JA et al., (1992), concluded that administration of IGF-I peptides improved gastrointestinal absorptive function following partial gut resection, probably due in part, to an increase in absorptive area.

Administration of the glucocorticoid, dexamethasone inhibits intestinal growth and induces a mild villous atrophy (Burrin DG et al., 1999). Concurrent administration of IGF-I for 7 days to rats made catabolic by co-treatment with dexamethasone (**SP13**) induced an increase in total gut weight of up to 60% compared to dexamethasone-treated controls receiving vehicle infusion. Weight increases were apparent in all regions of the gut examined, including the stomach, small intestine and colon. Histological and biochemical analyses of the intestine showed that cross-sectional mass, rather than gut length, was increased, and proportional increases in wet weight, protein and DNA content per unit length were measured in both the mucosa and muscularis layers. IGF-I was thus able to reverse the gastrointestinal manifestations of the catabolic state produced by administration of the glucocorticoid, dexamethasone.

Although diseases and malabsorptive disorders of the gastrointestinal tract can be broadly categorised into disorders characterised by diminished bowel mass, mucosal atrophy,

epithelial ulceration and motility, there are a wide variety of specific disease conditions which fall under each of these headings. Since evidence of IGF-I efficacy, indicated by promotion of gut growth and function, had been derived from studies of catabolism and reduced bowel mass (**SP12**, **SP13**), further investigations were focussed on the therapeutic potential of IGF-I in experimental models of specific types of bowel dysfunction. The following sections (5.2, 5.3. 5.4) describe the development and utilisation of animal models of radiotherapy-induced, chemotherapy-induced, and chemically-induced bowel damage to achieve respective models of dysmotility, mucosal atrophy and colonic ulceration for investigations into the potential efficacy of IGF-I.

5.2 IGF-I and radiation enteritis [**PP1,** SP14, SP15]

High dose abdominal irradiation is a common form of therapy for the ablation of abdominal neoplasms such as in gynaecological and pelvic malignancy (Yeoh, EK et al., 1988). However, abdominal symptoms such as diarrhoea, abdominal cramps and vomiting are common during and after radiotherapy for these conditions (Fyles A et al., 1996). Although small intestinal dysmotility and mucosal effacement contribute to these symptoms, the underlying mechanisms are unclear. The dysmotility accompanying radiation enteritis has been characterised in a model system described in **SP14**: *Small intestinal dysmotility following abdominal irradiation in the isolated rat small intestine.* Fraser R, Frisby CL, Blackshaw LA, Schirmer MB, **Howarth GS**, Yeoh EK. [1998]).

There have been few investigations into the likely therapeutic benefits of IGF-I in radiation enteritis. **PP1**: *Effects of insulin-like growth factor-I administration on radiation*

enteritis in rats. **Howarth GS**, Fraser R, Frisby CL, Schirmer MB, Yeoh EK [1997] represented the first investigation into IGF-I as a treatment modality for radiation enteritis, although Farrell CL et al., (1998) and Dorr W et al., (2001) have since reported beneficial effects of KGF in this setting. **PP1** employed a rat model of radiation enteritis utilising a single high dose (10 Gray) of suitably delivered abdominal irradiation. The study concluded that IGF-I was capable of reducing the severity of acute mucosal damage following radiation-induced injury. Irradiated rats receiving IGF-I lost less body weight than vehicle-treated rats, whereas the wet weights of the stomach, small intestine, and colon were increased by 10%, 19%, and 21%. Similarly, crypt depth was increased in the duodenum, jejunum, and ileum. IGF-I administration after abdominal irradiation increased small-intestinal mass and improved indicators of mucosal integrity, suggesting acceleration of small-intestinal mucosal recovery from radiation injury. This study provided evidence that systemically-administered IGF-I held potential to promote restoration of normal bowel integrity following an episode of radiotherapy, thereby creating an opportunity for dose escalation and hence the likelihood of improved tumour ablation.

A subsequent follow-up study (**SP15**: *Divergence of mucosal and motor effects of IGF-I and LR^{3}IGF-I on isolated rat ileum following abdominal irradiation.* Fraser R, Frisby CL, Blackshaw LA, Langman J, **Howarth GS**, Yeoh EK. [2000]) utilised the model of radiation enteritis developed in **SP14,** to explore the potential mechanism of IGF-I action during the repair phase following radiation-induced injury. The effects of IGF-I and its more potent analogue, long-R^3-IGF-I on the independent processes of mucosal repair and

intestinal motility were compared. This study concluded that IGF-I peptide action did not appear to influence motor activity in the jejunum, but exerted its beneficial effects through a direct effect on mucosal re-growth following injury. Further insight into the expression of IGF binding proteins was gained from the finding that IGF-I and its analogue peptide were equipotent in experimental radiation enteritis. Although not definitive, a decrease in local IGFBP expression may have resulted in this effect. This is a potentially intriguing hypothesis since there is accumulating evidence that IGFBP-3 can also cause apoptosis in an IGF-independent manner (Shen L, 1995). p53, perhaps the single most important human tumour suppressor, is commonly mutated in human cancers and one of the genes induced by p53 has been identified as that encoding IGFBP-3 (Grimberg A, 2000). Thus, IGFBP-3 induction by p53 may constitute a new means of cross-talk between the p53 and IGF axes, suggesting that the ultimate function of IGFBP-3 may be to serve a protective role against the potentially carcinogenic effects of growth hormone and IGF-I.

Recently, Wang J et al., (1999) utilising a modification of the animal model developed in **SP14** described an up-regulation and spatial shift in the localisation of the mannose 6-phosphate/insulin-like growth factor II receptor during the development of radiation enteropathy in the rat. The therapeutic potential for manipulation of the IGF axis have been pursued by Vazquez I et al., (1999) who subsequently described the protective effect of an enriched diet supplemented with growth hormone on radiation-induced intestinal injury in the rat.

5.3 IGF-I and chemotherapy-induced mucositis [PP2]

The previous section (5.2) described the emergence of a 'proof-of-concept' for IGF-I as an agent capable of accelerating repair of the proximal bowel following radiation damage. However, the number of patients receiving chemotherapy for haematological or solid malignancy far exceeds the number receiving radiotherapy (Source: American Gastroenterological Association report on Burden of Gastrointestinal Disease AGA, Bethesda, MD, USA, 2001). The aetiology of this disorder is targeted at achieving cell death through biochemical disruption of factors controlling the cell-cycle in rapidly-dividing cells, similar in principle to the mechanism of radiation enteritis. Accordingly, the positive indications of IGF-I efficacy in radiation enteritis were extended to investigations into the treatment of bowel damage induced by chemotherapeutic agents.

In broad terms, chemotherapy agents target rapidly-dividing cells and induce a state of hypoplasia in the intestinal crypts. For example, the anti-metabolite, methotrexate achieves this effect through interfering with folate metabolism through inhibition of the enzyme dihydrofolate reductase. This hypoplasia results in a loss of villous architecture (atrophy) accompanied by severe malabsorption. The gastrointestinal side-effects of chemotherapy may be severe, and secondary sepsis is a major concern (Altmann GG, 1974). Furthermore, the incidence of intestinal mucositis is increasing in conjunction with recent strategies to improve tumour ablation through intensification of chemotherapy regimens (Source: American Gastroenterological Association report on Burden of Gastrointestinal Disease AGA, Bethesda, MD, USA, 2001). Historically, the treatment of intestinal and oral mucositis has been largely palliative, with the local anaesthetic effects of orally-applied ice cubes as effective as any drug intervention.

Since the therapeutic potential of growth factors had not been previously investigated in chemotherapy-induced intestinal mucositis, a modified rat model of upper bowel mucositis was developed by subcutaneous administration of the chemotherapy drug, methotrexate, for this purpose. This study (**PP2**: *Insulin-like growth factor-I (IGF-I) stimulates regrowth of the damaged intestine in rats when administered following, but not concurrent with, methotrexate.* **Howarth GS**, Cool JC, Bourne AJ, Ballard FJ, Read LC. [1998]) found that IGF-I administered following the completion of methotrexate treatment was effective at inducing precocious bowel re-growth. IGF-I administered post methotrexate stimulated regrowth of the damaged intestine, particularly the ileum, with 22%, 32% and 29% increases in small intestinal weight, ileal villus height and ileal crypt depth respectively compared to methotrexate-treated controls receiving vehicle administration. However, IGF-I administered coincident with methotrexate failed to restore mucosal integrity to the damaged small intestine. Indeed, there were indications, although not statistically significant ($p = 0.07$), that concurrent IGF-I treatment had exacerbated histological indices of villus and crypt damage. This study concluded that IGF-I administered following methotrexate-induced intestinal damage primarily induced growth of the distal small intestine. The ineffectiveness of concurrently administered IGF-I may have represented an IGF-I induced increase in the number of proliferating epithelial cells concurrently exposed to the anti-proliferative effects of methotrexate. This study provided clear indications for clinical application of IGF-I following acute intestinal injury, but contra-indications for its administration during periods of treatment with chemotherapeutic agents.

PP2 concluded that IGF-I held therapeutic potential when administered during the recovery phase immediately following the cessation of chemotherapy. In addition to this clinically-relevant information, the animal model of methotrexate-induced mucositis has since been further exploited to characterise the effects on the expression of other factors such as HGF (**SP5**), the HGF receptor, *c*-met (**SP5**), the trefoil peptide, TFF3 (**SP6**) and zinc (**SP16**), and hence their therapeutic potential.

5.4 IGF-I and inflammatory bowel disease [PP3]

Inflammatory bowel disease (IBD) afflicts approximately 1 in 8000 individuals with an estimated health burden of $1.2 billion ($US) [Source: American Gastroenterological Association report on Burden of Gastrointestinal Disease, AGA, Bethesda, MD, USA, 2001]. The two variants of IBD, namely ulcerative colitis (which affects primarily the distal bowel) and Crohn's disease (which tends to be more focal, and which may affect any alimentary region) are characterised by uncontrolled chronic bowel inflammation with a predisposition to the development of colonic carcinoma (Jewell DP et al., 1992). Consequently, the proposal of therapeutic intervention by growth factors in such a clinical setting has met with concerns that the processes of initiation and progression of neoplasia could be promoted. Moreover, the idiopathic aetiology of IBD, and its unpredictable remission and relapse has tended to confound interpretation of growth factor expression during the course of IBD, recently reviewed by Beck PL et al., (1999). Although a detailed discussion of individual growth factors and their role in IBD is outside the boundaries of this thesis, the interpretation of IGF-I expression in IBD, and its potential therapeutic application, remain somewhat controversial.

Chronic uncontrolled bowel inflammation in the Crohn's Disease variant of IBD results in transmural granuloma formation and fibrosis (Jewell DP et al., 1992). These areas of intestinal fibrosis may result in stricture formation and even perforation of the bowel. Indeed, there is a growing body of evidence that IGF-I expression is associated with areas of fibrosis and may be implicated in the pathogenesis of granulomatous enterocolitis and fibrosis (Zimmermann EM et al., 1993; Ghosh S et al., 1997). Moreover, expression of IGF-I receptors and binding proteins by colonic smooth muscle cells and an up-regulation of insulin-like growth factor I (IGF-I) binding sites has been described in the smooth muscle layer of inflamed rat colon (Zeeh JM et al., 1997). However, it remains unclear whether increased IGF-I expression is primarily associated with the aetiology of IBD, or alternatively is an epiphenomenon of the fibrotic process.

The proposed role and molecular basis of IGF-I action in IBD has been reviewed in some detail (Lund PK, 1994; Lund PK et al., 1996; Lund PK, 1998) although a complete understanding requires analyses of the role of IGF interaction with other growth factors, hormones and cytokines. However, in order to define the potential indications or contraindications for IGF-I therapy in IBD, it is important to distinguish between long-term and short-term effects. We therefore set out to investigate the effects of short- and long-term IGF-I administration in an animal model of ulcerative colitis that predisposed to the development of neoplasia (Okayasu I et al., 1990; Cooper HS et al., 1993).

The first description of the short-term effects of IGF-I administration in an acute model of IBD were described in **PP3**: *Insulin-like growth factor-I partially attenuates colonic*

damage in rats with experimental colitis induced by oral dextran sulphate sodium. **Howarth GS**, Xian CJ, Read LC. [1998]. This study utilised a rat model of ulcerative colitis induced by the oral ingestion of dextran sulphate sodium (DSS). Compared with vehicle-treated rats consuming DSS, IGF-I increased the numbers of goblet cells by 76%, reduced the proportion of lamina propria cells expressing TGF-β_1, and reduced the thickness of submucosal and muscularis externa layers in the colon by 26% and 20%, respectively. This novel study identified a potential new mechanism of IGF-I action in acute colitis, since it concluded that the effects of IGF-I treatment on the colonic epithelium may have been mediated directly, whereas the reduced inflammation evident in the mucosa and submucosa may have been mediated by a separate, as yet undefined, mechanism *not* associated with up-regulation of the anti-inflammatory cytokine, TGF-β_1.

As described previously, increased expression of IGF-I has been associated with areas of colonic fibrosis. Fibrosis is not a feature of the DSS-colitis model used in **PP3**, however, suggesting therapeutic potential for IGF-I in a setting of acute, non-fibrotic IBD. However, IBD is generally a chronic condition, hence the likely duration of therapy would also be, presumably, correspondingly lengthy. Accordingly, in a follow-up study to **PP3**, the therapeutic potential of IGF-I was further assessed in a model of *chronic* colitis. The animal model of colitis proposed for the chronic study was selected on the basis that it exhibited neoplastic potential, to also determine if chronic IGF-I administration affected the initiation or progression of neoplasia (described in 6.2).

Failure to thrive is a common feature of inflammatory bowel disease (IBD) in infants and adolescents and although the aetiology of IBD remains obscure, there has been on-going development of dietary and parenterally-administered supplements to improve nutrition and hence growth in IBD-sufferers. Ballinger AB et al., (2000) in a recent experimental study, concluded that growth failure occurred through a decrease in serum insulin-like growth factor-I levels which was independent of under-nutrition in a rat model of colitis, a finding which lends further support to a likely benefit of therapeutic intervention with IGF-I in IBD. Furthermore, Savendahl L et al., (1997) described a decrease in serum IGF-I levels associated with experimental colitis which was abrogated by fasting. Taken together with the findings of **PP3** these studies would suggest that IGF-I administration in combination with periods of fasting could promote linear growth in IBD sufferers, whilst partially ameliorating the inflammatory disease process.

6. IGF-I and bowel cancer

In recent times there has been a proliferation of information on the role of IGF-I and IGF binding proteins in cancer (Reviewed in Camacho-Hubner C, 2000; Pollak M, 2000a; Pollak M, 2000b) and more specifically, in colon cancer (Reviewed in Singh P et al., 1993). Moreover, activation of the IGF-I/IGF-1 receptor system (IGF1/IGF1-R) has recently emerged as a critical event in the transformation and tumourigenicity of several murine and human tumours (Hakam A et al., 1999). The current scientific literature is divided with respect to IGF-I and its potential promotion of malignancy. Until recently, little information had been accumulated on the relative risk of IGF-I treatment on cancer

development (Pollak M, 2000b). In general terms, it would appear that IGF-I levels tend to correlate, and BP3 levels inversely correlate, with cancer risk (Pollak M, 2000b).

IGF-I has been reported to facilitate the growth of a number of malignant neoplasms (Daughaday WH, 1999; Macaulay VM, 1992). In colorectal carcinoma, IGF-I has been reported to induce vascular endothelial growth factor, an angiogenic hormone that is produced by, and which supports the growth of, many types of malignancies (Warren RS et al., 1996). Furthermore, Jenkins PJ et al., (1997) concluded that patients with acromegaly and elevated blood levels of IGF-I had an increased risk of developing colorectal cancer and tubulovillous adenomas.

The evidence supporting an active role for IGF-I in tumour promotion is balanced by studies suggesting a more passive role for IGF-I in cancer risk. Although increased expression of the EGF (epidermal growth factor) receptor has been strongly associated with malignancy (Malecka-Panas E et al.,1997). Such an association has not been investigated for the type 1 IGF-I receptor, despite its presence in tumours of the colon in humans (Guo YS et al., 1992). Furthermore, up-regulated expression of IGF-II, but not IGF-I, has been demonstrated in a number of tumour types, including colonic carcinoma (Macaulay VM, 1992; Warren RS et al., 1996. Remacle-Bonnet M et al., (1992), in an *in vitro* study, actually described a promotion of differentiation and an inhibition of tumour growth in a human colon-carcinoma cell line following treatment with the IGF-I analogue des-(1-3)-IGF-I. Although there is now a considerable body of information either implicating, or refuting, an active role for IGF-I in carcinogenesis, in 1996 there was little

information available on IGF-I and cancer *risk*. This question of IGFs and cancer development was investigated in **PP4**, as described in more detail in Section 6.2.

6.1 IBD and colon cancer

As introduced in Section 5.4, the uncontrolled chronic bowel inflammation that characterises IBD predisposes to the precocious onset of colon cancer (Jewell DP et al., 1992). Long-standing IBD-sufferers are thus considered to be at high risk for the subsequent development of small bowel adenocarcinomas, or more commonly, colon cancer (Edwards, PC et al., 1987). This strong association has resulted in justifiable safety concerns for therapeutic interventions which may impact on the subsequent risk of developing bowel cancer.

