EFFECTS OF GH ON THE IGFs AND IGFBPs IN CHILDREN WITH CHRONIC RENAL FAILURE AND TRANSPLANTATION

A thesis submitted to

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This thesis is dedicated to the memory of

JEAN DOROTHY VAN RENEN (1925-1996)

My beloved mother, who was always there for me. Her courage and strength in the face of insurmountable adversity has been a great inspiration to me.

and

WENDY KAYE JUREIDINI (1947-1994)

A very special lady, whose unselfish caring for others enabled me to become involved in the clinical work which preceeded, and eventually led to the work in this thesis.

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STATEMENT OF ORIGINALITY

The work presented in this thesis contains no material which has been accepted for the award of any other qualification in any university or other tertiary institution. To the best of my knowledge and belief, this thesis does not contain any material previously published or written by another person, except where due reference is made in the text. I consent to a copy of this thesis, when deposited with The University of Adelaide Library, being available for photocopy and loan.

Dated at Adelaide ----------23/12/96----------

Signed

Margaret Jean van Renen MB BCh

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PUBLICATIONS ARISING FROM THESIS RESEARCH

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ABSTRACTS:

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ABSTRACT

The work contained in this thesis involves the retrospective investigation of the insulin-like growth factors and their binding proteins in the serum of children with chronic renal failure (CRF) and transplantation, before and after treatment with recombinant human growth hormone (rhGH). Quantitation of serum IGF-I and IGF-II concentrations, carried out by specific radioimmuno- and radioreceptor-assay respectively, after extraction of the IGFs by means of acid chromatography, revealed that, before rhGH therapy, IGF-I levels were low-normal and IGF-II levels were elevated. Before rhGH treatment, normal IGFBP-3, ALS and elevated IGFBP-1 serum concentrations were found by specific radioimmunoassays. rhGH treatment resulted in a significant increase in serum IGF-I, a moderate increase in IGF-II, IGFBP-3 and ALS concentrations and a substantial fall in IGFBP-1 levels. The technique of Western ligand blotting (WLB) was employed to identify the IGF-binding proteins present in serum by virtue of their ability to bind to the ligands ^{125}I-IGF-I and/or ^{125}I-IGF-II. Five protein bands were found both before and after rhGH treatment, which were identified by Western immunoblotting (WIB) using the immunological properties of the IGFBPs and specific antibodies, to IGFBP-1, -2, -3, -4. A 30 kDa form of IGFBP-3, which apparently has reduced affinity for the radioligand, was only identified by WIB.

IGF-IGFBP complexes in pooled serum from prepubertal and pubertal children of both sexes with CRF and renal transplantation, before and after treatment with rhGH, were analysed by fast protein liquid chromatography under neutral conditions. The distribution of these complexes in the patients was compared and contrasted with age and sex-matched children with normal renal function and the specific effects of rhGH treatment and transplantation were

noted. An increase in ^{125}I-IGF-I binding capacity in the small molecular weight IGF-IGFBP complex was seen in all CRF and transplant sera before treatment. rhGH therapy saw a decrease in the small molecular weight binding complex in all patients.

These findings suggest that an increased small molecular weight binding capacity in CRF, due in particular to increased IGFBP-1, in association with low-normal IGF-I levels results in a reduction in IGF bioavailability and may be responsible for the growth retardation of CRF, which can be reversed by rhGH treatment.

CHAPTER 1

LITERATURE REVIEW

GENERAL INTRODUCTION

Growth retardation is a serious clinical manifestation associated with irreversible renal insufficiency (Fine, 1992). This relationship was first noted at the end of the last century by Guthrie and Oxon (Guthrie and Oxon, 1897). However, even as recently as 10 to 15 years ago, when the life expectancy of a child with chronic renal failure (CRF) was relatively short, growth was of little importance and children were often growth retarded (Broyer, 1982). Advances in renal replacement therapies such as dialysis and transplantation now mean that the majority of children with chronic renal failure do survive and the promotion of growth has become far more important (Rigden, Rees and Chantler, 1990). These treatments have, however, not resulted in the amelioration of growth retardation in many patients. For example, in 1974, when the European Dialysis and Transplantation Association (EDTA) analysed the heights of 474 European children at the start of haemodialysis, or at the first transplantation, it was found that 39% of all patients displayed a height below the third percentile for normal children (Scharer, Mehls, Schaefer, *et al*, 1993). In addition, it has been shown that the psychological consequences of short stature may be profound in both children and adults (Henning, Tomlinson and Rigden, 1988). Short stature in children may have profound psychological effects on social integration and, in adolescents and adults, may have an effect on their professional lives, even after successful renal transplantation (Mehls, Schaefer and Tonshoff, 1991; Rosenkranz, Bonzel, Bulla, *et al*, 1992). Henning *et al*, report that 35% of long-term survivors of renal failure failed to reach or complete secondary school, while only 10% reached a more advanced level of education (Henning, Tomlinson and Rigden, 1988). Therefore, the growth retardation of chronic renal failure has far-reaching consequences, and

the possibility that further research into this area could ultimately improve the quality of life for those affected was the stimulus for the work carried out in this thesis.

The research has concentrated on the insulin-like growth factors (IGFs) and insulin like growth factor-binding proteins (IGFBPs) in short children with CRF, as well as the effects on these parameters of recombinant human growth hormone treatment and transplantation. However, in order to understand the abnormalities of the GH/IGF axis in CRF, it is first necessary to have an understanding of the pathophysiology of growth involved and this is discussed in Chapter 1.1. The normal endocrinological roles of human growth hormone (hGH) and the IGFs in human growth physiology are discussed briefly in Chapter 1.2 and 1.3 respectively. This is followed by a discussion of the corresponding features found in CRF in Chapter 1.5 and 1.6