The known mitogenic and anti-apoptotic properties of IGF-I would indicate a mandatory requirement to gain further long-term safety information before IGF-I could be considered as a potential therapy for bowel conditions other than those involving acute bowel damage. On this basis, we elected to investigate the effects of chronic IGF-I administration in a setting of chronic bowel inflammation with pre-malignant potential.

6.2 IGF-I and IBD-related colon cancer [PP4]

A proof-of-concept had been established for IGF-I to stimulate bowel regrowth following acute colonic injury (**PP3**). Consequently, the effects of IGF-I on cancer risk were investigated in the more chronic disease setting (**PP4**: *Predisposition to Colonic Dysplasia is Unaffected by Continuous Administration of Insulin-like Growth Factor-I for*

Twenty Weeks in a Rat Model of Chronic Inflammatory Bowel Disease. **Howarth GS**, Xian CJ, Read LC. [2000]). At the time **PP4** was commenced there was scant information available on the safety of long-term IGF-I administration in any setting, although a number of clinical conditions were identified as candidates for long-term IGF-I therapy. These conditions included Laron dwarfism (Laron Z et al., 1992, Laron Z et al., 1994), insulin-resistant diabetes mellitus (Vestergaard H et al., 1997), osteoporosis (Eriksen EF et al., 1993), peripheral neuropathy (Apfel SC et al., 1996), the motoneuron disease, amyotrophic lateral sclerosis (Lange DJ et al., 1996), and gastrointestinal diseases such as the short bowel syndrome (**SP12**).

The aims of **PP4** included an examination of the capacity of chronic IGF-I administration to promote hyperplasia, and so contribute to the pool of knowledge on long-term IGF-I treatment and cancer risk. This study utilised an animal model of chronic colitis that had been modified from the acute colitis model described in **PP3**. The chronic colitis resulting from continuous oral consumption of dextran sulphate sodium (DSS) for 6 months induces features of colonic dysplasia and low-level neoplasia. Although 20 weeks IGF-I administration increased body weight by 19% and total gut weight by 43%, colonic crypt depth, proliferative compartment, labelling index, dysplasia, neoplasia and other indices of colitis were not altered. This study suggested that IGF-I does not actively influence the predisposition to colon cancer in this single animal model, providing preliminary safety information for other conditions in which long-term IGF-I treatment may be indicated.

This information, together with the results derived from **PP4** would tend to reinforce the suitability of short-term IGF-I therapy for acute bowel injury, although further information will be required to alleviate safety concerns for other conditions in which long-term IGF-I therapy is indicated.

7. Clinical applications of IGF-I

The previous sections have described a number of acute candidate conditions affecting the digestive tract for which IGF-I therapy could be beneficial. Moreover, we have gained some insight into the influences of IGF-I treatment on cancer risk in a disease condition (IBD) with pre-malignant potential. This section describes the current status of clinical testing of recombinant human IGF-I in the short gut condition, one of the candidate bowel disorders identified in Section 6.

7.1 IGF-I and the short bowel syndrome [SP12]

As described in Section 6, IGF-I has been under clinical, or pre-clinical, development for the potential treatment of a number of acute catabolic and chronic disease conditions including Laron dwarfism insulin-resistant diabetes mellitus, osteoporosis, peripheral neuropathy and the motoneuron disease, amyotrophic lateral sclerosis. More recently, the nitrogen-sparing capacity of IGF-I has been demonstrated clinically in acute settings such as following abdominal surgery (Jiang ZM et al., 2000) and in the critically-ill patient (Gelato MC et al., 2000). Taken together with he accumulated experimental data regarding the intestinotrophic properties of IGF-I, this information would strongly support a role for IGF-I in conditions in which bowel mass, length and function are

severely compromised. Bowel resection, resulting in development of the short bowel syndrome (SBS) represents such a condition.

The short bowel syndrome (SBS) may result from either a congenital deficiency in bowel mass or the necessity for surgical reduction of bowel mass as a consequence of bowel disease or other abdominal trauma. Congenital deficiencies in bowel mass and necrotizing enterocolitis (NEC) account for the highest proportion of SBS in newborn infants whilst inflammatory bowel disease and malignancy account for the highest incidence of SBS in juveniles and adults, respectively [Source : American Gastroenterological Association report on Burden of Gastrointestinal Disease, AGA, Bethesda, MD, USA, 2001].

In recent years, parenteral administration of the amino acid glutamine (the preferred substrate of enterocytes) (Wiren M et al., 1999), growth hormone (Ellegard L et al., 1997, Wilmore DW, 1999) or the combination thereof (Gu Y et al., 2001) have been incorporated into parenteral nutritional support formulations with proven effectiveness in the short bowel condition. In an attempt to enhance bowel rehabilitation, Wilmore DW, (1999) administered glutamine and growth hormone to more than 300 patients with the short bowel syndrome. Initially, 60% of the patients were weaned from TPN and an additional 30% had reduced TPN requirements. At long-term follow up (2 years) 40% of the group remained off TPN, 40% had reduced TPN requirements, and 20% had the same requirements. Similarly, Ellegard L et al., (1997) described an increase in body weight and lean body mass following treatment with recombinant human growth hormone in

patients with the short bowel syndrome. The demonstrated efficacy of growth hormone in these studies would therefore support a potential role for IGF-I as a mediator of some growth hormone actions in the treatment of short bowel syndrome.

Most studies on growth factor efficacy following bowel resection have been derived from studies with epidermal growth factor (Erwin CR et al., 1999; Chaet MS et al., 1994; Schwartz MZ et al., 2000). Indeed, Iannoli P et al., (1997) have reported accelerated adaptation in an additive, nutrient-dependent, and site-specific fashion after massive enterectomy, following treatment with epidermal growth factor and human growth hormone. Although more recent developments in improving adaptation after resection have focussed on the potential of glucagon-like peptide-2 (GLP-2) to increase mucosal mass, the remainder of this section will be restricted to the investigation of the therapeutic application of IGF-I for this disorder.

Section 4.2 detailed the effects of IGF-I treatment on linear and cross-sectional bowel growth in normal adult and neonatal animals, providing support for a likely beneficial effect in the treatment of short bowel syndrome. Furthermore, the significant increase in linear growth of the small intestine of neonatal rats following IGF-I administration would identify short bowel syndrome in the newborn as a likely target condition for IGF-I therapy. Indeed, in experimental studies in young adult rats, improved bowel adaptation and nitrogen excretion has been described following IGF-I administration after massive bowel resection (Lemmey AB et al., 1991; **SP12**; Vanderhoof JA et al., 1992; Lukish J et al., 1997). Moreover, in more recent studies in rats, therapeutic potential for IGF-I in

short gut has been derived from reports of its efficacy in combination with glutamine (Zhang W et al., 1995a; Ziegler TR, 1996). Indeed, Zhang et al., (1995a), utilising a recirculating perfusion method, demonstrated a 60% increase in glucose absorption following 10 days' administration of 2.4 mg/kg/day IGF-I to allotransplanted rats, accompanied by a 41% increase in mucosal mass.

The mechanism of improved bowel function following IGF-I treatment in experimental SBS is believed to be a primary consequence of increased mucosal mass, and hence absorptive surface area, as evidenced by effects on intestinal glucose absorption in settings of allotransplantation (Zhang W et al., 1995b) and normal development (*Long-R^3-IGF-I promotes intestinal absorption of 3-O-methyl-D-glucose in normal rats.* SM Garnaut, **GS Howarth** and LC Read. Submitted to Growth Factors [2001]).

In addition to defining therapeutic indications for IGF-I in short bowel syndrome, additional information on the mechanism of IGF-I action on bowel resection has been derived from studies with the des (1-3) analogue of IGF-I, which binds poorly to IGF binding proteins (Lemmey AB et al., 1991; **SP12**). These studies revealed that des-(1-3)-IGF-I does not exhibit any increase in intestinotrophic potency compared to native IGF-I, suggesting that IGFBP expression following resection may have been decreased. This has since been confirmed in a report by Albiston AL et al., (1992) which concluded that decreased ileal synthesis of IGFBP-3 enhances the ability of IGF-I to stimulate the adaptive response. These authors examined changes in the levels of ileal IGF-I and IGFBP-3 mRNA in the rat small intestine following small bowel resection utilising an S1

nuclease assay. IGF-I mRNA levels varied little over seven days following resection; whereas IGFBP-3 mRNA levels decreased to one-third after only seven hours, and remained suppressed for the length of this study. More recently, Gillingham MB et al., (2000) developed an experimental model of TPN-feeding following bowel resection in which differential adaptive responses in the colon and jejunum of IGF-I treated TPN-fed rats following ileal resection have been observed. The adaptive responses were evidenced by increases in jejunal mucosal mass, enterocyte proliferation, and migration rates following IGF-I treatment.

In summary, there is a substantial body of evidence that IGF-I, either alone, or in combination with glutamine, has the potential to improve nutrient uptake and utilisation following bowel resection by virtue of its intestinotrophic properties. This has now culminated in the recent assignment of Orphan Drug status to IGF-I by the United States Food and Drug Administration. Orphan Drug status for IGF-I has been designated for "the treatment of SBS as a result of resection of the small bowel or as a result of congenital dysfunction of the intestines" and was ascribed to GroPep Incorporated, a South Australian-based biotechnology company, on February 16th, 2000. At the time of preparation of this thesis, a multi-centre patient recruitment strategy is in progress for an impending clinical trial of IGF-I for the treatment of the short bowel syndrome.

7.2 Side-effects of IGF-I treatment

As described in Section 6.2, IGF-I has been investigated as a potential treatment modality in a broad range of disease states affecting humans, including Laron dwarfism, insulin-

resistant diabetes mellitus, osteoporosis, peripheral neuropathy and amyotrophic lateral sclerosis. However, during the course of clinical trials of IGF-I for these conditions, a number of adverse side-effects of IGF-I therapy have been described. A brief discussion of these adverse effects is therefore warranted.

Generalised oedema has been a well-documented side-effect of IGF-I therapy. For example, both GH and IGF-I have been shown to have dramatic effects on body composition of normal weight postmenopausal women even in the absence of dietary change (Thompson JL et al., 1995; Thompson JL et al., 1998). However, generalised oedema, necessitating anti-diuretic treatment with furosemide, was reported in many women in the study. A study by Usala AL et al., (1994) investigating the effects of insulin-like growth factor-I therapy on insulin sensitivity in severely resistant type I diabetes mellitus, however, revealed potentially more serious consequences of IGF-I therapy.

In the study by Usala AL et al., (1994), low doses (250 micrograms/kg-iv) of IGF-I were ineffective in acutely lowering serum glucose or inducing sustained insulin sensitivity. However, this IGF-I dose resulted in acute symptomatic hypophosphatemia, which could be prevented by co-administration of potassium phosphate. With sc administered IGF-I (up to 10 mg twice daily), insulin appeared to control patient glucose concentrations, but severe insulin resistance returned within 72 h of discontinuing IGF-I therapy. However, severe arthropathy and neurological symptoms including multiple cranial nerve palsies in one patient were associated with chronic therapy. These authors concluded that both iv

and sc administered IGF-I could precipitate acute symptomatic hypophosphatemia, whilst chronic low dose sc therapy was associated with severe neuropathy and arthropathy. Further evidence of a neuro-pathological effect of IGF-I therapy was revealed in the study by Thompson JL et al., 1995, which described the development of carpal-tunnel syndrome following IGF-I therapy for postmenopausal obesity.

More comprehensive studies of adverse effects following IGF-I therapy have been described in studies of growth hormone and IGF-I therapy in frail elderly patients (Sullivan DH et al., 1998) and in obese insulin-resistant type II diabetic patients (Jabri N et al., 1994) in which the range of adverse side-effects has been expanded to include oedema, primarily on the face and hands, mild weight gain, occasional dyspnea, bilateral jaw tenderness, arthralgias and myalgias, fatigue, tachycardia, flushing, orthostatic hypotension, and local burning at the injection site.

8. Treatment of gut disease with bioactive formulations

Although the previous sections have defined clear therapeutic potential for several growth factors in the treatment of a range of gastrointestinal disorders, these experimental studies have generally been restricted to factors administered by the systemic route. Clearly, however, an enteral mode of administration would be preferential for human applications, both for ease of administration and to maximise absorption across the gut. . In addition, there has been a recent focus on the development of natural, non-recombinant, sources of bioactive molecules in order to circumvent safety issues associated with administration of

recombinantly-produced molecules to humans. There are numerous sources of bioactive molecules, ranging from plant through to fungal extracts in addition to a variety of mammalian-sourced fluids. The current discussion, however, will focus on bioactive formulations derived from mammalian milk.

8.1 Milk and colostrum

Milk and colostrum contain a widely diverse milieu of bioactive factors including hormones, peptide growth factors, cytokines and immunoglobulins in addition to anti-microbial factors including lactoferrin and lactoperoxidase (Pakkamen R et al., 1997). Bovine-sourced milk and colostrum can be readily processed to provide formulations either enriched or depleted in specific components. Recently, the role of milk- and colostrum-derived peptide growth factors in the treatment of gastrointestinal disorders has been reviewed comprehensively (Playford RJ et al., 2000) and beneficial effects of bovine colostrum have been described in small bowel enteropathy (Playford RJ et al., 1999). Although the growth factor composition of milk and colostrum varies between species, these biological fluids contain an abundance of growth factors including epidermal growth factor family peptides, fibroblast growth factors, nerve growth factor, transforming growth factors, colony stimulating factors, and of particular relevance to this discussion, IGF-I. Moreover, the recent identification of betacellulin in bovine milk and colostrum (Bastian SE et al., 2001) expands the number of known intestinotrophic growth factors in bovine milk which possess therapeutic potential in bioactive formulations.

The baso-lateral localisation of many epithelial growth factor receptors (EGF and IGF-I for example) would tend to diminish the likelihood of growth factor bioactivity following the enteral route of administration. However, there is now evidence that enterally-administered IGF-I can exert beneficial effects on gut function in the immature gut associated with the pre-weaning period. For example, in neonatal piglets, small bowel disaccharidase activity and ileal villus height are increased by enteral formulae containing IGF-I (Houle VM et al., 1997), Ma L et al., (1997) have also described significantly increased aminopeptidase activity in the proximal small intestine of neonatal rats following three daily doses of recombinant human IGF-I. In addition, evidence is accumulating that accessibility to growth factor receptors could be enhanced by enteral administration of growth factor rich formulations containing alternative substrates able to minimise degradation by digestive enzymes. For example, Staley MD et al., (1998) have described enhanced intestinal growth in neonatal rats following *enteral* application of long-R^3-IGF-I. Alternatively, administration of bioactive formulations at stages of development associated with under-development of the mucosal barrier, such as in neonates could be effective in promoting growth and repair of the intestine at these ages. For example, Blattler U wt al., 2001 fed high amounts of first colostrum to neonatal calves resulting in enhanced survival of mature epithelial cells in the small intestine.

Although human milk is particularly rich in EGF, this peptide has not been detected in bovine milk, although high concentrations of IGF-I are present in the latter (Read LC, 1988). This suggests that bovine milk as a suitable and economical source of IGF-I for an enteral mode of administration of preparations to treat gut disease. On this basis, an

extract of growth factors enriched in IGF-I (and other growth factors) was developed from bovine cheese whey for potential therapeutic application (Francis GL et al., 1995; Belford DA et al., 1995; Smithers GW et al., 1996). This bioactive formulation has since been designated as WGFE (whey-derived growth factor extract).

8.2 Whey-derived growth factor extract (WGFE)

Milk growth factors were extracted by cation-exchange chromotography from bovine cheese whey obtained as a by-product of the cheese making process (Francis GL et al., 1995). The resultant growth factor extract (WGFE) contains IGF-I, IGF–II, IGF binding proteins, acidic and basic fibroblast growth factors, transforming growth factor-β, platelet-derived growth factor and more recently, betacellulin (Dunbar AJ et al., 1999). There is also a component of unknown growth factor activity in WGFE which has not yet been accounted for, on the basis of cell growth activity (Belford DA et al., 1997).

8.3 WGFE and gastrointestinal disease

Since many of the growth factors in WGFE have been shown to individually possess therapeutic activity in experimental settings of gastrointestinal disease (see previous sections), it was hypothesised that WGFE could have clinical utility in such conditions when administered by the enteral route. Accordingly, efficacy of orally-administered WGFE was initially evaluated in experimental settings of intestinal mucositis, oral mucositis and ulcerative colitis, in order to determine its potential for clinical application in disorders of the gastrointestinal tract.

8.3.1 WGFE and mucositis [**PP5**, **PP6**, SP16]

The initial identification of IGF-I as a promoter of intestinal re-growth following methotrexate-induced intestinal mucositis (**PP2**) resulted in investigations of the therapeutic potential of WGFE in a similar experimental setting. WGFE was therefore administered as an enteral dietary supplement to rats with chemotherapy-induced intestinal mucositis (**PP5**: *Milk growth factors enriched from cheese whey ameliorate intestinal disease by methotrexate when administered orally to rats.* **Howarth GS**, Francis GL, Cool JC, Xu X, Byard RB, Read LC. [1996]).

From a therapeutic perspective, at the time this study was commenced, there was no effective therapy for the treatment of chemotherapy-induced intestinal mucositis (described previously in Section 5.3). Various agents as diverse as vitamin A (Tsurui T et al., 1990), prostaglandin E_2 [PGE_2] (Bernard A et al., 1989), soy products (Funk MA et al., 1991), dietary lipids (Vanderhoof JA et al., 1990) dietary modification (Shou J et al., 1991) had been reported to reduce methotrexate-induced mucosal injury in rats. However, **PP5** represented the first investigation into the efficacy of a mixture of milk-derived growth factors on gut mucositis.

PP5 exploited the intestinal mucositis model developed in **PP2** in which rats were subcutaneously injected with the chemotherapeutic drug methotrexate on d 1, 2 and 3 to induce severe damage in the small bowel and bacterial translocation across the gut. WGFE was given orally for 5-12 days, starting on the first day of experimentation. WGFE at doses up to 514 mg/day for five days, increased villus surface length indices in

the jejunum and ileum by 52% and 56%, respectively compared with controls not receiving the whey extract. The crypt area index was 64% greater in the jejunum, but not significantly greater in the duodenum or ileum compared with controls not receiving whey extract. Similarly, sucrase activity was significantly higher in the ileum, whereas bacterial translocation (incidence and number of colonies) was significantly reduced compared with controls not receiving whey extract. Enteral administration of WGFE had therefore decreased the severity of methotrexate-induced intestinal damage, particularly in the ileum, suggesting clinical application for the treatment of intestinal mucositis. The results of **PP5** also shed an interesting perspective on the possible mechanism of WGFE action in the setting of chemotherapy-induced mucositis.

Transforming growth factor-β is a potent inhibitor of epithelial cell proliferation and is one of the major growth factors present in WGFE (Rogers ML et al., 1996). One could therefore speculate that the beneficial effects of WGFE in intestinal mucositis may have resulted from decreased susceptibility of the epithelium to methotrexate by virtue of the TGF-β component of WGFE, or alternatively, an accelerated rate of repair induced by IGF-I following withdrawal of the chemotherapeutic agent. Although each of these mechanisms was feasible, it was likely that the true mechanism was complex, given the likelihood of growth factor synergy and the contribution of other, as yet unidentified growth factor activities in WGFE to protection and repair of the epithelium. Independent studies in which WGFE was administered either prophylactically, or alternatively, during the repair phase would be desirable to test these hypotheses.

Although these factors in WGFE, responsible for the improved intestinal architecture described in **PP5** could not be identified, it was hypothesised that the reduced incidence of bacterial translocation following WGFE administration may have resulted from a WGFE-induced maintenance of mucosal barrier function. It was therefore hypothesised that WGFE treatment could reduce small bowel permeability, an indicator of mucosal barrier function, in chemotherapy-induced mucositis. The impact of WGFE on permeability of the intestine was assessed from the urinary appearance of orally-administered ^{51}Cr-EDTA (**SP16**: *Zinc in combination with a growth factor extract from bovine whey promotes recovery from methotrexate-induced small bowel damage in rats.* Tran CD, Butler RN, **Howarth GS**.[1999]).

Although a full discussion of zinc in relation to protection and repair of the bowel is beyond the scope of this thesis, zinc has a proven role in bowel homeostasis (reviewed in MacDonald RS, 2000). Indeed, a beneficial role of zinc on intestinal permeability has been demonstrated in vitro (Connell P et al., 1997). The availability of the rat mucositis model provided an opportunity to investigate potential additive or synergistic effects of WGFE and zinc on intestinal permeability in vivo.

Intestinal permeability is associated with both paracellular and transcellular passage of molecules of appropriate size. The urinary appearance of orally-administered, non-metabolisable sugar molecules (for example the sugars lactulose and rhamnose) is being employed increasingly as an indication of intestinal permeability (Bjarnason I et al., 1995). ^{51}Cr-EDTA is believed to permeate the small intestine primarily by the

paracellular route. Accordingly, its permeation represents an indicator of tight junction integrity. ^{51}Cr-EDTA permeability was improved by the combination of WGFE and zinc (1.8 ± 0.2%) compared to control (5.2 ± 0.9%) in the early phase of methotrexate-induced bowel damage, indicating a beneficial effect on enterocyte tight junctions and therefore, mucosal barrier function in this experimental setting. **SP16** thus provided some insight into the possible mechanism of WGFE action in chemotherapy-induced mucositis.

Mucositis confined to the oral cavity is a more readily accessible clinical presentation associated with chemotherapy or radiotherapy. In order to investigate the potential efficacy of WGFE in this setting we developed a hamster model of oral mucositis induced by the chemotherapeutic drug, 5-flurouracil (*Exposure of oral mucosa to bioactive milk factors reduces severity of chemotherapy-induced mucositis in the hamster.* Clarke JC, Butler RN, **Howarth GS**, Read LC, Regester GO. Oral Oncology [In Press, 2001]). This study investigated the potential protective effects of a modified form of WGFE known as WGFE-a. WGFE-a is an acid-activated form of WGFE in which TGF-β levels are 30-fold higher than in WGFE due to release from TGF-β binding proteins at low pH. Treatment of the hamster oral mucosa with WGFE-a resulted in a 21% reduction in the proliferative labelling index in the buccal epithelium, an effect consistent with the known inhibition of epithelial proliferation by TGF-β. This decrease in epithelial cell cycling rate presumably rendered the epithelium less susceptible to the effects of 5-FU, resulting in a significant decrease in the severity and duration of ulceration in the oral cavity.

The findings of the previously-described experimental studies of WGFE efficacy in settings of intestinal and oral mucositis culminated in the submission of a patent application. The patent (**PP6** : *Method of preventing or treating alimentary tract damage due to chemotherapy or radiation.* Inventors: Leanna C Read and Gordon S Howarth) was issued in Australia in 1996 and has since been granted in the United States and New Zealand. WGFE entered a Phase II clinical trial for the treatment of oral mucositis at the Peter MacCallum Cancer Institute in Melbourne in July 2001. The trial is expected to be complete within one year.

8.3.2 WGFE and IBD [SP17]

The beneficial effects of enterally-administered WGFE in chemotherapy-induced mucositis and the concomitant improvement in ileal architecture (**PP5**) suggested that sufficient bioactive material had reached the distal regions of the small bowel. Accordingly, it was hypothesised that sufficient WGFE could survive transit through the small intestine to protect the colon from experimentally-induced colitis. The effects of dietary WGFE on gut function and integrity were therefore investigated in an experimental model of inflammatory bowel disease in which epithelial barrier function was chronically compromised.

Inflammation of the colon results in epithelial damage through a mechanism involving lipid peroxidation. Ethane is produced as a product of this process and can be detected in the breath (Kneepkens CM et al., 1994). Utilising breath ethane as an indicator of colonic inflammation in the dextran sulphate sodium (DSS) model of chronic colitis, Porter SN et

al., (**SP17**: *An orally administered growth factor extract derived from bovine whey suppresses breath ethane in colitic rats*. Porter SN, **Howarth GS**, Butler RN. [1998]), demonstrated that dietary WGFE supplementation was as effective as conventional IBD therapies at limiting ethane production in the early phase of experimental chronic colitis. Ethane breath tests were conducted on days 2, 4, 6, 8, and 10 (acute phase) and weeks 3, 6, and 9 (chronic phase) after the commencement of DSS consumption. Although ethane production was not affected during the acute phase of colitis, breath ethane production was significantly increased in the chronic phase. WGFE and conventional therapy by aminosalicylates and corticosteroids were equally effective at suppressing ethane production by 30% ($p < 0.05$) in the chronic phase of colonic disease compared to colitic controls. This study represented the first experimental evidence that oral growth factor supplementation could influence chronic inflammation of the colon.

DISCUSSION and CONCLUSIONS

Gastrointestinal disease or disorder can manifest through defects at the molecular, cellular or tissue levels, resulting in compromised gut function through effects on physiological processes as diverse as peristaltic motility, mucosal barrier function, nutrient absorption and normal or dysregulated bowel growth. The studies contained within this thesis describe the applicability of peptide growth factors, and in particular, of IGF-I, to the modulation of these processes (Reviewed in **SP18**: *Enhancement of intestinal growth and repair by growth factors.* **Howarth GS** and Shoubridge CA. [2001]).

To date, there has been no demonstrable evidence that growth factors such as IGF-I directly influence peristaltic motion. This is supported by in situ studies from our own laboratory in which intestinal motor function was unaffected by IGF-I treatment in rats following abdominal irradiation (SP15). There is now clear evidence, however, including that from our further experimental studies, that IGF-I can impact positively on other parameters of gut function.

Specific receptors which bind IGF-I and initiate signalling events, are present throughout the gastrointestinal tract and their concentration along the baso-lateral enterocyte surface is consistent with access of IGFs to enterocytes by the systemic route. Indeed, the combined effects of luminal digestion and trans-epithelial passage have resulted in scant evidence that enterally-administered IGF-I can exert a significant biological effect through receptor-ligand interactions at this location. This is supported by studies in

which systemically-administered IGF-I improved bowel growth in adult and neonatal rats but enterally-delivered IGF-I did so only in neonates. These developmental differences presumably reflect differences in survival and access of enterally-delivered ligand to receptors between adults and neonates. In neonates, IGF-I may better survive in the lumen and be absorbed, presumably as a consequence of increased intestinal permeability and the limited appearance of brush border enzymes.

Growth of the small bowel is promoted by IGF-I, particularly when administered by the systemic route. Its capacity to stimulate linear and cross-sectional growth of both mucosal and muscular bowel components suggests IGF-I may be effective in treatment of conditions such as the short bowel syndrome. However, clinical application of IGF-I in bowel resection could be limited owing to the requirement for a systemic mode of administration, necessitating either daily injection or continuous infusion via subcutaneously implanted slow-release devices. Moreover, recent advances in intestinal transplantation and stem cell technology in which stem cell fate can be manipulated toward the development of specific tissue types, may provide an alternative therapeutic approach and further restrict the scope of IGF-I therapy. The development of GLP-2 as an intestinotrophic factor and the paucity of long-term safety data for IGF-I also need to be considered. Taken together, it would appear likely that clinical indications for IGF-I therapy would be limited to the acute period immediately following an episode of intestinal injury. For example, the increased sensitivity and responsiveness of the neonatal gut to IGF-I peptides would suggest therapeutic application following surgical intervention necessitated by an episode of acute necrotising enterocolitis.

.Breached or compromised mucosal barrier function can result from a variety of initiating agents or events. These include drug or pathogen-induced disruption of the epithelium, in addition to more severe conditions such as intestinal mucositis and inflammatory bowel disease. There has been a clear need to develop new agents capable of maintaining intestinal barrier function and accelerating epithelial regrowth following episodes of gastrointestinal injury. IGF-I has been demonstrated to improve bowel re-growth following injury and to restore barrier function as evidenced by an increased rate of re-epithelialisation and a reduced incidence of bacterial translocation. The accumulated evidence of IGF-I actions on barrier function supports the hypothesis that optimal therapeutic application of IGF-I would be as an inducer of epithelial regeneration in the acute period immediately following the development of an intestinal injury.

Diminished absorptive function of the bowel can occur in the absence of frank mucosal damage through reductions in absorptive surface area resulting from more subtle effects on brush border enzyme activity, or the induction of villous atrophy. There have been few studies of IGF-I and its effects on absorptive function. The data accumulated to date, however, including investigations into glucose uptake from our own laboratory, would suggest that IGF-I improves absorptive capacity through an increase in mucosal mass, and hence, absorptive surface area, rather than by an up-regulation of specific transporters or brush border enzymes. The IGF-I-induced increase in absorptive capacity may be responsible for other indicators of improved gut function, such as fat and nitrogen excretion, described in experimental studies of gut adaptation following bowel resection. Further studies of IGF-I and its effects on specific aspects of gut function are indicated

utilising recently-developed non-invasive in vivo breath tests. The principle of these tests relies on the metabolism of enterally-delivered, ^{13}C-labelled substrates and the subsequent appearance of $^{13}CO_2$ in the breath. These tests could be used to investigate the effects of IGF-I on parameters including gastric emptying rates, intestinal transit time, overall absorptive function and bacterial over-growth.

Receptors for IGF-I are present on many tumour cells, and although there is little evidence of an active promotion of tumour growth by IGF-I, there are still precautionary safety concerns for its chronic therapeutic use in neoplastic settings. Circulating IGF-I levels have been correlated with some neoplastic conditions such as breast carcinoma, and the incidence of colonic carcinoma is known to be increased in acromegalics over-expressing growth hormone, suggesting a possible role for IGF-I, albeit by association, in transformation. Further long-term studies of IGF-I administration in experimentally-induced colonic carcinoma, such as that induced by dimethyl hydrazine (DMH) will be required, before we are able to more clearly define the relative contribution of IGF-I therapy to cancer risk and address concerns for its use in conditions associated with dysplasia or neoplasia.

Analogues of growth factors such as KGF and GLP-2 have been demonstrated to be efficacious in experimental settings of gut disease. Future studies could address the capacity to further potentiate IGF-I bioavailability by developing protease-resistant IGF-I analogues or, alternatively, other agonists of the type 1 IGF-I receptor. Historically, EGF has been the growth factor best characterised with respect to its role in the gastrointestinal

tract. However, the discovery that intestinal expression of EGF is lower than that of its peptide family homologue, TGF-α indicates a broader role for EGF family peptides in epithelial homeostasis. This has been supported by preliminary indications of intestinotrophism following administration of betacellulin, a further member of the EGF peptide family.

In recent years, bioactive formulations have been prepared from milk and colostrum, and significant advances into the targeted delivery of enterally-administered agents have been achieved. Whey-derived growth factor extract (WGFE) represents an example of an orally-administered bioactive formulation which has demonstrated efficacy in experimental settings of acute ulcerative colitis, intestinal- and oral-mucositis. WGFE is currently undergoing Phase II clinical trial for oral mucositis. Growth factor formulations such as WGFE retain enteral bioactivity, presumably via the incorporation of alternative substrates for proteolytic enzymes. However, the development of acid- stable and/or protease-resistant growth factors, or more potent growth factor analogues is indicated, to optimise efficacy by the more desirable, enteral, mode of growth factor administration. Future strategies for the therapeutic implementation of bioactive formulations could involve the supplementation of growth factor-enriched preparations with analogue peptides exhibiting greater potency or stability.

The recent advent of microarray gene analysis and proteomic technologies could provide a valuable approach to target the development of enterally-delivered growth factor mixtures or formulations more specifically. This approach could occur at two levels.

Genomic and proteomic methodologies could be utilised to characterise the expression of growth factor ligands and receptors during the phases of gastrointestinal damage and repair. Secondly, utilising this information on ligand/receptor expression, under different conditions, candidate factors may be identified to direct the development of potentially more bioactive formulations specifically enriched in, or depleted of, indicated growth factor ligands or their receptors.

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APPENDIX A

PRINCIPAL PUBLICATIONS

PP1. Howarth GS, Fraser R, Frisby CL, Schirmer MB, Yeoh EK. Effects of insulin-like growth factor-I administration on radiation enteritis in rats. *Scand J Gastro* 1997, 32:1118-1124.

Hypothesis and Aims

Acute radiation-induced damage to the small bowel occurs frequently during abdominal radiotherapy. Since the small intestine is selectively responsive to the growth-promoting effects of insulin-like growth factor-I (IGF-I), we investigated the effects of IGF-I administration on mucosal recovery from radiation enteritis in the rat.

Outcome and Contribution to Scientific Understanding

Irradiated rats receiving IGF-I lost less body weight than vehicle-treated rats, whereas the wet weights of the stomach, small intestine, and colon were increased by 10%, 19%, and 21%, respectively, and crypt depth was increased in the duodenum, jejunum, and ileum. IGF-I administration after abdominal irradiation increased small-intestinal mass and improved indicators of mucosal integrity, suggesting acceleration of small-intestinal mucosal recovery from radiation injury. This represented the first study of exogenous IGF-I efficacy in a model of radiation enteritis. The outcome of this study also provided a sound rationale for the investigation of the effects of IGF-I administration in other experimental gastrointestinal conditions associated with mucosal insufficiency.

Personal Contribution

In consultation with Dr Leanna Read, I developed the hypothesis that IGF-I could potentially accelerate the repair of the small intestinal mucosa following acute injury and subsequently initiated the collaborative study with the Oncology and Medicine Departments of the Royal Adelaide Hospital. I was primarily responsible for the planning of experiments and preparation of all aspects of the final manuscript including data collation, tables, figures and micrographs.

Howarth, G.S., Fraserab, R., Frisbyab, C.L., Schirmerab, M.B. and Yeoh, E.K. (1997) Effects of Insulin-like Growth Factor-I Administration on Radiation Enteritis in Rats. *Scandinavian Journal of Gastroenterology, v. 32 (11), pp. 1118-1124, 1997*

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://dx.doi.org/10.3109/00365529709002990

PP2. Howarth GS, Cool JC, Bourne AJ, Ballard FJ, Read LC. Insulin-like growth factor-I (IGF-I) stimulates regrowth of the damaged intestine in rats when administered following, but not concurrent with, methotrexate. ***Growth Factors*** **1998, 15:279-292.**

Hypothesis and Aims

Methotrexate is a chemotherapeutic drug which inhibits folate metabolism, preferentially killing rapidly-dividing cells such as those lining the small bowel. We tested the ability of insulin-like growth factor-I (IGF-I) to reduce damage to the intestinal mucosa (mucositis) in rats injected with methotrexate. IGF-I was infused concurrent with methotrexate administration and compared to IGF-I administered following the withdrawal of methotrexate.