1.1 Growth retardation in chronic renal failure

Growth retardation in children with CRF occurs as a result of multiple factors interfering with one or more of the variables involved in normal growth in children, namely genetic potential, nutrition, metabolic balance and hormonal control (Chesney, 1987; French and Genel, 1984; Powell, 1988). Results of analyses of the contributions of these variables have been disappointing in identifying a specific underlying mechanism. Betts and Magrath (Betts and Magrath, 1974), described a growth pattern for children with CRF which links growth failure in infancy with a lack of energy intake, resulting in a falling away from the birth height percentile, followed by a normal height velocity during childhood. This velocity slows again during adolescence, where puberty is often delayed, and may coincide with end stage renal failure. Recent longitudinal growth data compiled by Schaefer *et al* (Schaefer, Wingen and al,

1996) on 321 prepubertal children with CRF due to congenital disorders, confirmed that the children had normal heights at birth, but dropped to below the third normal percentile during the first 15 months of life. Karlberg *et al*, speculate that the growth failure during fetal life and the first post-natal months reflects metabolic and/or nutritional influences, while the poor growth between 0.75 and 1.5 years of age is related to a partial insensitivity to growth hormone (Karlberg, Schaefer and Hennicke, 1996). However, in distinct contrast to these findings, Polito *et al* reported that a normal growth rate was possible in CRF children regardless of the degree of reduction of glomerular filtration rate (GFR) (Polito, Greco and Totino, 1987), and more recently, that the pubertal growth spurt was normal in their subjects who were treated conservatively (Polito, La Manna and Iovene, 1995).

A high protein diet in uremia can be toxic, due to the retention of phosphate and products of protein degradation, with the additional possibility of additional direct renal damage due to hyperfiltration of the kidneys (Brenner, Meyer and Hostetter, 1982). We have previously shown that dietary manipulations, involving a low-protein and low-phosphate diet, together with keto-acid supplements of the essential amino acids and histidine, with additional energy, can improve growth over a period of time (Jureidini, Hogg, van Renen, *et al*, 1990). In addition, disturbances of water and electrolyte metabolism, sodium chloride in particular, in patients with obstructive uropathies and renal hypoplasia, may be involved in growth retardation during the years of moderate chronic renal failure. Potassium aberrations, in children with tubular abnormalities, particularly nephropathic cystinosis, may have similar consequences (Broyer, 1982). Metabolic acidosis is commonly seen in CRF in cases where the renal function falls below 50% and workers in 1956, had already observed that growth retardation was most pronounced in those children with severe acidosis (West and Smith, 1956). The physiology responsible for this poor growth is not clear, but "catch-up" growth

often occurs with oral bicarbonate therapy (Powell, 1988). Metabolic acidosis has also been shown to accelerate skeletal muscle protein breakdown in normal humans (Mitch, 1996).

Although it is not certain that chronic anemia leads to growth impairment, pronounced anemia in children with CRF can lead to poor general health. However, studies of blood transfusions and recombinant erythropoietin treatment in uremic rats (Mehls, Scheafer and Tonshoff, 1991) have not shown improved growth. Hormonal derangements have also been described in CRF (Scharer, Mehls, Schaefer, *et al*, 1993). Characteristically, patients with CRF exhibit elevated basal insulin levels, but normal or slightly elevated serum glucose concentrations, indicating tissue resistance to the hypoglycemic effect of insulin (Holliday, Kulin and Lockwood, 1986; Mehls, Scheafer and Tonshoff, 1991). A slight reduction in the GFR to approximately 60%, results in an elevated serum parathyroid (PTH) concentration which is secondary to a decreased production of 1,25, dihydroxycholecalciferol by the kidney (Reichel, Deibart and H, 1991). Renal osteodystrophy, resulting from secondary hyperparathyroidism, can delay linear growth (Betts and White, 1976; Stickler and Bergen, 1973) and radiological evidence of renal osteodystrophy is more often seen in short children and in those with less than 20% renal function (Powell, 1988).

Over the last decade, much scientific and clinical research has focused on the endocrine abnormalities associated with growth retardation in CRF and in particular the growth hormone (GH)- insulin like growth factor (IGF) system. In recent years, following initial experimental research in the uremic rat model, using supraphysiological doses of porcine growth hormone (Mehls and Ritz, 1983) and more recently, recombinant human growth hormone (rhGH) (Mehls, Ritz, Hunziker, *et al*, 1988), several clinical trials, each involving small numbers of children, have reported improved growth in short children with CRF treated with rhGH

(Hokken-Koelega, Stijnen, De Muinck Keizer-Schrama, *et al*, 1991; Lippe, Fine, Koch, *et al*, 1988; Rees, Rigden, Ward, *et al*, 1990; Tonshoff, Mehls, Schauer, *et al*, 1989; van Renen, Hogg, Sweeney, *et al*, 1992) and in short children following renal transplantation (Hokken-Koelega, Stijnen, Keizerschrama, *et al*, 1993; Tonshoff, Haffner, Mehls, *et al*, 1993).

1.2 Growth Hormone

The isolation of GH from bovine pituitary glands was first reported in 1944 (Li and Evans, 1944) and was followed in 1956 by isolation from human pituitary glands (Li and Papkoff, 1956). The major physiological form of circulating human growth hormone (hGH) is a single chain polypeptide made up of 191 amino acids with a molecular weight of 22 kilodaltons (kDa) (Martha and Krieg, 1991). A 20 kDa hGH molecule is the second most common form found in the circulation, while fragments and an acidic form make up the rest.

1.2.1 Regulation of synthesis

Human growth hormoneis produced, stored and secreted by specialised cells called somatotropes in the anterior lobe of the pituitary gland. Regulatory factors produced by the hypothalamus and released into the hypophyseal portal system control the activity of these cells, namely GH-releasing hormone (GHRH) (Frohman and Jansson, 1986) and an inhibitory factor known as somatostatin (Brazeau, Vale, Burgus, *et al*, 1978). These two factors are alternately released and inhibited, leading to the intrinsic hypothalamic-somatotroph rhythm controlling GH secretion. hGH, itself, appears to maintain this rhythm by feeding back to the hypothalamus to stimulate somatostatin and inhibit GHRH (Kelijman and Frohman, 1991).