Outcome and Contribution to Scientific Understanding

IGF-I administered coincident with methotrexate failed to restore mucosal integrity to the damaged small intestine. IGF-I administered post methotrexate stimulated regrowth of the damaged intestine, particularly the ileum, with 22%, 32% and 29% increases in small intestinal weight, ileal villus height and ileal crypt depth respectively. This study concluded that IGF-I administered following methotrexate-induced intestinal damage primarily induced growth of the distal small intestine. The ineffectiveness of concurrently administered IGF-I may have represented an IGF-I induced recruitment of proliferating epithelial cells to the anti-proliferative effects of methotrexate. This represented the first study to describe promotion of intestinal repair following administration of chemotherapeutic agents, providing clear indications for clinical

application following acute intestinal injury, but contra-indications for its administration during periods of treatment with these agents.

Personal Contribution

Together with co-investigator, Dr Leanna Read, I extended the hypothesis developed in **PP1**, to hypothesise that IGF-I could accelerate repair of the small intestinal mucosa following chemotherapy-induced injury. Under the supervision of Dr Read, I was responsible for the planning of experiments and assisted in conducting the in vivo experimentation. I collated the results for the study and prepared the data for publication. I was involved in all aspects of the final manuscript including data collation, tables, figures, micrographs and critical interpretation of results.

Howarth, G.S., Cool, J.C., Bourne, A.J., Ballard, F.J. and Read, L.C. (1998) Insulin-like growth factor-I (IGF-I) Stimulates regrowth of the damaged intestine in rats, when administered following, but not concurrent with, methotrexate.
Growth Factors, v. 15 (4), pp. 279-292, 1998

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PP3. Howarth GS, Xian CJ, Read LC. Insulin-like growth factor-I partially attenuates colonic damage in rats with experimental colitis induced by oral dextran sulphate sodium. ***Scand J Gastro*** **1998, 33:180-190.**

Hypothesis and Aims

Administration of insulin-like growth factor-I (IGF-I) results in selective growth of the gastrointestinal tract. In order to investigate the therapeutic potential of IGF-I to induce re-growth of the large bowel (colon) in inflammatory bowel disease (IBD), we investigated the effects of IGF-I on the colonic damage induced by oral dextran sulphate sodium (DSS) in the rat.

Outcome and Contribution to Scientific Understanding

Compared with the colon of vehicle-treated rats consuming DSS, IGF-I increased the numbers of goblet cells by 76%, reduced the proportion of lamina propria cells expressing TGF-beta1, and reduced the thickness of submucosal and muscularis externa layers by 26% and 20%, respectively. This study concluded that the effects of IGF-I treatment on the colonic epithelium are mediated directly, whereas the reduced inflammation in the mucosa and submucosa may be mediated by a mechanism other than up-regulation of TGF-beta1-mediated immunosuppression. This represented the first report of the actions of exogenous IGF-I in experimental colitis, supporting its therapeutic potential and further describing a novel mechanism of IGF-I action on submucosal inflammation.

Personal Contribution

Together with chief investigator, Dr Leanna Read, I extended the hypotheses developed in **PP1** and **PP2** to determine whether IGF-I could promote re-epithelialisation of the colon in a setting of experimental colonic inflammation. Under the supervision of Dr Read, I was responsible for the planning of experiments and assisted in conducting the in vivo experimentation. I collated the results for the study and prepared the data for publication. I was involved in all aspects of the final manuscript including data collation, tables, figures, micrographs and critical interpretation of results.

Howarth, G.S., Xian, C.J. and Read, L.C. (1998) Insulin-like Growth Factor-I Partially Attenuates Colonic Damage in Rats with Experimental Colitis Induced by Oral Dextran Sulphate Sodium.
Scandinavian Journal of Gastroenterology, v. 33 (2), pp. 180-190, 1998

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PP4. Howarth GS, Xian CJ, Read LC. Predisposition to Colonic Dysplasia is Unaffected by Continuous Administration of Insulin-like Growth Factor-I for Twenty Weeks in a Rat Model of Chronic Inflammatory Bowel Disease. ***Growth Factors*** **2000, 18:119-133.**

Hypothesis and Aims

We investigated the cancer risk of chronic IGF-I administration in a rat model of inflammatory bowel disease which predisposes to the development of colonic dysplasia.

Outcome and Contribution to Scientific Understanding

Rats consumed 2% DSS for 4 weeks when pumps were implanted to deliver either vehicle or IGF-I for 15 or 20 weeks while rats continued to consume DSS. Compared to vehicle, 20 weeks IGF-I significantly increased body weight by 19% and total gut weight by 43%. Colonic crypt depth, proliferative compartment, labelling index, dysplasia, neoplasia and other indices of colitis were not significantly affected. This study concluded that twenty weeks administration of IGF-I to rats induced growth of the intestine but did not affect the severity of experimentally-induced colitis or the incidence or progression of colonic dysplasia. This study concluded that the partial amelioration of acute colitis by IGF-I treatment, described in **PP3**, was not extended to the chronic setting of colonic inflammation. This study further represented the first description of long-term IGF-I administration in a pre-neoplastic setting, providing valuable safety information on IGF-I and cancer risk applicable to other conditions in which chronic IGF-I administration is indicated.

Personal Contribution

Together with chief investigator, Dr Leanna Read, I developed the hypothesis that IGF-I would not actively promote neoplasia in a setting of chronic colonic inflammation. Under the supervision of Dr Read, I was responsible for the planning of experiments and assisted in conducting the in vivo experimentation. I collated the results for the study and prepared the data for publication. I was involved in all aspects of the final manuscript including data collation, tables, figures, micrographs and critical interpretation of results.

Howarth, G.S., Xian, C.J. and Read, L.C. (2000) Predisposition to Colonic Dysplasia is Unaffected by Continuous Administration of Insulin-like Growth Factor-1 for Twenty Weeks in a Rat Model of Chronic Inflammatory Bowel Disease.
Growth Factors, v. 18 (2), pp. 119-133, 2000

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

http://dx.doi.org/10.3109/08977190009003238

PP5. Howarth GS, Francis GL, Cool JC, Xu X, Byard RB, Read LC. Milk growth factors enriched from cheese whey ameliorate intestinal disease by methotrexate when administered orally to rats. *J Nutrition* 1996, 126:2519-2530.

Hypothesis and Aims

At the time that this study commenced, various agents as diverse as vitamin A, prostaglandin E_2, soy products and dietary lipids had been investigated for their potential to reduce methotrexate-induced mucosal injury. This study represented the first investigation into the efficacy of milk-derived growth factors on gut mucositis.

Outcome and Contribution to Scientific Understanding

Rats were injected with the chemotherapeutic drug methotrexate to induce severe damage to the small bowel. Whey extract was given orally for 5-12 days, starting on day 1. Indices of villus and crypt integrity were utilized to assess potential efficacy of the extract. Administration of the whey extract for 5 days increased the villus surface length indices in the jejunum and ileum by 52% and 56%, respectively ($P< 0.001$). The crypt area index was 64% greater ($P < 0.001$) in the jejunum, but not in the duodenum or ileum whilst sucrase activity was significantly higher in the ileum ($P < 0.001$) but not significantly elevated in the jejunum. Bacterial translocation was significantly reduced. This study concluded that oral whey growth factor extract reduced methotrexate-induced damage in the small bowel, suggesting clinical applications for the treatment of intestinal mucositis. This study represented the first report of the efficacy of enterally-delivered milk growth factors in reducing damage/accelerating repair in intestinal mucositis,

resulting in the successful granting of an Australian patent and providing the foundation for clinical studies for WGFE in oral mucositis.

Personal Contribution

Together with chief investigator, Dr Leanna Read, I developed the hypothesis that WGFE treatment would reduce susceptibility of the small intestine to chemotherapeutic agents. Under the supervision of Dr Read, I was responsible for the planning of experiments and assisted in conducting the in vivo experimentation. I collated the results for the study and prepared the data for publication. I was involved in all aspects of the final manuscript including data collation, tables, figures, micrographs and critical interpretation of results.

Howarth, G.S., Francis, G.L., Cool, J.C., Xu, X., Byard, R.B. and Read, L.C. (1996) Milk growth factors enriched from cheese whey ameliorate intestinal damage by methotrexate when administered orally to rats.
Journal of Nutrition, v. 126 (10), pp. 2519-2530, October 1996

NOTE: This publication is included in the print copy of the thesis held in the University of Adelaide Library.

PP6. Patent: Method of preventing or treating alimentary tract damage due to chemotherapy or radiation. Inventors: Leanna C Read and Gordon S Howarth. Australian Patent No. 689719. Duration: 20 years from May 2nd 1996.

Perhaps the most significant outcome of the studies of the efficacy of WGFE in the treatment of gastrointestinal disease was the granting of a patent for the use of WGFE (coded PV701) as a therapeutic formulation for the treatment of damage to the alimentary tract induced by radiotherapy or chemotherapy. The patent was issued in Australia to GroPep Inc. (Adelaide, South Australia) on May 2nd, 1996 and has since been granted in the United States and New Zealand. Patents are currently pending in Europe, Canada and Japan. WGFE entered a Phase II clinical trial for the treatment of oral mucositis in July 2001. The trial was commenced at the Peter MacCallum Cancer Institute in Melbourne and is expected to be complete within one year.

Personal Contribution

Although Dr Leanna Read was principally responsible for preparation of the actual patent application, I was involved in development of the underlying hypothesis (**PP5**) and was listed as co-inventor, accordingly.

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(54) Title: METHOD OF PREVENTING OR TREATING ALIMENTARY TRACT DAMAGE DUE TO CHEMOTHERAPY OR RADIATION

(57) Abstract

The present invention provides a method for preventing, ameliorating and/or treating damage to the lining of the alimentary tract resulting from chemotherapy and/or radiation, which method includes administering to a patient in need thereof an effective amount of a milk product extract. The present invention also provides a pharmaceutical or veterinary composition for preventing, ameliorating and/or treating damage to the lining of the alimentary tract resulting from chemotherapy and/or radiation, said composition including an effective amount of a milk product extract and a pharmaceutically or veterinarily acceptable diluent, carrier or excipient, therefor.

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METHOD OF PREVENTING OR TREATING ALIMENTARY TRACT DAMAGE DUE TO CHEMOTHERAPY OR RADIATION

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(57) Claim

1. A method for preventing damage to the lining of the alimentary tract resulting from chemotherapy and/or radiation, which method includes administering to a patient in need thereof an effective amount of a milk product extract.

2. A method according to claim 1 wherein the milk product extract is a cheese whey extract, a colostral whey extract, a skim milk extract or an acid (casein) whey extract.

14. A method for ameliorating and/or treating damage to the lining of the alimentary tract resulting from chemotherapy and/or radiation, which method includes administering to a patient in need thereof an effective amount of a milk product extract.

METHOD OF PREVENTING OR TREATING ALIMENTARY TRACT DAMAGE DUE TO CHEMOTHERAPY OR RADIATION

This invention relates to the prophylaxis, amelioration and/or treatment of damage to the lining of the alimentary tract resulting from chemotherapy and/or radiation.

Chemotherapy and/or radiotherapy are effective at destroying tumours because they target fast-growing tissues. The mechanism involves impairment of DNA synthesis or interference with metabolic processes required for rapidly dividing cells. While tumour cells are selectively targeted by anticancer treatments, the fast-growing tissues of the host are also susceptible, particularly the immune cells of the body and the lining of the alimentary tract. Epithelial cell division in the alimentary tract occurs in the crypt zone of the mucosa. The newly synthesised cells then acquire their functional properties (such as digestive enzymes) as they migrate towards the luminal surface and finally, they are extruded into the lumen of the alimentary tract. This entire process takes only several days so that the mucosal epithelium of the alimentary tract has one of the most rapid rates of cell division of any body tissue, and is therefore a major site of toxicity for anticancer regimens.

The linings of the mouth and oesophagus are particularly sensitive to chemotherapy and radiation. The oral ulcerations characteristic of mucositis (also referred to as 'stomatitis') are a major clinical problem causing considerable pain, increased susceptibility to infection and inability to eat. Damage to the intestinal lining also occurs commonly in the small bowel, and less frequently in the large bowel, leading to severe diarrhoea and pain. (Verdi CJ 1993 Cancer therapy and oral mucositis. An appraisal of drug prophylaxis. Drug Safety 9:185-195; Sonis ST 1993 Oral complications of cancer chemotherapy In VT DeVita Jr, S Hellman and SA Rosenberg (ed) Cancer, Principles and Practice of Oncology, pp 2385-2391. Philadelphia, JB Lippencott Co).

Mucositis occurs by two distinct mechanisms: by direct damage to the alimentary lining by anticancer drugs or radiation, and indirectly as a result of opportunistic infections associated with neutropenia in patients with a compromised immune system. As a result, any drug that causes significant

neutropenia can precipitate indirect mucositis (Verdi CJ 1993). Direct damage to the gut barrier would also increase susceptibility to opportunistic infections by allowing bacterial translocation across a damaged gut lining.

In general, mucositis is manifest within 5 to 10 days of the drug or radiation treatment and can last several weeks. The severity of mucositis can vary from mild to so severe that it limits the dose of chemotherapy or radiation. For patients undergoing high-dose chemo/radiation therapy, mucositis is the chief source of infection and the resultant sepsis, the main cause of morbidity and mortality, and the primary reason for their hospitalisation. Patients suffering mucositis may need several weeks or more of intravenous feeding as a result of the mouth ulcers, cramps, extreme pain, gut denuding, and severe diarrhoea (Verdi 1993; Sonis 1993)

Mucositis can delay retreatment of patients with chemotherapy or radiotherapy or necessitate a subsequent dose reduction, thereby compromising the overall efficacy of anticancer treatment. With some anticancer regimens, mucositis is the limiting toxicity. Overcoming this toxicity would improve quality of life, reduce susceptibility to secondary infection, obviate the need for intravenous feeding, and importantly, improve the efficacy of tumour ablation through increased tolerance to higher doses of chemotherapy or radiation (Verdi CJ 1993). Costs of hospitalisation would be substantially reduced as more patients could be managed as out-patients.

About 40% of all patients receiving chemotherapy develop significant mucositis, with up to 100% incidence in some forms of chemotherapy or radiotherapy. Clinically significant mucositis develops with a range of standard chemotherapy drugs that are used, either alone or in combination, to treat various cancers including those of the colon, breast, prostate, head, neck and haemopoetic system. Examples of drugs that frequently cause direct mucositis include, but are not limited to, alkylating agents such as mechlorethamine, melphalan and busulphan, antimetabolites including cytarabine, floxuridine, 5-fluorouracil, mercaptopurine, methotrexate and thioguanine, cytotoxic drugs such as bleomycin, actinomycin-D, daunorubicin, etoposide, mitomycin, vinblastine and vincristine, and other chemotherapy drugs such as hyroxyurea and procarbazine

(Sonis 1993). Direct exposure of the alimentary tract to high-dose radiotherapy, as occurs for example with total body irradiation, treatment of head and neck tumours or radiotherapy of abdominal tumours, will also cause a high incidence of mucositis.

Mucositis is particularly severe with high-dose chemotherapy or when two or more drugs are used in the one course of treatment, for example the ablative therapy prior to bone marrow transplant or peripheral stem cell transplant. The combination of high-dose chemotherapy with aggressive radiotherapy can also cause severe mucositis (Sonis 1993).

The prior art suffers from the lack of an effective drug to prevent, reduce or treat damage to the lining of the alimentary tract resulting from chemotherapy and/or radiation. At present, patient treatment is mainly palliative to control pain through analgesics, prevent infection and provide intravenous nutritional support.

The prior art includes a number of approaches aimed at reducing the severity of mucositis. Low energy laser treatment of the mouth has been reported to reduce the severity of oral mucositis in patients given high dose chemotherapy prior to bone marrow transplantation (Ninth Annual Meeting of the International Soc. Oral Oncology, June 1994, NIH, Bethesda, USA). Numerous drugs have been evaluated in the prevention of mucositis, with some degree of efficacy for cytoprotectants (e.g. sucralfate) and antimicrobial drugs such as chlorhexidine and benzydamine (reviewed in Verdi CJ 1993). A somatostatin analogue (octreotide acetate) has been shown to inhibit secretory diarrhoea in patients with mucositis induced by the chemotherapy drug, 5-fluorouracil. The mechanism of action is probably secondary to inhibition of pancreatic and gastrointestinal function (Petrelli NJ, Rodriguez-Bigas M, Rustum Y, Herrara L, Creaven P 1993 Bowel rest, intravenous hydration and continuous high-dose infusion of octreotide acetate for the treatment of chemotherapy-induced diarrhoea in patients with colorectal carcinoma. Cancer. 72:1543-1546).

Recombinant transforming growth factor-beta 3 (TGF-b 3) has been shown to reduce the severity of oral mucositis induced by injection of hamsters with 5-fluorouracil (Sonis ST, Lindquist L, Van Vugt A, Stewart AA, Stam K, Qu G-Y, Iwata KK, Haley JD 1994 Prevention of chemotherapy-induced ulcerative

mucositis by transforming growth factor-b3. Cancer Res. 54:1135-1138). The effects of other growth factors are less clear. For example, recombinant epidermal growth factor (EGF) does not appear to relieve oral mucositis (Sonis ST, Costa JW, Evitts SM, Linquist LE, Nicolson M 1992 Effect of epidermal growth factor on ulcerative mucositis in hamsters that receive cancer chemotherapy. Oral Surg Oral Med Oral Pathol. 74:749-755), but may enhance intestinal recovery following abdominal radiation (McKenna KJ, Ligato S, Kauffman GL, Abt AB, Stryker JA, Conter RL 1994 Epidermal growth factor enhances intestinal mitotic activity and DNA content after acute abdominal radiation. Surgery. 115:626-632).

The prior art also includes International Patent Application PCT/SE93/00503) to Kabi Pharmacia. This application discloses the use of insulin-like growth factor-II (IGF-II) or effective analogues thereof for the manufacture of a medicament for prevention or treatment of nutritional or gastrointestinal diseases and for promoting human or animal neonatal growth. However, utility in the prophylaxis, amelioration and/or treatment of damage to the lining of the alimentary tract resulting form chemotherapy and/or radiation is neither disclosed or suggested.

The prior art also includes WO92/00994 A1 to GroPep Pty Ltd. Th. application discloses the use of a milk product extract for the treatment of gastrointestinal injury, disease or ulcers. However, utility in the prevention or treatment of damage to the alimentary tract resulting from chemotherapy or radiotherapy is neither disclosed nor suggested in this citation. Prior art in the field of mucositis, the most common injury from chemotherapy and/or radiotherapy predicts that it would be beneficial to reduce rather than increase the growth rate of the lining of the alimentary tract, and thereby render the cells less susceptible to chemotherapy or radiotherapy that targets rapidly growing tissues. This is the opposite effect to that which is predicted to occur with agents that heal or promote the regrowth of gastrointestinal tissues such as the plurality cell growth stimulating agents described in WO92/00994 A1. Accordingly the approach taken by the applicants in treating mucositis is contrary to the normal approach.

WO92/00994 A1 does not teach or disclose the use of a milk product extract for the amelioration or treatment of mucositis because the mechanism of alimentary tract damage in mucositis (inhibition of cell proliferation and neutropenia) is different from that which occurs in other alimentary tract conditions. For example, gastric ulcers, the treatment of which by milk products is described in the prior art, are not caused by an inhibition of cell proliferation nor are they accompanied by neutropenia. Rather, they result from acid-induced destruction of the lining of the stomach.

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Mucositis of the mouth and esophagus is outside the region of the alimentary tract also claimed in WO92/00994A1, which only refers to the gastrointestinal tract (stomach and intestines). Since the structure of the epithelial lining of the mouth and esophagus is different from that in the stomach and intestines, an agent that reduces damage to the lining of the gastrointestinal tract cannot be predicted to have the same action in the mouth and esophagus.

It is an object of the present invention to overcome or at least alleviate one or more of the difficulties or deficiencies related to the prior art.

In a first aspect, the present invention provides a method for preventing, ameliorating and/or treating damage to the lining of the alimentary tract resulting from chemotherapy and/or radiation, which method includes administering to a patient in need thereof an effective amount of a milk product extract.

In the second aspect, the present invention provides a pharmaceutical or veterinary composition for preventing, ameliorating and/or treating damage to the lining of the alimentary tract resulting from chemotherapy and/or radiation, said composition including an effective amount of a milk product extract and a pharmaceutically or veterinary acceptable diluent, carrier or excipient, therefor.

The present invention may be useful for research purposes including administration of milk product extract to animals with experimental damage to the lining of the alimentary tract. For example, the invention may be used in

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hamsters with 5-fluorouracil-induced oral or large bowel mucositis, or rodents with intestinal mucositis induced by radiation or chemotherapy drugs such as cytarabine or etoposide. The present invention may also be useful, for example, in cell culture to protect or treat epithelial cells cultured from the oral, oesophageal or gastrointestinal lining from chemotherapy or radiation-induced damage.

Accordingly in a third aspect, the present invention provides a method for preventing, ameliorating and/or treating damage to epithelial cells cultured from the lining of the alimentary tract resulting from treatment of said cells with a chemotherapeutic agent and/or radiation, which method includes culturing said cells in the presence of a milk product extract.

By "damage" is meant any alteration in normal structure or function. Such damage includes mucositis, at least partial loss of mucosal crypt area and/or mucosal villus length, or an increase in bacterial translocation across the alimentary tract.

The term "alimentary tract" as used herein refers to the digestive passage in any animal from mouth to anus and includes mouth, oesophagus and gastrointestines (including stomach, small and large bowel). In a preferred aspect, the present invention is particularly applicable to the mouth and/or oesophagus.

By "lining" is meant any biological material which covers a surface or lines a cavity or the like and which performs protective, screening and/or other functions. The lining of the alimentary tract includes the oral, oesophageal and gastrointestinal epithelia.

By "an effective amount" is meant a quantity of milk product extract which will upon single or multiple dose administration to the patient be effective in the prophylaxis, amelioration and/or treatment of damage to the lining of the alimentary tract resulting from chemotherapy and/or radiation.

By "preventing, ameliorating and/or treating" is meant

(a) in the case where the milk product extract is administered before the damage occurs, a reduction or elimination of subsequent damage compared with the damage which would have occurred if the milk product extract was not administered; and

(b) in the case where the milk product extract is administered after the damage has occurred, a reduction or elimination of such damage.

By a "pharmaceutically or veterinarily acceptable diluent, carrier or excipient" is meant a diluent carrier or excipient which is compatible with the other ingredients of the composition and not injurious to the patient.

The term "milk product" as used herein refers to a derivative from human or animal milk in which the proportions of fat and/or protein constituents thereof are altered. Examples of milk products include milk whey, skim milk, colostral whey, cheese whey and acid (casein) whey. In a preferred aspect, the milk product may be from an ungulate mammal.

The term "milk product extract" as used herein refers to an extract from human or animal milk product in which the proportions of salt, fat and/or main protein constituents thereof are altered. The milk product extract may be a cheese whey extract, a colostral whey extract, a skim milk extract or an acid (casein) whey extract. Examples of milk product extracts include ultrafiltrates of milk products or milk products that have been subjected to adsorption and to elution from chromatography matrices. Preferably the milk product extract is prepared by subjecting a milk product to cation exchange chromatography, for example by the method described in Australian Patent 645,589.

Preferably the milk product extract is a milk product extract composition including a plurality of cell growth stimulating factors, extracted from milk product, in concentrated form; said factors having basic to approximately neutral isoelectric points. More preferably the milk product extract is a milk product extract composition including a mixture of cell growth factors having basic to about neutral isoelectric points (eg. isoelectric points between approximately 6.0 and approximately 10.5), wherein the mixture of cell growth factors is obtained from a milk product of an ungulate mammal, and wherein the milk product is subjected to a cation exchange matrix under conditions whereby casein, α lactalbumin, and β lactoglobulin present in the milk product are not adsorbed to the matrix, after which the adsorbed growth factor mixture is eluted with a substantially aqueous salt solution and then optionally concentrated.

Preferably the milk product extract composition is a cheese whey extract composition.

The cheese whey extract composition may be formed from cheese whey wherein the proportions of the main protein constituents thereof are altered.

The milk product extract may include less than approximately 1% w/w casein, α lactalbumin or ß lactoglobulin, based on the total weight of the extract.

More preferably the milk product extract is a cheese whey extract prepared by the method described in Australian Patent 645589, the entire disclosure of which is incorporated herein by reference. This includes GFE and GFE-2 as described in Australian Patent 645589.

The milk product extract may include lactoperoxidase and/or lactoferrin. Preferably the milk product extract including lactoperoxidase and/or lactoferrin is prepared by adsorption of a milk product to and elution from one or more chromatography matrices, for example a cation exchange matrix. Those familiar with the art will recognise that lactoperoxidase and lactoferrin are major protein components in GFE and lactoperoxidase is a major protein component in GFE-2 as described in Australian Patent 645,589.

The milk product extract may be modified to enhance activity, including but not limited to transient acidification and/or purification under acidic conditions, for example using molecular sieve chromatography or controlled pore ultrafiltration, as described in International Patent Application No. PCT/AU95/00237, the entire disclosure of which is incorporated herein by reference.

Accordingly, in an alternative preferred form the milk product extract is a milk product extract composition including a plurality of modified milk growth factors having isoelectric points above approximately 6.0 and molecular weights in the range of approximately 5000 to 30,000, the milk growth factors being modified by transient acidification.

Alternatively or in addition, the milk product extract may be modified to enhance activity by the addition of one or more growth factors including but not limited to IGF-I, IGF-II, TGFβ, EGF, transforming growth factor α (TGFα), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) and keratinocyte growth factor (KGF).

The present invention may be applied in relation to any type of chemotherapy or radiation treatment that causes damage to the lining of the alimentary tract. Examples include, but are not limited to, alkylating agents such as mechlorethamine, melphalan and busulphan, antimetabolites including cytarabine, floxuridine, 5-fluorouracil, mercaptopurine, methotrexate and thioguanine, cytotoxic drugs such as bleomycin, actinomycin-D, daunorubicin, etoposide, mitomycin, vinblastine and vincristine, and other drugs such as hyroxyurea and procarbazine, as well as tissue-specific or total body irradiation. Any combination of these drugs and radiation regimens may be applicable to the present invention.

The milk product extract may be administered by any suitable route, including the oral, enteral or systemic route. Preferably, the milk product extract is administered directly into the alimentary canal by oral delivery or other means of direct enteral administration, in order to maximise the effective dose reaching the affected tissue.

Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, sachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; as a mouthwash or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

Compositions suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The compositions may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

It should be understood that in addition to the ingredients particularly mentioned above, the compositions of this invention may include other agents conventional in the art having regard to the type of composition in question, for example, those suitable for oral administration may include such further agents as sweeteners, thickeners and flavouring agents.

The milk product extract may be administered at any appropriate time including prior to, during or after chemotherapy or radiation.

The milk product extract may be useful in combination with known chemotherapeutic agents. If formulated as a fixed dose, such combination products employ the milk product extract in an appropriate dosage range and the other pharmaceutically active agent within its approved dosage range. Compositions of the invention, may be used sequentially with known chemotherapeutic agents when a combination formulation is inappropriate.

When the milk product extract is administered to a human subject the daily dosage can be determined by the attending physician with the dosage generally varying according to the age, weight, and response of the individual patient, as well as the severity of the patient's symptoms. In general a suitable dose of the compound of the invention will be in the range of 10mg to 10g per kilogram body weight of the recipient per day, preferably in the range of 100mg to 1g per kilogram body weight per day. However, the dose will also depend on the formulation and purity of the milk product extract used. The abovementioned doses are calculated on GFE or GFE-2 and could be modified accordingly by a person skilled in the art if a product of different activity or purity was used.

The present invention will now be more fully described with respect to the following examples. It should be understood, however, that the description following is illustrative only, and should not be taken in any way as a restriction of the generality of the invention described above.

In the Figures

Figure 1. Oral administration of a milk product extract (GFE-2) for 5 days to methotrexate-injected rats reduces (a) the loss of mucosal crypt area in the jejunum and ileum, and (b) loss of mucosal villus length in the jejunum and ileum.

Figure 2. Oral administration of a milk product extract (GFE-2) for 5 days to methotrexate-injected rats increases the sucrase activity of the mucosa in the ileum.

Figure 3. Oral administration of a milk product extract (GFE-2) for up to 12 days to methotrexate injected rats reduces (a) the incidence of rats showing bacterial translocation, and (b) the number of bacterial colonies per gram of intestinal lymph node.

Figure 4. Administration of milk product extracts (GFE-2, transiently acidified GFE-2 and permeate GFE-2) concurrently with methotrexate to cultured intestinal cells (IEC-6) in culture for 24 hours enhances the survival of the cells in a dose dependent manner.

Figure 5. Daily treatment of the cheek pouch in hamsters with a milk product extract (GFE-2) reduces the severity of oral mucositis ulcers caused by 5-fluorouracil.

Figure 6. Daily treatment of the cheek pouch in hamsters with a milk product extract (GFE-2) reduces the body weight loss induced by 5-fluorouracil.

Introduction to Examples

Surprisingly, a milk product extract from cheese whey, equivalent to GFE-2 in Australian Patent 645589 has been found by the applicants to reduce the severity of intestinal mucositis caused by injection of rats with the chemotherapy agent, methotrexate.

This same milk product extract has been found by the applicants to reduce the severity of oral mucositis in the hamster cheek pouch caused by injection in the hamsters with the chemotherapy agent, 5-fluorouracil.

In accordance with the above, treatment of chemotherapy or radiotherapy patients with GFE-2 would at least partially alleviate the symptoms of gut mucositis including mucosal damage, functional impairment, and susceptibility to infection, as well as at least partially alleviating the symptoms of oral and oesophagael mucositis, thereby facilitating recovery and potentially increasing tolerance to higher doses of chemotherapy drug or radiation.

Example 1

Oral administration of a milk product extract from bovine cheese whey (GFE-2) partially prevents loss of small intestinal crypts and villi in rats with methotrexate-induced small bowel mucositis

In this Example, rats were injected with high doses of the chemotherapy agent, methotrexate, as an experimental model of gut mucositis. In rats, methotrexate damages the small bowel, but not the oral or colonic mucosa (Vanderhoof JA, Park JHY, Mohammadpour H, Blackwood D 1990 Effects of dietary lipids on recovery from mucosal injury. Gastroenterology. 98:1226-1231.) Oral administration to methotrexate-injected rats of a milk product extract purified from bovine cheese whey (GFE-2 as described in Australian Patent 645,589) provided evidence that the milk product extract can ameliorate chemotherapy damage to the small bowel.

Male Sprague Dawley rats, weighing on average 140 g and maintained in metabolism cages were fed a high-carbohydrate diet. Control rats received no milk product extract, whereas experimental rats were treated for 5 days with a milk product extract purified from bovine cheese whey. The whey-derived milk product extract (Whey Growth Factor Extract) was prepared as described for GFE-2 in Australian Patent 645,589. GFE-2 treated rats were fed a modified diet containing 31.2 g GFE-2/kg diet in place of the equivalent amount of casein. In addition, the GFE-2 fed rats were given GFE-2 by stomach gavage on days 3, 4 and 5 of the experimental period so that the total dose of GFE-2 per day averaged 514 mg/day GFE-2. Control rats were fed the unmodified diet and gavaged by an identical protocol on days 3, 4 and 5 with an equivalent amount of bovine serum albumin to ensure an isonitrogenous diet.

One group of control rats and the GFE-2 treated rats (8 rats per group) were injected subcutaneously with 2.5 mg/kg methotrexate at the start of days 1, 2 and 3 according to the protocol described by Vanderhoof et al (1990), the entire disclosure of which is incorporated herein by reference. An additional control group ('pair-fed') received sham methotrexate injections, and was pair-fed to the methotrexate-injected control group.

Rats were maintained in the metabolism cages for 5 days, at which time they were killed for collection of the gastrointestinal tract. Tissue samples were collected from the proximal small bowel (duodenum and jejunum) as well as the distal small bowel (ileum). Tissue samples were fixed in methacarn, embedded in paraffin, sectioned and stained with haematoxylin-eosin for histological analysis using methods described in Read et al (1992), the entire disclosure of which is incorporated herein by reference (Read LC, Tomas FM, Howarth GS, Martin AA, Edson KJ, Gillespie CM, Owens PC, Ballard FJ 1992 Insulin-like growth factor-I and its N-terminal modified analogues induce marked gut growth in dexamethasone-treated rats. J Endocrinol. 133:421-431).

Compared with the pair-fed controls, the methotrexate-injected control group showed loss of mucosal crypts in the jejunum, and to a lesser extent in the ileum. This is illustrated in Figure 1 (a) as the area of intact crypts per unit area of total mucosa, and demonstrates that methotrexate causes loss of mucosal crypts (which contain the dividing cells of the epithelium) characteristic of chemotherapy damage. Also characteristic of chemotherapy damage in the small bowel, methotrexate injection caused stunting and loss of intestinal villi, being the functional compartment of the small bowel mucosa. This is illustrated in Figure 1(b) by a reduction in the surface length of the finger-like villi per unit length of intestinal circumference in methotrexate-treated controls compared with the pair-fed group receiving no methotrexate.

Oral administration of GFE-2 for 5 days starting at the time of the first methotrexate injection partially prevented the loss of mucosal crypts and villi in both regions of the small bowel (Figure 1). The effects of GFE-2 were statistically significant ($P < 0.05$ by ANOVA) in the jejunum, where methotrexate-induced damage was more severe, and in the ileum for villus surface length. The example demonstrates that oral administration of GFE-2 is able to partially prevent or accelerate repair of chemotherapy damage in the small bowel.

Example 2

Sucrase activity in the mucosa of rats from Example 1

From the same experiment as described in Example 1, 4 cm lengths of small bowel were frozen for measurement of the activity of mucosal sucrase, an enzyme located on the surface of epithelial cells of the villus. Because sucrase is essential for digestion of dietary sucrose, the sucrase activity per unit length of intestine provides a measure of the functional capacity of the small bowel (Read et al, 1992).