Thyroid hormone, glucocorticoids and gonadal steroids also have an influence on GH secretion (Devesa, Lima and Tresguerres, 1992). Nutrition is an important factor in GH regulation (Ketelslegers, Maiter, Maes, *et al*, 1996), with nutritional deprivation of 2-5 days having been shown to increase GH secretion in healthy young men (Vance, Hartman and Thorner, 1992). Undernourished or fasted men, or patients with anorexia nervosa, display increased basal and peak serum GH levels, which can be reversed by refeeding (Hochberg, Phillip, Youdim, *et al*, 1993). The mechanisms involved in this regulation are not fully understood. A further factor affecting GH levels is metabolic acidosis. Experimental rats, rendered acidotic using ammonium chloride, display significantly reduced levels of serum IGF-I, inhibition of hepatic IGF-I gene expression and hepatic GH receptor messenger ribonucleic acid (mRNA) (Challa, Chan, Krieg, *et al*, 1993). IGF-I, itself, regulates growth hormone secretion by a negative feedback circuit which operates at both the pituitary and hypothalamic levels (Kelijman and Frohman, 1991).

1.2.2 Receptor

The GH receptor in man and rabbits has been purified and the protein sequence determined. (Leung, Spencer, Cachianes, *et al*, 1987). The cloned cDNA sequences predict a single centrally located membrane-spanning structure consisting of 620 amino acids with a molecular weight of 70 kDa. It has an extracellular domain as well as a transmembrane and intracellular domain and it's structure is closely related to the prolactin receptor and shows some homology with various cytokine receptors (Carlsson, Eden, Nilsson, *et al*, 1991). The intracellular domain has recently been shown to be important for signal transduction (Moldrup, Allevato, Dyrberg, *et al*, 1991) and the extracellular domain is responsible for highly specific hGH

binding. It appears that the receptor undergoes cycles of internalization and recycling that are synchronous with the frequency of the GH pulses (Bick, Youdim and Hochberg, 1989). GH binds to the receptor in a 1:2 stoichiometrical manner and dimerization of two GH receptor molecules after hormone binding may be the initial step in hormone induced signal induction (Norstedt, Enberg, Francis, *et al*, 1994). Recent studies by Werther *et al* support evidence for direct effects of GH in multiple mesenchymal tissues, including the growth plate, bone, hemopoietic tissue and skin (Werther, Haynes and Oakes, 1993). The gene for the human growth hormone receptor has been localised to chromosome 5 and its structure has been characterised (Godowski, Leung, LR, *et al*, 1989).

1.2.3 GH binding protein (GHBP)

The GHBP is a high-affinity, low-capacity binding protein with a molecular weight of 60-65 kDa (Baumann, Stolar, Amburn, *et al*, 1986; Herington, Ymer and Stevenson, 1986). Leung *et al*, established the structural identity of the GHBP as the extracellular, hormone-binding domain of the membrane-bound receptor, suggesting that it may be derived by specific proteolysis from the extracellular portion of the GH receptor in man and rabbits (Leung, Spencer, Cachianes, *et al*, 1987). A second, lower-affinity, high-capacity GHBP has been identified, which does not appear to be related to the GH receptor and binds the 20kDa hGH peptide preferentially (Baumann and Shaw, 1990). The exact physiological role of the GHBP is not clear (Martha and Krieg, 1991), however, it may modulate the interaction between GH and its receptor by altering the half-life of the hormone, or by modulating the free GH concentration to which target cells are exposed during pulses of secretion (Carlsson, Eden, Nilsson, *et al*, 1991; Herington, Tiong and Ymer, 1991).

1.2.4 Growth Hormone actions

hGH plays a vital regulatory role in determining the rate of postnatal growth and ultimately determines the final statural size of the individual (Isakkson, Lindahl, Nilsson, *et al*, 1987). In addition, hGH has physiological effects on body composition which include anabolic and lipolytic actions and effects on extracellular water (Bengtsson, Brummer and Bosaeus, 1990), as well as potent effects on bone and mineral metabolism (Salusky and Goodman, 1995). GH secretion also increases transiently after exercise, and in response to stress. In addition, obesity, depression and hyperglycemia can reduce basal and stimulated secretion of GH (Corpas, Harman and Blackman, 1993). The fact that it continues to be produced after cessation of growth, remaining the most abundant pituitary hormone, suggests that it continues to exert these important metabolic functions into adult life (Ho, Kelly and O'Sullivan, 1993). The mechanism by which hGH causes cell proliferation and longitudinal growth in bone has been elucidated (Isaksson, Ohlsson, Nilsson, *et al*, 1991), and it appears to have both direct and indirect actions. GH can induce the differentiation of precursor cells by stimulating the production of local growth factors, for example IGF-I.

1.2.5 Growth hormone secretion

Growth hormone secretion is pulsatile. In man, secretory bursts of the hormone are released 4-8 times in a 24 hour period and this pattern of release seems to be related to the optimal induction of peripheral physiological effects (Devesa, Lima and Tresguerres, 1992). It was noted that GH target tissues appear to be more sensitive to the frequency of the hormone over

a period of time, rather than the total amount secreted during that time (Robinson and RG, 1987). hGH pulsatility also varies with age. Multiple-parameter deconvolution analysis of 24 hour serum GH concentrations in normal boys at various stages of puberty was performed by Martha *et al* (Martha, Gorman, Blizzard, *et al*, 1992). This analysis mathematically removed the influence of hormone clearance kinetics, so that the secretory events leading to the changes in serum GH concentration could be measured. The augmented serum GH concentration observed at puberty appears to be due to an increased amount of GH released at each secretory episode, with the secretion rate being elevated 2- to 3- fold. Rising levels of gonadal steroid hormones during puberty appear to be involved in the regulation of GH at this time (Rogol, 1992).

1.3 Insulin-like growth factors

In 1957, a hormonally controlled serum factor, called "sulphation factor", which stimulated sulphate incorporation by cartilage *in vitro* was described (Salmon and Daughaday, 1957). In 1963, Froesch, *et al*, described factors in human serum which could exert insulin-like effects, but could not be suppressed by the simultaneous addition of anti-insulin antibody; these factors were called nonsuppressible insulin-like activity (NSILA) (Froesch, Burgi, Ramseier, *et al*, 1963). A phenomenon, which was termed "multiplication-stimulating activity," in the conditioned medium of rat liver cells was discovered in 1973 (Dulak and Temin, 1973), and it later became evident that all three terms were describing a similar, if not identical, group of substances with growth promoting and insulin-like actions and researchers in the field agreed on the term "somatomedin" in 1972.

In 1978, Rinderknecht and Humble described the structural sequence of NSILA and showed it to be 48% homologous with human proinsulin (Rinkerknecht and Humbel, 1978). It was therefore renamed insulin-like growth factor-I (IGF-I). Following this, another biologically active insulin-like molecule was found by Rinderknecht and Humble to be similar to, but not identical to IGF-I, and it was named insulin-like growth factor-II (IGF-II) (Rinderknecht and Humbel, 1978a). IGF-I and IGF-II are single-chain polypeptides, made up of 70 and 67 amino acids respectively, with molecular weights of 7.6 and 7.4 kilodaltons (Sara and Hall, 1990). These two peptides display a 65% homology with one another and a 43% and 41 % homology with insulin (Daughaday and Rotwein, 1989).

1.3.1 Receptors

The IGFs exert their effects by binding to three different types of receptors situated on the cell membranes, *viz*, the insulin receptor, and the type I and type II receptors (Cohick and Clemmons, 1993c). In 1972, Hintz *et al* showed that IGF-I competed with insulin for binding to insulin receptors (Hintz, Clemmons, Underwood, *et al*, 1972), however, once purified, iodinated IGF-I was also found to bind to a receptor that was distinct from the insulin receptor, the IGF-I or type I receptor (Marshall, Underwood, Voina, *et al*, 1974). Further binding studies uncovered the existence of a receptor which had preferential affinity for IGF-II over IGF-I, and did not bind insulin. This IGF-II, or type II, receptor, is identical to the cation-independent mannose 6-phosphate receptor (Rechler and Nissley, 1990).

1.3.2 Genes

The human IGF-I gene was first characterised in 1983 (Jansen, Van Schaik, Ricker, *et al*, 1983) and the IGF-II gene in 1988 (De Pagter-Holthuizen, Jansen, Van Schaik, *et al*, 1988). The single gene locus for IGF-I has been identified on the long arm of human chromosome 12 and the IGF-II locus on the short arm of chromosome 11 (Bell, Gerhard, Fong, *et al*, 1985). Both IGF-I and IGF-II genes have been found to be expressed in a great variety of cells and organs, suggesting not only an endocrine, but also paracrine and autocrine functions (Sara and Hall, 1990).

1.3.3 Biological actions

The insulin-like growth factors make up a family of peptides which have cell proliferation, cell differentiation and insulin-like metabolic effects (Froesch, Zenobi and Hussain, 1994a). Circulating IGFs have endocrine functions, while those IGFs synthesised locally by many other individual tissues are thought to act by autocrine and/or paracrine mechanisms (Holly and Wass, 1989). IGF-I appears to be one of the primary regulators of post-natal growth and has been shown to act as a progression factor in the DNA synthesis and replication of cells. As yet, however, the intracellular mechanisms involved in these events have not been defined (Cohick and Clemmons, 1993c). IGF-I has been shown to be a potent hypoglycemic hormone in healthy human volunteers, also causing falls in serum triglyceride levels, and in the ratio of total to high density lipoprotein cholesterol (Guler, Zapf and Froesch, 1987). In addition, increased renal plasma flow and GFR have been observed (Guler, Schmid, Zapf, *et al*, 1990). Froesch *et al*, demonstrated that IGF-I increases energy expenditure and lipid oxidation, and has a protein-sparing effect in normal subjects (Froesch, Zenobi and Hussain, 1994a). Lewitt

suggests that the IGFs may play a greater role in glucose homeostasis than had previously been considered, and that the actions of the IGFs and insulin may, in fact, be complementary (Lewitt, Saunders, Phuyal, *et al*, 1994). IGF-II has been associated with fetal and placental growth and development (Rutanen and Pekonen, 1990), as well as in association with tumours *e.g.* Wilms tumour (Renqiu, Ruelicke, Hassam, *et al*, 1993). The IGFs circulate throughout the body bound to a family of carrier proteins known as insulin like growth factor binding-proteins (IGFBPs) so that the amount of unbound or "free" IGF in the circulation at any one time is probably less than 1% (Cohick and Clemmons, 1993c).

1.4 IGF binding proteins

The complete structures of six different IGFBPs have been determined (Shimasaki, Gao, Shimonaka, *et al*, 1991b; Shimasaki, Shimonaka and Zhang, 1991a) and they have been named IGFBP-1 through to -6 (Drop, Brinkman, Kortleve, *et al*, 1991). None of these proteins binds to insulin and they may therefore be responsible for some of the different physiological responses between insulin and the IGFs, despite their binding to similar receptors (Clemmons, 1993). The IGFBPs have been proposed to have four major functions that coordinate and regulate the biological activities of the IGFs. Jones and Clemmons report these as: acting as transport proteins in plasma and, in addition, controlling the movement of the IGFs from the intravascular compartment; prolonging the half-lives of the IGFs and regulating their metabolic clearance; providing a way to ensure tissue- and cell type-specific localization; and to regulate the biological actions of the IGFs by modulating their interactions with their receptors (Jones and Clemmons, 1995). Two different sized molecular weight complexes are formed by the IGF-IGFBP associations *viz.*, a large 150kDa molecular weight unit and smaller 30 - 50 kDa

forms. The 150 kDa complex is made up of an IGF peptide, a IGFBP-3 subunit and an acid-labile subunit (Baxter, Martin and Beniac, 1989). The function of this ternary complex appears to be as a storage reservoir in the circulation, by prolonging the half-life of the growth factors from approximately 10 minutes in the unbound form, to 10-15 hours when complexed (Baxter, 1991). The role of the smaller IGF-IGFBP, binary complex may be in the transport of the IGFs to their specific target cells and modulation of their receptor binding, growth-promoting and metabolic actions (Clemmons, 1990). The IGFBPs may serve to inhibit (Zapf, Schoenle, Jagars, *et al*, 1979), or in some cases potentiate, the actions of the IGFs (Elgin, Busby and Clemmons, 1987). The mechanism by which this occurs may be regulated by specific proteases for the IGFBPs, which may cleave the proteins into forms which have a reduced affinity for the IGFs. Furthermore, phosphorylation or differential localisation of the IGFBPs, may affect the actions of the IGFs (Clemmons, 1993). In addition, the IGFBPs themselves, may have direct effects on the cells which are independent of, but possibly modulated by, the IGFs (Jones and Clemmons, 1995).