Five days' oral administration of GFE-2 to methotrexate-injected rats significantly improved ($P < 0.05$) the sucrase activity per unit length of ileum compared with the methotrexate-injected control group, or the pair-fed control group (Figure 2).

This example demonstrates that GFE-2 improves the functional capacity of the chemotherapy-damaged small bowel.

Example 3

Oral administration of GFE-2 to rats for 5 to 12 days reduces bacterial translocation across the gut.

The ability of the gut epithelium to provide a barrier against bacterial invasion provides another measure of gut function that is compromised by gut mucositis.

In Example 3, 140 g male Sprague Dawley rats were injected with methotrexate for three consecutive days as described in Example 1. Methotrexate-injected rats were administered oral GFE-2 by an identical protocol to that described in Example 1. One group of rats was killed on day 5 after the start of methotrexate injections (as in Example 1), while in other groups, GFE-2 treatment was continued for a total of 8 or 12 days (8 rats per group). Control methotrexate-treated rats and pair-fed control rats identical to those in Example 1 were killed on days 5, 8 and 12 (8 rats per group).

Rats were maintained in metabolism cages as in Example 1 until exsanguination on day 5, 8 or 12. The abdominal skin was soaked in 70% ethanol before the intestine was removed under aseptic conditions. All visible mesenteric lymph nodes were placed into a sterile pre-weighed container.

Samples were then weighed and brain heart infusion solution was added to a final concentration of 100 mg/ml. Tissues were homogenised in this solution with sterile glass-reinforced grinders. For measurement of translocation of gram negative bacteria into mesenteric lymph nodes, 40 or 60 mg of each tissue homogenate was placed onto MacConkey agar II or blood agar plates and incubated aerobically at 35°C for 48 hours. Enteric gram negative bacterial colonies were identified using API 20E strips, then counted. The incidence (proportion of animals exhibiting detectable bacterial translocation) and mean number of bacterial colonies per gram of tissue were calculated for each treatment group.

Pair-fed control animals receiving no methotrexate showed no incidence of bacterial translocation across the gut. Methotrexate injection impaired the intestinal barrier so that all rats in the methotrexate-injected control group (Figure 3: 'No GFE-2') had positive bacterial cultures from mesenteric lymph nodes on day 5. The incidence in this group diminished over the next 7 days, but remained at 60% of rats on day 12 (Figure 3a). The number of colonies per gram of mesenteric lymph node was maximal on day 5, and then diminished thereafter in parallel with the incidence (Figure 3b).

Oral administration of GFE-2 resulted in a lower incidence of translocation on days 8 and 12, with the difference between GFE-2 treated and control methotrexate-injected rats reaching statistical significance by χ^2 test ($P < 0.05$) on day 12. The number of colonies per gram of mesenteric lymph node was also significantly lower in the GFE-2 treated group on both day 5 and 8.

The example demonstrates that oral administration of the milk product extract partially prevents chemotherapy-induced loss of barrier function in the gut. This could be expected to decrease the incidence of infection and sepsis following chemotherapy.

Example 4

Milk product extracts protect intestinal cells in culture against damage by the chemotherapy agent, methotrexate.

The milk product extracts evaluated in this Example were GFE-2 prepared as described in Australian Patent 645,589, transiently acidified GFE-2 prepared as in Example 2 of International Patent Application PCT/AU95/00237 and the permeate fraction obtained by controlled pore ultrafiltration under acid conditions of GFE-2 as described in Example 5 of International Patent Application PCT/AU95/00237.

Intestinal epithelian cells (IEC-6) were plated on to plastic 96-well plates at a density of 2.5 x 10^4 cells/ml in Dulbecco-Modified Eagle's Minimal Essential Medium (DMEM) containing 10% fetal bovine serum. The plates were incubated in a humidified atmosphere at 37°C in the presence of 5% CO_2 for 1 day after which the medium was replaced and the incubation continued for a second day.

On the third day the medium in each well was replaced by 100µl of a methotrexate solution (10^{-6}M in DMEM plus 10% fetal bovine serum) plus 100µl of a milk product extract solution containing either GFE-2, transiently acidified GFE-2 or permeate GFE-2 at various dilutions in DMEM plus 10% fetal bovine serum.

The cells were left in contact with these solutions for one day. The wells were then washed twice with DMEM and incubated for a further day in DMEM containing 10% fetal bovine serum.

After incubation of the cells in this fresh medium for one day the cells were washed, fixed and the cell numbers quantified using an automated methylene blue method (MH Oliver *et al.*, J. Cell Sci. 92, 513, 1989, the entire disclosure of which is incorporated herein by reference). Growth is expressed as the percentage survival of cells relative to cells not exposed to methotrexate. The results are illustrated in Figure 4.

The experiment demonstrates dose-dependent increases in survival of the intestinal cells with all three examples of milk product extract.

Example 5

Continuous topical application of GFE-2 to the hamster cheek pouch reduces the severity of 5-fluorouracil (5-FU)-induced chemotherapy-induced mucositis

This experiment investigated the effects of GFE-2 administered topically on chemotherapy-induced oral mucositis in male Golden Syrian hamsters. The trial included continuous treatment of GFE-2 to the cheek pouch of 10 hamsters treated with 5-fluorouracil.

Hamsters were divided into two groups of five animals. The initial mean body weight of each group was similar. All hamsters were given intraperitoneal injections of 90mg/kg of 5-FU on day 1, and 60mg/kg on day 3. The cheek pouch was scratched on days 1, 2 and 3 with six strokes of a wire brush in one direction and six strokes in the other perpendicular direction to achieve a uniform wound.

Groups were treated with either a commercial mouthwash as vehicle, or 0.3ml of GFE-2 at 40mg/ml protein concentration. The cheek pouch liquid treatments were applied daily for one minute, during which time the hamsters were anaesthetised using isoflurane anaesthesia.

The cheek pouch was assessed on days 5, 7, 8, 11, 13 and 15. Monitoring was based on a visual assessment of the cheek pouch (graded on a 1-10 scale) taking into account the overall severity of the lesion, degree of bruising, swelling and scarring. Body weight was recorded as a percentage of the day 0 value.

Animals given a topical treatment of GFE-2 showed reduced mucositis compared to the vehicle treated group, measured as overall visual score (Figure 5), total ulcer area and body weight loss (Figure 6). Each of these effects was statistically significant by paired t-test favouring GFE-2 treatment.

This example suggested that topical administration of GFE-2 may reduce the severity of oral mucositis and related symptoms such as body weight loss.

Finally, it is to be understood that various other modifications and/or alterations may be made without departing from the spirit of the present invention as outlined herein.

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CLAIMS:

1. A method for preventing damage to the lining of the alimentary tract resulting from chemotherapy and/or radiation, which method includes administering to a patient in need thereof an effective amount of a milk product extract.

2. A method according to claim 1 wherein the milk product extract is a cheese whey extract, a colostral whey extract, a skim milk extract or an acid (casein) whey extract.

3. A method according to claim 2 wherein the milk product extract is prepared by subjecting a milk product to cation exchange chromatography.

4. A method according to claim 3 wherein the milk product extract is a milk product extract composition including a mixture of cell growth factors having basic to about neutral isoelectric points, wherein the mixture of cell growth factors is obtained from a milk product of an ungulate mammal, and wherein the milk product is subjected to a cation exchange matrix under conditions whereby casein, α lactalbumin, and β lactoglobulin present in the milk product are not adsorbed to the matrix, after which the adsorbed growth factor mixture is eluted with a substantially aqueous salt solution and then optionally concentrated.

5. A method according to claim 2 wherein the milk product extract includes lactoperoxidase and/or lactoferrin.

6. A method according to claim 2 wherein the milk product extract is GFE or GFE-2 as hereinbefore described.

7. A method according to claim 1 wherein the milk product extract is modified by transient acidification to enhance activity.

8. A method according to claim 1 wherein the milk product extract is modified to enhance activity by the addition of one or more growth factors including IGF-I, IGF-II, TGFβ, EGF, transforming growth factor α (TGFα), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) or keratinocyte growth factor (KGF).

9. A method according to claim 1 wherein the damage includes damage to the lining of the mount and/or oesophagus.

10. A method according to claim 9 wherein the damage includes mucositis.

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11. A method according to claim 1 wherein the damage includes at least partial loss or mucosal crypt area and/or mucosal villus length.

12. A method according to claim 1 wherein the damage includes an increase in bacterial translocation across the alimentary tract.

13. A method according to claim 1 wherein the damage results from chemotherapy including administration to the patient of mechlorethamine, melphalan, busulphan, cytarabine, floxuridine, 5-fluorouracil, mercaptopurine, methotrexate, thioguanine, bleomycin, actinomycin-D, daunorubicin, etoposide, mitomycin, vinblastine, vincristine, hyroxyurea or procarbazine alone or in combination.

14. A method for ameliorating and/or treating damage to the lining of the alimentary tract resulting from chemotherapy and/or radiation, which method includes administering to a patient in need thereof an effective amount of a milk product extract.

15. A method according to claim 14 wherein the milk product extract is a cheese whey extract, a colostral whey extract, a skim milk extract or an acid (casein) whey extract.

16. A method according to claim 15 wherein the milk product extract is prepared by subjecting a milk product to cation exchange chromatography.

17. A method according to claim 16 wherein the milk product extract is a milk product extract composition including a mixture of cell growth factors having basic to about neutral isoelectric points, wherein the mixture of cell growth factors is obtained from a milk product of an ungulate mammal, and wherein the milk product is subjected to a cation exchange matrix under conditions whereby casein, α lactalbumin, and β lactoglobulin present in the milk product are not adsorbed to the matrix, after which the adsorbed growth factor mixture is eluted with a substantially aqueous salt solution and then optionally concentrated.

18. A method according to claim 15 wherein the milk product extract includes lactoperoxidase and/or lactoferrin.

19. A method according to claim 15 wherein the milk product extract is GFE or GFE-2 as hereinbefore described.

20. A method according to claim 14 wherein the milk product extract is modified by transient acidification to enhance activity.

21. A method according to claim 14 wherein the milk product extract is modified to enhance activity by the addition of one or more growth factors including IGF-I, IGF-II, TGFβ, EGF, transforming growth factor α (TGFα), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF) or keratinocyte growth factor (KGF).

22. A method according to claim 14 wherein the damage includes damage to the lining of the mouth and/or oesophagus.

23. A method according to claim 22 wherein the damage incudes mucositis.

24. A method according to claim 14 wherein the damage includes at least partial loss of mucosal crypt area and/or mucosal villus length.

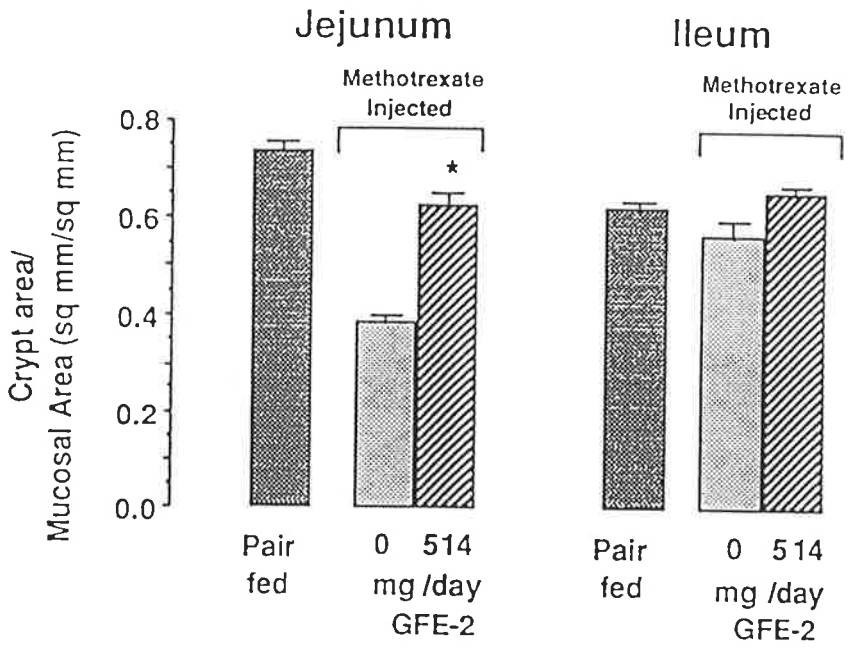
25. A method according to claim 14 wherein the damage includes an increase in bacterial translocation across the alimentary tract.

26. A method according to claim 14 wherein the damage results from chemotherapy including administration to the patient of mechlorethamine, melphalan, busulphan, cytarabine, floxuridine, 5-fluorouracil, mercaptopurine, methotrexate, thioguanine, bleomycin, actinomycin-D, daunorubicin, etoposide, mitomycin, vinblastine, vincristine, hyroxyurea or procarbazine alone or in combination.

Dated: 6 March 1997

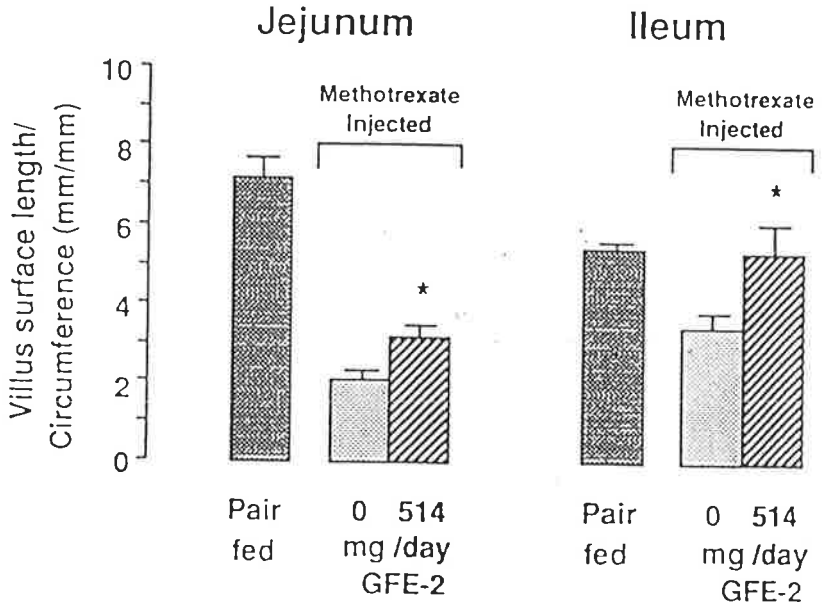
PHILLIPS ORMONDE & FITZPATRICK

Attorneys for: GROPEP PTY LTD



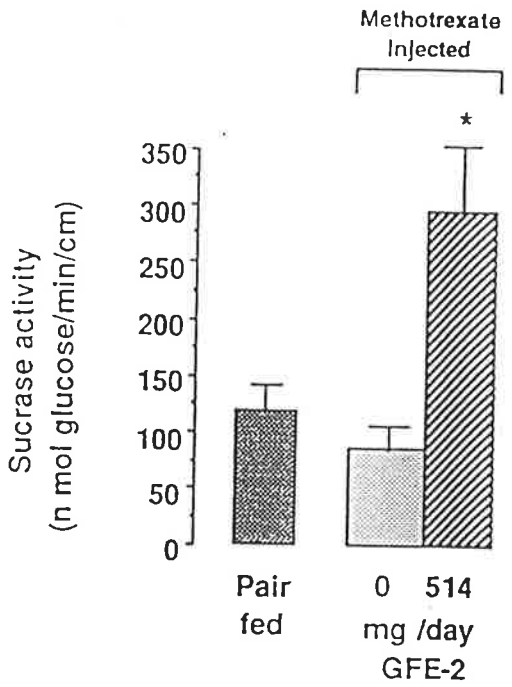
*: P<0.05 vs Methotrexate - treated Controls (0 mg/day GFE-2)

Fig 1a



*: P<0.05 vs Methotrexate - treated Controls (0 mg/day GFE-2)

Fig 1b



*: P<0.05 vs Methotrexate - treated Controls (0 mg/day GFE-2)