1.4.1 IGFBP-3

IGFBP-3 is the most abundant IGFBP in the circulation in postnatal life and is the only IGFBP to appear in the 150 kDa complex (Martin and Baxter, 1992). It is GH dependent, and is synthesised in a variety of tissues other than the liver and can therefore modulate the actions of the IGFs in extracellular fluids as well as in the circulation. It is an acid stable glycoprotein which appears as a doublet on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) with a mass corresponding to a molecular weight of 47-53 kDa (Baxter and Martin, 1986). IGFs have a much higher affinity for soluble IGFBP-3 than for the IGF type-I

receptor and thus IGFBP-3 can prevent receptor interaction (Clemmons, 1992). Cell-associated IGFBP-3, however, which may be non-glycosylated, has a reduced affinity for the IGFs, making the growth factors more available for binding and enhancing receptor interactions (Conover, Bale, Clarkson, *et al*, 1992). In addition, Lamson and co-workers, have demonstrated that proteolysis of IGFBP-3 may be a common regulatory mechanism of IGF action in vivo (Lamson, Giudice and Rosenfed, 1991).

The acid-labile subunit (ALS), in association with IGFBP-3 and an IGF peptide, plays a vital role in the 150 kDa complex formation in the circulation and therefore needs to be included in discussions of IGFBP-3. The ALS is an 85 kDa growth hormone dependent glycoprotein, produced by hepatocytes (Baxter, 1990). It is found in the circulation in concentrations in excess of those of the IGFs and IGFBP-3, which are present in approximately equimolar concentrations (Baxter and Martin, 1986). It contains a sequence of leucine-rich amino-acids, commonly found in those proteins which are involved in protein-protein interactions (Leong, Baxter, Camerato, *et al*, 1992). The ALS appears to have no effect on the binding of the IGFs to IGFBP-3, but has a high affinity for IGFBP-3 when associated with an IGF peptide (Baxter, Martin and Beniac, 1989). The main function of the ALS appears to be its binding with the IGF-IGFBP-3 complex, increasing its molecular weight, thereby preventing access by the IGFs to the tissues. However, Barreca and co-workers, have recently, postulated that the ALS may have a more complex function than was previously thought, possibly by also acting to enhance the binding of IGFs to IGFBP-3 (Barreca, Ponzani, Arvigo, *et al*, 1995).

1.4.2 IGFBP-2

SDS PAGE analysis of human serum revealed a band at 34 kDa which preferentially bound IGF-II (Hardouin, Hossenlopp, Segovia, *et al*, 1987). Subsequently, human IGFBP-2 has been sequenced by Binkert *et al* and Zapf *et al* (Binkert, Landwehr, Mary, *et al*, 1989; Zapf, Kiefer, Merryweather, *et al*, 1990). IGFBP-2 is nonglycosylated and is found in the smaller 30-50 kDa IGF-IGFBP complex in the serum (Baxter, 1991), displays a 10-20 times greater affinity for IGF-II over IGF-I (Rogani, Hossenlopp, Lepage, *et al*, 1989) and is regulated primarily by IGF-II (Blum, Horn, Kratzsch, *et al*, 1993). Serum concentrations of IGFBP-2 demonstrate a marked age-dependence, with high levels at birth and senescence, and low levels at puberty. In addition, markedly raised levels of IGFBP-2 were found in CRF, non-islet cell tumour induced hypoglycemia and in leukaemias (Zapf, Kiefer, Merryweather, *et al*, 1990). IGFBP-2 may interact with and permeate the capillary endothelium, serving to transport the IGFs to their target cells (Ooi, 1990). The molecular weight of IGFBP-2 in plasma can be altered by proteolysis induced by starvation in young children (Pucilowska, Davenport, Kabir, *et al*, 1993) and its half life indicates that it is cleared much more rapidly from the circulation than the large ternary complex (Guler, Schmid and Froesch, 1989). Baxter suggests that IGFBP-2 may serve to regulate IGF availability when the 150 kDa complex is not able to carry all the available IGFs (Baxter, 1991).

1.4.3 IGFBP-1

IGFBP-1 has a predicted molecular mass of 25.3 kDa and is produced primarily by the liver, but also by pregnant human endometrium (Lee, Conover and Powell, 1993). It appears on

SDS PAGE at about 28 kDa (Baxter, Martin and Wood, 1987) and although it shares sequence homology with the other known IGFBPs, it is unique in that its levels may vary more than 10-fold over a very short period of time (Cotterill, Holly, Amiel, *et al*, 1993). It is rapidly cleared from the circulation in response to food intake and appears to be under the regulation of insulin by rapid inhibition of transcription (Lee, Jensen, Divertie, *et al*, 1993; Suikkari, Koivisto, Rutanen, *et al*, 1988). It is also thought to be a potential transporter of IGFs from the circulation to the tissues (Holly, 1991). IGFBP-1 is an important modulator of the insulin-like activity of the IGFs and plays a role in glucose counterregulation by linking the availabilty, and thereby the actions of, the IGFs with acute, albeit temporary, changes in nutritional status (Cotterill, Holly, Amiel, *et al*, 1993; Lewitt, 1994).

1.4.4 IGFBPs -4, -5 and -6

Although IGFBPs -4, -5, and -6 have been purified from human fluids and their sequences delineated, not much is known of their actions in vivo at this stage. As these IGFBPs do not pertain to most of the work presented in this thesis, they will not be discussed in detail. IGFBP-4 was purified from human serum (Kiefer, Schmid, Waldvogel, *et al*, 1993) where it appears as a band at 24 kDa on SDS PAGE and has a possible role in bone metabolism (Mohan, 1993). IGFBP-5 has also been purified from human bone (Bautista, Baylink and Mohan, 1991) and from human CSF and is thought to be involved in bone formation and resorption (Mohan, 1993). IGFBP-6 was purified from human serum (Zapf, Kiefer, Merryweather, *et al*, 1990) and CSF in adults and children (Rogani, Hossenlopp, Lepage, *et al*, 1989). Serum concentrations of IGFBP-6 have been found to be similar to those for IGFBP-2 (Baxter and Saunders, 1992).

1.5 Effects of Chronic Renal Failure on GH and IGFs

1.5.1 Growth Hormone

Studies of circulating levels of growth hormone in children with CRF have been found to reveal conflicting results, with both normal (Hokken-Koelega, Hackeng, Stijnen, *et al*, 1990; van Renen, Hogg, Sweeney, *et al*, 1992) and elevated (El-Bishti, Counahan, Bloom, *et al*, 1978) concentrations having been reported. The inconsistency in these results is likely to be due to the different methods of measuring GH levels, by stimulation or 24 hour excretion, and the different populations of children with renal disease investigated. Hokken-Koelega and co-investigators found that 66% of children with severe CRF, treated both conservatively and by means of dialysis, studied over a 24 hour period, displayed normal GH secretion, albeit with a wide variation. These children showed evidence of regular pulses and diurnal pattern, indicating that GH regulation in CRF remains intact. However, elevated GH levels were noted in a small group, particularly girls, with end-stage nephrotic syndrome, who also displayed an abnormal secretory pattern. Increased urinary GH excretion was found in the CRF subjects compared to normal controls (Hokken-Koelega, Hackeng, Stijnen, *et al*, 1990).

Higher mean GH values and secretion peaks have been observed in children on dialysis compared with those on conservative therapy, whilst the lowest levels were found in transplanted patients (Schaefer, Hamill, Stanhope, *et al*, 1991). An increased number of nocturnal GH secretory bursts in children with end stage renal failure have been reported as

determined by deconvolution analysis (Tonshoff, Veldhuis, Heinrich, *et al*, 1995). The authors also describe an increase in the half-life of GH in both pre-terminal, but more so in end-stage, renal failure. This finding is attributable to the fact that renal metabolism and clearance are major routes of removal of endogenous GH from the circulation. To further substantiate these results, the authors also described an inverse correlation between GH half-life and declining renal function as measured by glomerular filtration rate (GFR) (Tonshoff, Veldhuis, Heinrich, *et al*, 1995).

A delayed start to the pubertal growth spurt in CRF, with low minimal and peak height velocities has been reported (Scharer, Mehls, Schaefer, *et al*, 1993), although the absolute increase in peak height velocity was normal. Therefore, poor pubertal growth, together with progressive skeletal maturation, eventually results in an irreversible loss of growth potential. A diminished GH response to the normal stimulatory effects of increasing circulating gonadal steroids occurs in and around puberty in CRF, particularly in boys (Schaefer, Veldhuis, Stanhope, *et al*, 1994). An inverse relationship between GH peak amplitude and corticosteroid dosage was observed in patients with renal grafts, leading to the conclusion that glucocorticoid therapy and obesity in transplanted children were the main factors contributing to the low levels of GH secretion (Schaefer, Hamill, Stanhope, *et al*, 1991). More recently, Schaefer *et al* have found a reduced GH production, but a normal GH half-life in transplanted peripubertal boys (Schaefer, Veldhuis, Stanhope, *et al*, 1994).

1.5.1 GHBP

Postel-Vinay and co-workers, found that plasma GH binding activity was reduced in children with CRF and suggested that this finding may result from a reduction in hepatic GH receptors (Postel-Vinay, Tar, Crosnier, *et al*, 1991). A similar conclusion was drawn from a study in adults with CRF (Maheshwari, Rifkin, Butler, *et al*, 1992). The mechanism for this proposed reduction in the number of GH receptors in CRF has yet to be elucidated. Recently, a decrease in hepatic GH receptor gene expression in pair-fed experimental uraemic rats has been demonstrated (Tönshoff, Eden, Weiser, *et al*, 1994), however analogous GH receptor studies in human tissues cannot be conducted because of inaccessibility and ethical reasons (Bauman, 1996). Preliminary clinical data from one study, which conflicts with previous findings, suggests increased levels of immunoreactive GHBP in some children with end-stage renal failure (Kratzsch, Blum, Selisko, *et al*, 1993). It may be possible, therefore, that both the increased GHBP levels and decreased GH receptor numbers may play a part in the "GH resistance" described both in humans (Blum, Ranke, Kietzmann, *et al*, 1991) and experimental animals with CRF (Mehls, Ritz, Hunziker, *et al*, 1988).

1.6 Insulin-like growth factors

Circulating levels of the IGFs in CRF have been found to vary considerably depending on the method of analysis employed. Low serum IGF-I levels by RIA, and elevated IGF-II levels by RRA, have been reported (Goldberg, Trivedi and Delmez, 1982), while other investigators describe elevated concentrations of both IGF-I and -II (Enberg and Hall, 1984). In 1984, a low

molecular weight inhibitor was found in uraemic serum, containing normal IGF levels, which affected the stimulatory activity of the IGFs on rat cartilage in bioassay experiments (Phillips, Fusco, Unterman, *et al*, 1984). Unsaturated carrier proteins in unextracted and even acid-ethanol extracted serum, were found to interfere with the RIA for IGF-I, as well as the RRA for IGF-II, in uraemic adult patients (Powell, Rosenfeld, Baker, *et al*, 1986) and once the binding proteins were removed by acid chromatography before assay, normal serum concentrations of IGF-I and -II in patients with CRF were found. However, elevated IGF-II values by RRA, after acid chromatography separation, were found in a group of children undergoing dialysis (Powell, Rosenfeld, Sperry, *et al*, 1987.) These results led to the conclusion that an increase in total IGF binding, due to an excess of binding proteins over total IGF concentration, accounted for the decreased IGF bioactivity, and that this was probably due to reduced renal clearance in CRF (Blum, Ranke, Kietzmann, *et al*, 1991). This hypothesis is further supported by Mehls and colleagues who have reported that IGF bioactivity is reduced in patients on haemodialysis compared with pre-terminal renal failure, and that the bioactivity increases after renal transplantation (Mehls, Scheafer and Tonshoff, 1991).