Fig 2

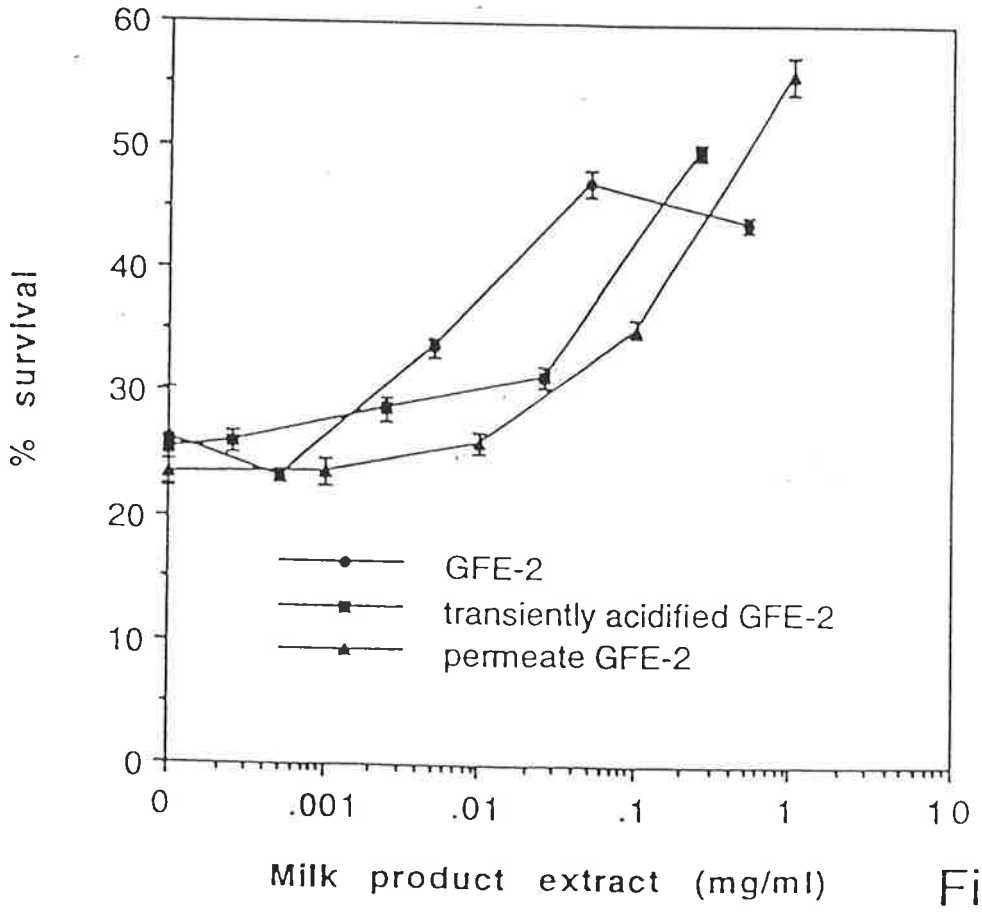
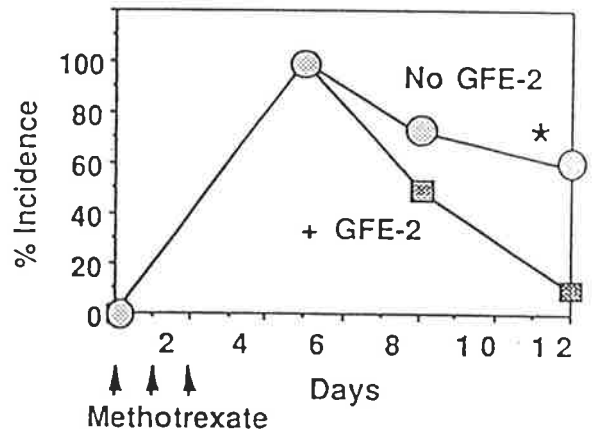
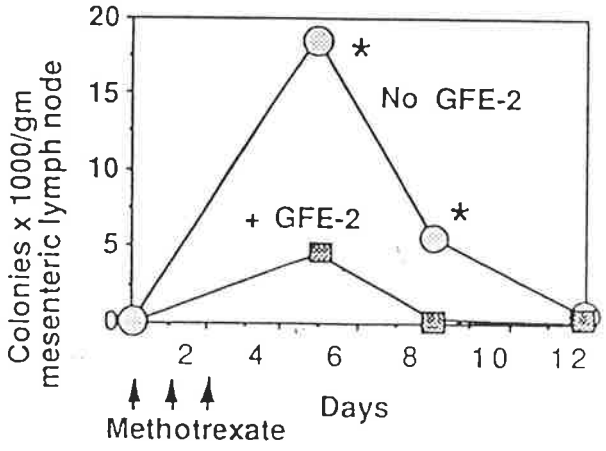


Fig 4



* P<0.05 vs
+ GFE-2 Group

Fig 3a



* P<0.05 vs
+ GFE-2 Group

Fig 3b

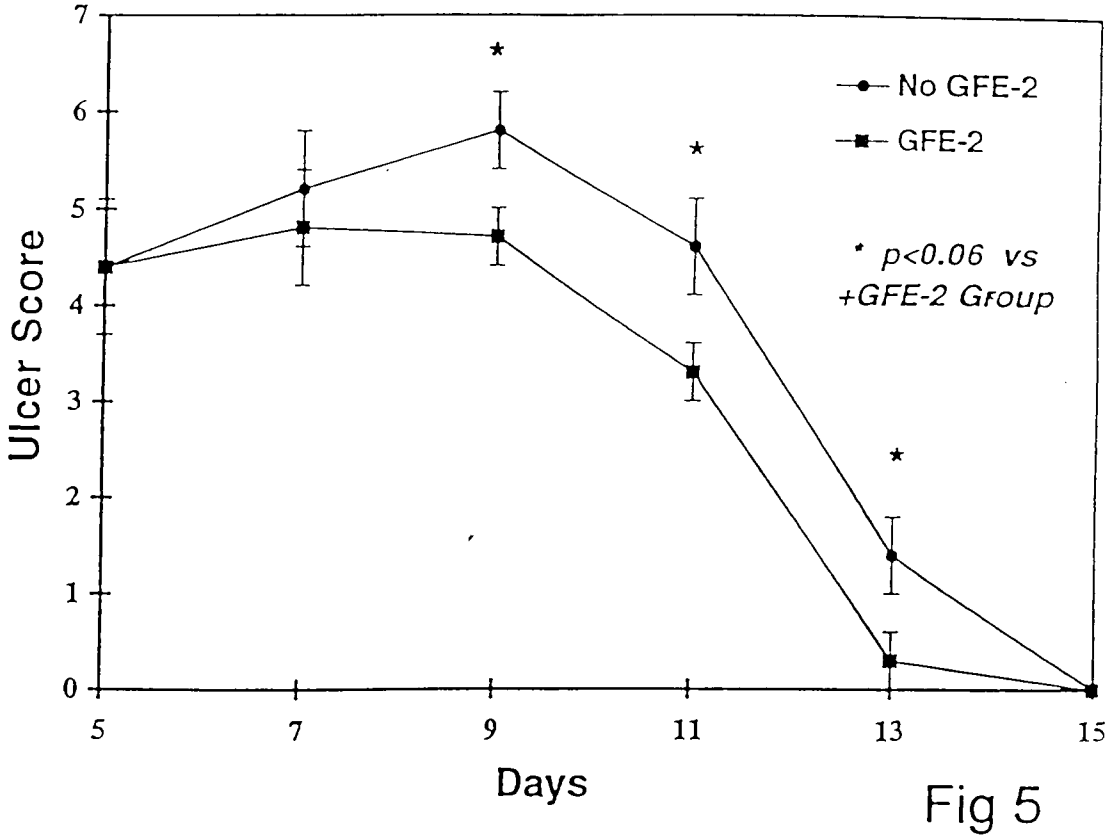

Ulcer Score
Days
No GFE-2
GFE-2
* p<0.06 vs
+GFE-2 Group
0
1
2
3
4
5
6
7
5
7
9
11
13
15

Fig 5

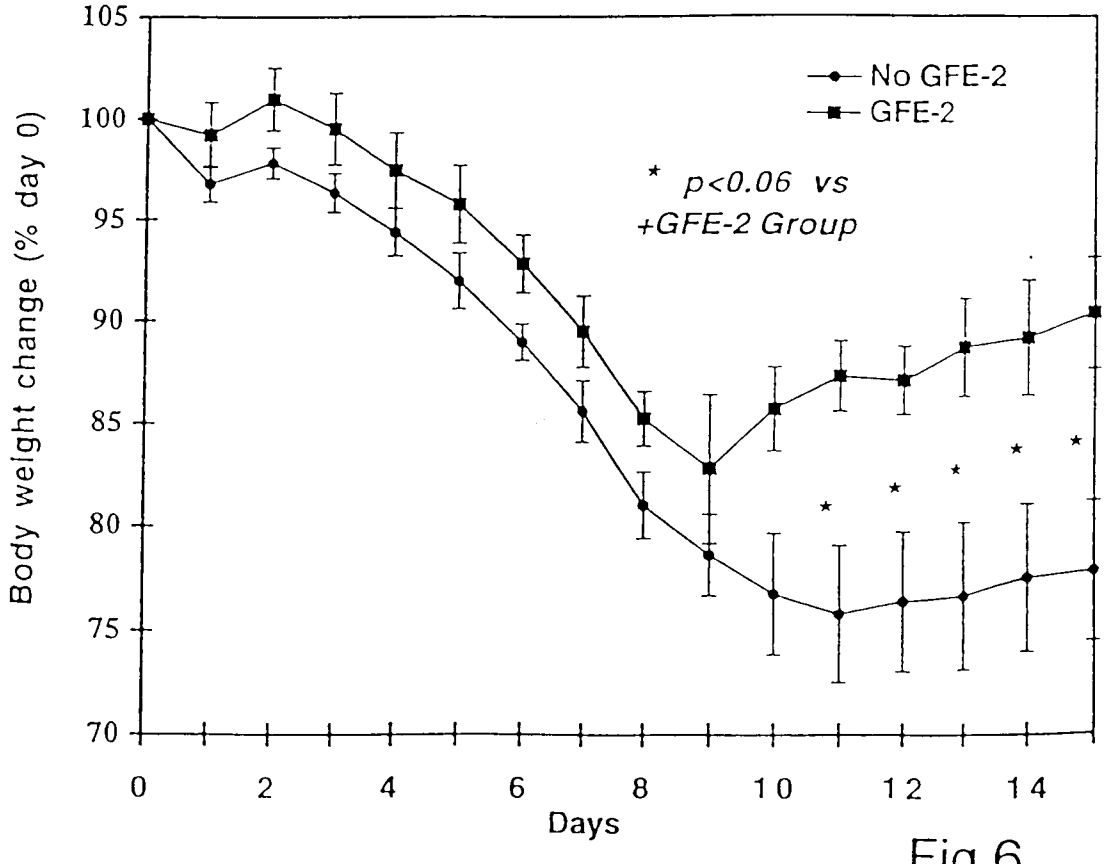

Body weight change (% day 0)
Days
No GFE-2
GFE-2
* p<0.06 vs
+GFE-2 Group
70
75
80
85
90
95
100
105
0
2
4
6
8
10
12
14

Fig 6

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00253

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: A61k 035/20, 038/18, 038/40, 038/30, 038/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC A61K 035/20

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

AU: IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Chem Abs. Milk or whey or colostrum and aliment: or mucos:
Derwent: Growth factors and milk and gast: or intest: or muco:
Medline: Milk or whey or colostrum and pharmaceutical or therapy or treatment

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 95/29933 A1 (GROPEP PTY LTD) 9 November 1995 Claims 22 and 24	1-7, 9-14
Y		8
X	WO 92/00994 A1 (GROPEP PTY LTD) 23 January 1995 Claims 18 and 21	1-7, 9-14
Y		8
X	WO 93/25227 A1 (KABI PHARMACIA) 23 December 1993 page 1, paragraph 1, pages 3-4	1, 8, 14

[X] Further documents are listed in the continuation of Box C [ ] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
3 July 1996

Date of mailing of the international search report
11 JUL 1996

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 96/00253

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 527283 A1 (SOCIETE DES PRODUITS NESTLE SA) 17 February 1993 Claims 1, 2, Table 1 page 4	1, 3, 8
X	WO 95/00155 A1 (VALIO BIOTUOTTEET) 5 January 1995 Page 1, page 5, lines 19-28	1, 14
Y	AU 17353/88 A (BAYLOR COLLEGE OF MEDICINE) 8 December 1988 Page 6, lines 17-23	1, 5, 14
Y	WO 94/23032 A1 (AMGEN INC) 13 October 1994 Claims 12-17	8
Y	WO 92/18153 A1 (CREATIVE BIOMOLECULES) 29 October 1992 Whole document	8
Y	WO 92/08480 A1 (CELTRIX LABORATORIES INC) 29 May 1992 Whole document	8
Y	WO 90/01941 A1 (CHILDREN'S MEDICAL CENTRE CORPORATION) 8 March 1990 Whole document	8
X	Milchwissenschaft 47(11) 1992 "Gastroprotection with milk phospholipids" Kinnen et al. pages 694-696	1, 14
X	Pharmacological Research 29 March 1994 "The effects of milk and calcium on ethanol induced gastric mucosal damage" M W L Kloo pages 217-224. See particularly page 220	1, 14

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No.
PCT/AU 96/00253

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	9529933	AU	5347/94				
WO	9200994	AU	900713	EP	545946	JP	5508542
WO	9325227	AU	920608	EP	602211	JP	6510066
		US	5482926				
EP	527283	AU	19521/92	EP	91810629	EP	527283
		JP	5284936	US	5461033		
WO	9500155	AU	68478/94	EP	711171		
AU	17353	EP	295009	JP	1093534	US	4977137
WO	9423032	AU	65243/94	EP	619370		
WO	9218153	AU	17959/92	EP	584286	JP	6506939
		US	5234908				
WO	9208480	AU	90467/91	EP	557418	JP	6505965
		US	5462925				
WO	9001941	AU	42189/89	EP	360006	JP	3505736
		US	5175147				

END OF ANNEX

APPENDIX B

SUPPORTING PUBLICATIONS

SP1. Ribbons KA, Howarth GS, Davey KB, George-Nascimento C, Read LC. Subcutaneous but not intraluminal epidermal growth factor stimulates colonic growth in normal adult rats. ***Growth Factors*** **1994, 10(3):153-162.**

Hypothesis and Aims

At the time this paper was published, EGF receptors were thought to be localised to the baso-lateral surface of intestinal enterocytes and there were preliminary indications of EGF efficacy in bowel protection. We therefore hypothesised that systemic-delivery of EGF would be more effective than enteral delivery at inducing colonic growth. EGF was administered to normal adult rats by chronic subcutaneous or intra-colonic infusion to determine and compare the effects on colonic growth.

Outcome and Contribution to Scientific Understanding

Subcutaneous infusion of 200 micrograms EGF/kg/day for 7 days increased the cross-sectional mass and protein content of the muscularis and mucosal layers of the proximal colon, with the distal colon showing less responsiveness. The results demonstrated that the normal adult colon is responsive to subcutaneously delivered EGF, whereas EGF may not be active when presented from the luminal direction, supporting the baso-lateral localisation of the EGF receptor. This was confirmed by Playford et al, (1996) further supporting the therapeutic potential for EGF to induce colonic re-growth following injury.

Personal Contribution

My role in this study was to assist in the in vivo aspects of experimentation, to conduct the qualitative and quantitative histological analyses, and to assist in preparation of the Methods section of the final manuscript.

SP2. Ribbons KA, Howarth GS, Ford WDA, George-Nascimento C, Bourne AJ, Read LC. Effects of epidermal growth factor administration on repair of acetic acid-induced colonic ulcerations in rats. ***Growth Factors*** **1997, 14(2-3):89-101.**

Hypothesis and Aims

Following the discovery that systemically-delivered EGF held the potential to induce growth of the proximal colon (**SP1**), we administered EGF to rats with experimentally-induced colonic ulceration and hypothesised that EGF would promote colonic regrowth following damage.

Outcome and Contribution to Scientific Understanding

Systemic administration of exogenous EGF for up to 6 days failed to enhance re-epithelialization of acetic acid-induced colonic ulcerations but attenuated the associated inflammatory response. This study described a novel mechanism of EGF action not associated with enhanced epithelial repair, but involving an attenuation of the thickening of the submucosa and muscularis externa layers of the colon.

Personal Contribution

My role in this study was to assist in the in vivo aspects of experimentation, to conduct the qualitative and quantitative histological analyses, and to assist in preparation of the Methods section of the final manuscript.

SP3. Xian CJ, Mardell CE, Howarth GS, Byard RW, Moore DJ, Miettinen P, Read LC. Site-specific changes in transforming growth factor-α and –β expression in colonic mucosa of adolescents with inflammatory bowel disease. ***Scand J Gastro*** **1999, 34:591-600.**

Hypothesis and Aims

This study investigated the role of TGF-α in colonic protection by comparing TGF-α distribution by immunostaining and mRNA expression methods in the colonic epithelium of specimens sourced from patients with active IBD.

Outcome and Contribution to Scientific Understanding

The finding that TGF-α expression was unchanged in active IBD implied a degree of functional redundancy within the members of the EGF peptide family. This finding was supported by further work from our own laboratory on the effects of targeted deletion of the TGF-α gene on intestinal damage induced by methotrexate in the mouse (*Effects of TGF-α gene knockout on epithelial cell proliferation, migration, apoptosis, and repair of acute damage in the small intestine.* Xian CJ, Cool JC, **Howarth GS,** Read LC. : Submitted to Cellular Physiology : 2001)

This second study on the role of TGF-α in the bowel demonstrated that the effects of TGF-α gene knock-out on bowel susceptibility to methotrexate-induced damage were minimal. TGF–α gene knockout did not affect crypt cell production, mitosis position, migration, or apoptosis in non-injured intestine, nor did it impair mucosal repair in methotrexate-induced acute damage in the small intestine, although there was a delayed

and a smaller crypt cell overproduction during repair. Gene expression analysis by RT-PCR revealed the presence of 4 out of 6 EGF family ligands in the normal intestine and dramatic upregulation of all ligands (except EGF) in the repairing intestine in both wild type and mutant mice, indicating a functional redundancy of the EGF family in maintenance and acute-damage repair in the small intestine.

This publication also contributed to our understanding of TGF-β and its role in the pathogenesis of IBD since it concluded that the expression of TGF-β was unaltered in the colonic mucosa of adolescents with IBD although an increased density of TGF-β-positive immune cells in the lamina propria during disease activity, suggested a role in modulation of inflammation in this disorder.

Personal Contribution

My role in this study was to aid in the collection of patient biopsy material and to assist in preparation and editing of the final manuscript. I was also responsible for assisting in the planning and interpretation of experiments conducted in the second (as yet, unpublished) study utilising TGF-α 'knock-out' mice.

SP4. Howarth GS, Bastian SEP, Dunbar AJ, Kallincos NC, Goddard C. Systemic administration of betacellulin to rats promotes growth of the gastrointestinal organs. ***Gastroenterology*** **2000, 118(4):2930.**

Hypothesis and Aims

Our understanding of betacellulin function in vivo remains incomplete. The capacity for betacellulin to induce pancreatic β-cell mitogenesis and EGF-like properties led us to speculate that betacellulin could have a physiological role in the processes of pancreatic function and gastrointestinal growth. Accordingly, we administered betacellulin systemically to rats in order to attain a state of betacellulin over-expression to facilitate investigations into its effects on physiological parameters including fluid balance, body growth and gastrointestinal growth.

Outcome and Contribution to Scientific Understanding

This study revealed that betacellulin induced a diuresis and appetite suppression, accompanied by epithelial cell proliferation and growth of the gastrointestinal organs, particularly the ileum and colon. For the first time, this study identified betacellulin as a potential treatment modality for conditions associated with damage to the distal bowel. This work is currently being prepared for full publication.

Personal Contribution

My role in this study was to assist in development of the central hypothesis, to plan the experiments, to collate and interpret the data.

SP5. Xian CJ, Couper RT, Howarth GS, Mardell CE, Read LC, Kallincos NC. Increased expression of HGF and its receptor c-met in rat small intestine during recovery from methotrexate-induced mucositis. ***Br Jour Cancer*** **2000, 82:945-952.**

Hypothesis and Aims

In order to investigate the mechanisms underlying intestinal repair in chemotherapy-induced gut damage, we examined the expression profiles of HGF and its receptor c-met, two molecules previously implicated in tissue repair, in comparison to the histopathological and proliferative changes in a rat model of methotrexate-induced small intestinal mucositis.

Outcome and Contribution to Scientific Understanding

Up-regulation of HGF and c-met coincided with crypt hyperproliferation and mucosal recovery, suggesting a role for HGF in intestinal repair following acute injury. The demonstration of HGF and c-met localization to the crypt epithelium represented the first indication of an autocrine or paracrine mechanism of HGF action.

Personal Contribution

My role in this study was to develop the animal model, to assist in development of the central hypothesis and the interpretation of results.

SP6. Xian CJ, Howarth GS, Mardell CE, Cool JC, Familari M, Read LC, Giraud AS. Temporal changes in TFF3 expression and jejunal morphology during methotrexate-induced damage and repair. ***Am J Physiol*** **1999, 277:G785-G795.**

Hypothesis and Aims

Trefoil factor TFF3 has been implicated in intestinal protection and repair. This study investigated the spatiotemporal relationship between TFF3 expression and morphological changes during intestinal damage and repair in a rat model of methotrexate-induced small intestinal mucositis.

Outcome and Contribution to Scientific Understanding

TFF3 mRNA levels increased marginally during histological damage whilst TFF3 peptide was depleted but normalized during repair, mirroring the disappearance and repopulation of goblet cells. The absence of a temporal relationship between TFF3 levels and crypt hyperproliferation, confirmed the non-mitogenic nature of TFF3. The coincidental normalisation of TFF3 peptide with repopulation of goblet cells and mucin production suggested that TFF3 may play a role in the remodelling phase of bowel repair.

Personal Contribution

My role in this study was to develop the animal model, to assist in development of the central hypothesis and the interpretation of results.

SP7. Conlon MA, Tomas FM, Owens PC, Wallace JC, Howarth GS, Ballard FJ. Long R^3-IGF-I infusion stimulates organ growth but reduces plasma IGF-I, IGF-II and IGF binding protein concentrations in the guinea pig. ***J Endocrinol*** **1995, 146:247-253.**

Hypothesis and Aims

This publication sought to investigate whether an animal with substantial amounts of both IGF-I and IGF-II in circulation, such as the guinea pig, would respond to chronic IGF infusion in the same manner as the adult rat.

Outcome and Contribution to Scientific Understanding

The study concluded that the analogue IGF-I peptide was capable of stimulating growth of the gastrointestinal organs, confirming earlier studies in rodents, providing further rationale for IGF-I application in candidate disease conditions affecting humans. However, the effects on overall body growth were less than that observed in rodents. This result was believed to be a consequence of reduced plasma IGF-I levels resulting from infusion of the analogue peptide.

Personal Contribution

My role in this study was to oversee histological analyses and the subsequent interpretation of histological results.

SP8. Conlon MA, Francis GL, Tomas FM, Wallace JC, Howarth GS, Ballard FJ. Continuous 14 day infusion of Insulin-like growth factor (IGF)-II increases the growth of normal female rats, but exhibits a lower potency than IGF-I. ***J Endocrinol*** **1995, 144:91-98.**

Hypothesis and Aims

The effects of continuous 14 day infusion of recombinant human IGF-I or IGF-II were compared in young female rats in order to establish the relative efficacies of these two growth factors.

Outcome and Contribution to Scientific Understanding

IGF-II infusion increased the length of absorptive villi lining the jejunum, suggesting an increased absorptive capacity of the gut. However there was no significant change in the amount of faecal nitrogen excretion when expressed as a percentage of nitrogen intake. Interestingly, IGF-II was at least as potent as IGF-I in increasing the depth of jejunal crypts. This study represented the first time that *IGF-II* had been administered to rats. This important study furthered our understanding of IGF-I and –II action in vivo by confirming that IGF-II signalling through the type I (IGF-I) receptor was indeed weak since the physiological effects, although qualitatively similar, were quantitatively less in comparison to IGF-I, thus confirming the decreased potency of IGF-II in the adult.

Personal Contribution

My role in this study was to oversee histological analyses and the subsequent interpretation of histological results.

SP9. Read LC, Lemmey AB, Howarth GS, Martin AA, Tomas FM, Ballard FJ. The gastrointestinal tract is one of the most responsive target tissues for IGF-1 and its potent analogs. In: *Modern Concepts of Insulin-like growth factors.* Ed. E. Martin Spencer. Elsevier Science Publishing Company Inc. London. 1991, 225-234.

Supporting publications **SP9**, **SP10** and **SP11** represented state-of-the-art reviews of the accumulated knowledge on IGF-I action in the gastrointestinal tract that was current as at 1991, 1994 and 1997, respectively. These published reviews were presented at the corresponding Second, Third and Fourth International Symposia on Insulin-like Growth Factors held in those years.

Personal Contribution

Dr Leanna Read was principally involved with the preparation of review studies SP9, SP10 and SP11. Aside from my role in the studies which comprised the reviews, my role was essentially editorial in determining the accuracy of information contained therein.

SP10. Read LC, Howarth GS, Steeb C-B, Lemmey AB. In vivo effects of IGF-I on gut growth and function. In : *The insulin-like growth factors and their regulatory proteins.* Proceedings of the Third International Symposium on Insulin-like Growth Factors. Ed. R.C Baxter, P.D. Gluckman and R.G. Rosenfeld. Elsevier Science Publishing Company Inc. London. 1994, 409-416.

See annotation for SP9.

SP11. Steeb C-B, Shoubridge CA, Lamb J, Howarth GS, Read LC. Role of IGF-I in gastrointestinal growth and repair. In : *Molecular Mechanisms to Regulate the Activity of Insulin-like Growth Factors.* Proceedings of the Fourth International Symposium on Insulin-like Growth Factors. Ed. K. Takano, N. Hizuka and S-I. Takahashi. Elsevier Science Publishing Company Inc. London. 1998, 331-339.

See annotation for SP9.

SP12. Lemmey AB, Ballard FJ, Martin AA, Tomas FM, Howarth GS, Read LC. Treatment with IGF-I peptides improves absorption of the remnant gut following small bowel resection in rats. ***Growth Factors*** **1994, 10:243-252.**

Hypothesis and Aims

The effects of IGF-I on gut growth and absorptive function were examined in growing rats following removal of 70 or 80% of the jejuno-ileum, and compared with the responses to the analogues, long-R^3-IGF-I and des-(1-3)-IGF-I, which bind poorly to IGF binding proteins.

Outcome and Contribution to Scientific Understanding

Administration of all IGF-I peptides following 70% jejuno-ileal resection significantly attenuated malabsorption of fat and nitrogen. Responses in rats with 80% resection were less substantial, but a dose-responsive reduction in malabsorption was apparent with long-R^3-IGF-I. Both IGF-I and long-R^3-IGF-I were shown to increase body weight gain and food conversion efficiency in a dose-dependent manner following 80% jejuno-ileal resection. Total gut weight was increased by up to 21%, due predominantly to increased weight of the stomach and proximal small bowel, with the latter effect attributable at least in part to an increased bowel length.long-R^3-IGF-I was more potent than IGF-I at stimulating body weight gain and food conversion efficiency, but its potency advantage on gut absorptive function and small intestinal re-growth was less marked. This important study concluded that administration of IGF-I peptides improved gastro-intestinal absorptive function following partial gut resection, most likely reflecting, at least in part,

an increase in gut absorptive surface area. This publication was one of the first studies to describe potential for IGF-I to induce linear growth of the bowel following resection.

Personal Contribution

My role in this study was to conduct and interpret all histological analyses, and to assist in preparing the histological data for publication.

SP13. Read LC, Tomas FM, Howarth GS, Martin AA, Edson K, Owens PC, Ballard FJ. IGF-1 and its N-terminal modified analogs induce marked gut growth in dexamethasone-treated rats. ***J Endocrinol*** **1992, 133:421-431.**

Hypothesis and Aims

The effects of insulin-like growth factor-I (IGF-I) on the gut of 150 g dexamethasone-treated rats were compared with those of two analogues with reduced affinity for IGF-binding proteins, des-(1-3)-IGF-I and long-R^3-IGF-I.

Outcome and Contribution to Scientific Understanding

Administration of IGF-I to rats made catabolic by co-treatment with dexamethasone increased total gut weight by up to 60%, and gut weight as a fraction of body weight by up to 32%, affecting all regions of the gut, including the stomach, small intestine and colon. Cross-sectional mass, rather than gut length, was increased, and proportional increases in wet weight, protein and DNA content per unit length were measured in both the mucosa and muscularis layers. IGF-I analogues were consistently several-fold more potent, providing evidence that IGF-binding proteins (IGFBPs) reduce the biological activity of exogenous IGF-I in the gut. This study represented the first indication that the gut was one of the most sensitive target tissues for IGF-I, and that potency in vivo correlated with a reduced interaction with IGFBPs, suggesting further therapeutic indications for IGF-I in conditions associated with gastrointestinal insufficiency.

Personal Contribution

My role in this study was to conduct and interpret all histological analyses, and to assist in preparing the histological data for publication.

SP14. Fraser R, Frisby CL, Blackshaw LA, Schirmer MB, Howarth GS, Yeoh EK. Small intestinal dysmotility following abdominal irradiation in the isolated rat small intestine. ***Neurogastroenterology and Motility*** **1998, 10:413-419.**

Hypothesis and Aims

Small intestinal dysmotility is believed to contribute to the symptoms of radiotherapy-induced enteritis although the underlying mechanisms are unclear in part because of the technical difficulties inherent in performing studies in irradiated small intestine. The aim of the current study was to develop an ex-vivo model of dysmotility to evaluate small intestinal motor activity using perfused micromanometric techniques in the ileum.

Outcome and Contribution to Scientific Understanding

Intestinal segments from rats were studied 4 days after treatment with10 Gray abdominal irradiation. For each experiment the total number of pressure waves, high-amplitude (> 20 mmHg, long-duration > 6 sec) pressure waves, and long (> 20 associated) bursts of pressure waves were determined. Irradiation had no effect on the overall number of pressure waves, but increased high-amplitude long-duration (HALD) pressure waves (248 vs 7, $P < 0.01$). Retrograde migration of HALD waves was seen in five segments following irradiation. Irradiation abolished bursts of > 20 pressure waves. The study represented the first validation of an ex-vivo model of radiation-induced dysmotility for potential investigations of agents capable of modifying intestinal motility.

Personal Contribution

Although I was intrinsically involved in the development of the animal model that formed the basis of this study (see **PP1**) my role in **SP14** was primarily advisory.

SP15. Fraser R, Frisby CL, Blackshaw LA, Langman J, Howarth GS, Yeoh EK. Divergence of mucosal and motor effects of IGF-I and LR^{3}IGF-I on isolated rat ileum following abdominal irradiation. ***J Gastro Hepatol*** **2000, 15 (10):1132-1137.**

Hypothesis and Aims

This study aimed to explore the potential mechanism of IGF-I action during the repair phase following radiation-induced injury, by comparing the effects of IGF-I and its more potent analogue, long-R^3-IGF-I on the independent processes of mucosal repair and neurally-mediated ileal dysmotility.

Outcome and Contribution to Scientific Understanding

Utilising the rat model system developed in **SP14**, this study concluded that IGF-I peptide action did not appear to influence motor activity in the jejunum, but exerted its beneficial effects through a direct effect on mucosal re-growth following injury. This study also provided further insight into the expression of IGF binding proteins by the finding that IGF-I and its analogue peptide were equipotent, suggesting that local IGFBP expression was decreased following radiation-induced injury.

Personal Contribution

I was involved in the development of the animal model that formed the basis of this study (see **PP1**). Otherwise, my role in **SP15** was to oversee the biochemical and histological analyses and to prepare these aspects of the study for publication.

SP16. Tran CD, Butler RN, Howarth GS. Zinc in combination with a growth factor extract from bovine whey promotes recovery from methotrexate-induced small bowel damage in rats. ***Gastroenterology*** **1999, 116(4, Pt. 2):G4089.**

Hypothesis and Aims

WGFE had previously been demonstrated to be efficacious against small intestinal mucositis although the mechanism was unclear. This study sought to investigate the effects of WGFE on intestinal permeability, an indicator of mucosal barrier integrity, following the induction of methotrexate-induced small bowel mucositis. In a second aspect of the study, the interaction between dietary zinc and WGFE on intestinal permeability was assessed.

Outcome and Contribution to Scientific Understanding

Utilising a similar experimental protocol to **PP5**, rats received either dietary zinc, WGFE, or the combination thereof, and the effects on intestinal permeability were assessed by the urinary recovery of orally-gavaged ^{51}Cr-EDTA. Bowel permeability was improved by the combination of WGFE and zinc in the early phase of bowel damage although WGFE alone was equally as effective as the combination during the recovery phase, results which suggested a beneficial interaction between zinc and WGFE during the phase of initial bowel damage. This preliminary study provided some insight into the mechanism of WGFE action in intestinal mucositis since the finding that WGFE had somehow reduced bowel permeability indicated a beneficial effect on enterocyte tight junctions and therefore, mucosal barrier function. A full manuscript has now been submitted (*Zinc and*

metallothionein in the methotrexate-damaged rat intestine. Tran CD, Butler RN, Philcox JC, Rofe AM, Coyle P and **Howarth GS**. Submitted to Scand. J. Gastro. [2001])

Personal Contribution

Together with Dr Ross Butler, I developed the hypothesis that WGFE and zinc could improve mucosal barrier function. In consultation with PhD student, Cuong Tran, I was responsible for the planning of experiments and the subsequent interpretation of results.

SP17. Porter SN, Howarth GS, Butler RN. An orally administered growth factor extract derived from bovine whey suppresses breath ethane in colitic rats. ***Scand J Gastro*** **1998, 33:967-974.**

Hypothesis and Aims

The effect of oral consumption of WGFE on lipid peroxidation, a potential mechanism of bowel damage in colitis, was assessed using the ethane breath test in the dextran sulphate sodium (DSS) model of ulcerative colitis (UC) in rats.

Outcome and Contribution to Scientific Understanding

Groups of rats consumed water (control), 2% DSS in drinking water, 2% DSS with a WGFE-supplemented diet, or 2% DSS plus prednisolone for 6 weeks, changing to sulphasalazine for the subsequent 4 weeks. Ethane breath tests were conducted on all animals on days 2, 4, 6, 8, and 10 (acute phase) and weeks 3, 6, and 9 (chronic phase) after commencement of DSS consumption. There were no significant differences in ethane production between any groups during the acute phase. Ethane was significantly increased ($P < 0.05$) in rats consuming DSS alone in week 6 compared with control but had decreased to control levels by week 9. WGFE and conventional therapy were effective in suppressing ethane production in week 3. This study concluded that WGFE was as effective as conventional therapies at limiting ethane production and thus colonic lipid peroxidation in the early phases of experimental chronic UC. This study represented the first evidence that oral growth factor supplementation could influence chronic inflammation of the colon.

Personal Contribution

Together with Dr Ross Butler, I developed the hypothesis that WGFE could reduce inflammation of the colon in experimental colitis. I was responsible for the planning of experiments and the subsequent interpretation of data.

SP18. Howarth GS and Shoubridge CA. Enhancement of intestinal growth and repair by growth factors. (Review) ***Curr Opinion Pharmacol*** **2001, 1(6):568-574.**

This invited review summarises the current understanding of growth factors in the gut, further describing their therapeutic potential for a number of diseases and disorders affecting the gastrointestinal tract.