1.7 IGFBPs

The decreased bioactivity of IGF in CRF may be due to both a significant excess of binding proteins over IGFs (Lee, Hintz, Sperry, *et al*, 1989; Powell, Rosenfeld, Sperry, *et al*, 1987), and a reduction in IGF secretion (Blum, 1991). This increase in the IGF binding capacity results in a reduction in IGF bioactivity compared with normal age and sex-matched controls (Blum, 1991). Removal of the excessive IGFBP by affinity chromatography resulted in a significant increase in IGF bioactivity (Blum, Ranke, Kietzmann, *et al*, 1991).

Increased amounts of small molecular weight IGFBPs have been identified by WLB (Liu, Powell and Hintz, 1990) and RIA (Blum, 1991) in the serum of uremic patients. Normal serum concentrations of IGFBP-3 have been reported (Hodson, Brown, Roy, *et al*, 1992), in contrast to the increased levels reported by others (Blum, Ranke, Kietzmann, *et al*, 1991; Lee, Hintz, Sperry, *et al*, 1989). These discrepancies are probably due to different antibody specificities. Normal amounts of 41 and 38 kDa IGFBP-3 forms, in the high molecular weight binding region of CRF serum have been found, however, IGFBP-3 fragments of 19 and 14 kDa were also discovered after cross-linking and immunoprecipitation of low molecular weight IGF-IGFBP fractions of uremic sera (Powell, Liu, Baker, *et al*, 1993). These fragments, supposedly derived by proteolysis, have been shown to be immunologically active, but have a reduced affinity for radioactive IGFs in that they are not able to be identified by Western ligand blotting. Elevated levels of IGFBP-1 have been found by radioimmunoassay, immunoprecipitation and ligand binding in CRF serum (Blum, Ranke, Kietzmann, *et al*, 1991; Lee, Conover and Powell, 1993; Lee, Hintz, Sperry, *et al*, 1989; Powell, Liu, Baker, *et al*, 1993). Serum IGFBP-2 levels have also been shown to be increased in CRF by WLB, and more recently by specific RIA (Blum, Horn, Kratzsch, *et al*, 1993; Powell, Liu, Baker, *et al*, 1993).

Children receiving renal replacement therapy by dialysis have been shown to have clearly increased levels of IGFBPs -1, -2 and -3, which were more pronounced than those noted in children with preterminal renal disease. This finding suggests that accumulation of the small molecular weight IGFBPs, in particular IGFBP-3 fragments, results from reduced renal clearance (Tonshoff, Blum and Mehls, 1996). Decreased IGF bioavailability, as a result of increased serum levels of IGFBP-3, together with corticosteroid immunosuppresive therapy

has been suggested to account for the poor growth velocity seen in children after renal transplantation (Hokken-Koelega, Stijnen, Keizerschrama, *et al*, 1993). Recombinant growth hormone treatment has successfully improved the growth velocity of short children with CRF, reportedly by overcoming the increased IGF binding capacity by stimulating IGF-I levels over and above those of the IGFBPs (Hokken-Koelega, Stijnen, De Muinck Keizer-Schrama, *et al*, 1991; Tonshoff, Mehls, Schauer, *et al*, 1989). Further research of the IGFBPs will no doubt help clarify how the alteration of these proteins in CRF affects IGF bioactivity and therefore growth in children.

CHAPTER TWO

MATERIAL AND METHODS

2.1 Introduction

In order to further increase our understanding of one of the most recently researched causes of growth failure in CRF, *i.e.*, disturbances of the GH - IGF axis, including the role of the IGFBPs, we analysed serum from children with varying degrees of renal failure who were followed by our Unit. In addition, we proposed to determine the effects of rhGH treatment and transplantation on IGF-I, -II and their binding proteins. To compare and contrast the IGFs and IGFBPs of these children with subjects without renal disease, we also obtained serum from normal controls. We aimed wherever possible to match the children by age and sex.

A variety of techniques were carried out on the samples in order to characterise the IGFs and IGFBPs in the serum. The methodology discussed in this Chapter is common to the subsequent experimental chapters, however further detailed information regarding composition of buffers *etc* is contained in appendix A.

2.2 Patients and controls

2.2.1 Renal failure patients

All of the renal failure children who were managed conservatively were on a reduced protein and reduced phosphate diet with additional calories, which has, in our hands, proved successful in moderating uremia and controlling secondary hyperparathyroidism (Jureidini, Hogg, van Renen, *et al*, 1990). This was in addition to the routine medical management of fluids and electrolytes, metabolic acidosis, anemia and control of hypertension. Those children who

received renal replacement therapy underwent hemodialysis, in general, three times per week. Those who had received transplants were managed with routine immunosuppresive therapy of prednisolone, cyclosporin and azothioprine. The children were from both sexes and ranged in age from 2 to 17 years. Most had been followed in the Unit for two or more years. Ten boys and one girl (Chapter 5), who were treated with conservative medical, as well as dietary management, were, in addition, treated for two years with recombinant human growth hormone for short stature as a consequence of CRF (van Renen, Hogg, Sweeney, *et al*, 1992). An additional aliquot of blood was collected from the children at the time of venepuncture for routine biochemical tests and was analysed retrospectively for IGF-I, IGF-II and IGFBPs 1-4. The blood was collected into plain tubes containing no anticoagulant and, after being allowed to clot at room temperature, was spun at 3,000 rpm at 4° C. The serum was aspirated immediately and stored at -80° C.

2.2.2 Normal controls

A small additional volume of blood was obtained from normally growing prepubertal male and female children who attended the hospital for a non-renal medical reason requiring a venepuncture. Signed parental consent was obtained and approval was given by the Hospital Ethics Committee. Serum from normal pubertal females was generously supplied by the Department of Chemical Pathology, Women's and Children's Hospital, Adelaide, from a batch of samples stored from a previous study. Pubertal male serum was obtained from the author's own teenage sons. The blood was allowed to clot at room temperature, spun at 3,000 rpm at 4° C and the aspirated serum stored at -80° C.

2.3 Radioligands

Radiolabelled peptides, both ^{125}I-IGF-I and ^{125}I-IGF-II, were required for ligand binding studies, radioimmunoassays for IGF-I, radioreceptor assays for IGF-II, and also for analytical gel filtration experiments. The growth factors were supplied by GroPep Pty. Ltd., Adelaide, Australia. Iodination of the peptides was performed by Mr. Spencer Knowles and Mr. Callum Gillespie using the chloramine T method as described by Van Obberghen-Schilling *et al* (Van Obberghen-Schilling and Poussegur, 1983). Sodium ^{125}I was obtained from Amersham Australia Pty. Ltd., Castle Hill, NSW, Australia.

2.4 Western ligand blotting (WLB)

Ligand blots were used to characterise the IGFBPs present in serum by determining their respective molecular masses and their binding of radiolabelled IGF-I and -II (Hossenlopp, Seurin, Segovia-Quinson, *et al*, 1986). 20 ml of serum was mixed with 180 ml 4x dissociating buffer (see Appendix A) and heated at 65° C for 15 minutes. The molecular mass standards (Rainbow ^{14}C protein molecular weight markers; Amersham International, UK), were also incubated in the dissociating buffer and heated for 15 minutes. The standards (10 ml) and samples (20 ml of sample plus dissociating buffer, resulting in an equivalent volume of 2 ml of serum) were then loaded onto a 1.5 mm thick discontinuous sodium dodecyl sulphate (SDS) polyacrylaminde gel (Laemmli, 1970) using a Hamilton syringe. The standards were loaded into the wells at each end of the gel, while the samples were loaded into the wells between these outside wells. The proteins were stacked through a 4% (w/v) polyacrylamide gel and separated in a 10 % (w/v) gel under non-reducing conditions overnight at 15 mA using a

Hoefer SE 600 Vertical Slab Unit (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.) and a PS 500X DC Power Supply (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). Electrophoretic transfer of the proteins to nitrocellulose filters (Schleicher and Schuell Inc., Keene, NJ, U.S.A.) followed the next day, according to the method of Towbin *et al* (Towbin, Staehelin and Gordon, 1979), for 3 hours at 300 mA using a Hoefer TE series Transphor (Hoefer Scientific Instruments). The nitrocellulose sheets were then air dried overnight before probing with radiolabelled IGF-I and/or -II. The sheets were washed in Western buffer (see appendix A) with 1% (w/v) Triton X-100 (Ajax Chemicals, Auburn, NSW, Australia) for 30 minutes at room temperature. Non specific binding sites were blocked using Western buffer with 1% (w/v) bovine serum albumin (BSA-RIA grade, Sigma Chemical Co., St. Louis, MO, U.S.A.) for 1.5 hours. The sheets were then washed for 10 minutes in Western buffer with 0.1 % (w/v) Tween 20 (BDH Ltd., Poole, Dorset, U.K.). Probing with ^{125}I-IGF-I or ^{125}I-IGF-II (approximately 10 x 10^6 cpm) in Western buffer, 1 % (w/v) BSA and 0.1 % (w/v) Tween 20, took place for 2 hours at room temperature. The nitrocellulose filters were finally washed for 2 hours in Western buffer with 0.1 % (w/v) Tween 20, with the solution being changed every 15 minutes, before being allowed to air dry. Autoradiography (X-ray film; Konica, Tokyo, Japan) took place at -80° C using cassettes with intensifying screens (Du Pont, Wilminton, MA, USA). In addition, proteins contained in pooled serum samples separated by fast protein liquid chromatography (2.6) into different molecular size IGF-IGFBP complexes, were further separated by SDS PAGE using the Hoefer Mighty Small II minigel system (Hoefer Scientific Instruments, San Francisco, CA, USA).

2.5 Western immunoblotting (WIB)

Immunoblotting was performed to identify specific IGFBPs occurring at different molecular weights after separation by SDS polyacrylamide gel electrophoresis (PAGE) of both whole serum, and serum fractionated by analytical gel filtration chromatography (see 2.6). In particular instances (see 5.3.2), the nitrocellulose filters used initially for WLB were further used for immunoblotting purposes. Specific antibodies to human IGFBP-1, -2, -3 and -4 were employed to identify each individual species of IGFBP. IGFBP-1 (A2) (Baxter, Martin and Wood, 1987) and IGFBP-3 (R 100) (Baxter and Martin, 1986) antibodies were both very generously donated by Prof. Robert C. Baxter, (Department of Endocrinology, Kolling Institute of Medical Research, St. Leonards, NSW, Australia), who also kindly provided pure hIGFBP-1 (Baxter, Martin and Wood, 1987) and -3 (Baxter and Martin, 1986) as standards. Anti Madin-Darby bovine kidney cell (MDBK) antiserum, used to identify IGFBP-2, was a kind gift from Dr. P.C. Owens (CSIRO, Division of Human Nutrition, Adelaide, S.A., Australia) and anti-ovine IGFBP-4 antiserum was generously donated by Dr. P.E. Walton (formerly of the Co-operative Research Centre, Kintore Ave, North Adelaide, S.A., Australia). Antibodies to hIGFBP-1, -2, -3 and -4 were also purchased from Upstate Biotechnology Inc. (UBI) (Lake Placid, New York, U.S.A.). The nitrocellulose filters were blocked in 3 % (w/v) BSA in Tris-buffered saline (TBS) -Tween 20 buffer (see appendix A) at 4° C overnight to prevent non-specific binding and then washed in TBS-Tween. All other reactions took place at room temperature. Incubation with the primary antibody was for 1 hour with a 1:5000 dilution used for anti-hIGFBP-3 (R 100) and anti-hIGFBP-1 (A2) and a 1:1000 dilution used in the case of the UBI anti-hIGFBP-1 and -3 antibodies. The diluent was TBS-Tween buffer. The anti-hIGFBP-2 antibody was diluted 1:2000 and the anti-hIGFBP-4 antibody was used in a 1:1000 dilution. The ECL (enhanced chemiluminescence) Western blotting method (Amersham

International plc.) was used to visualise the proteins after incubation with a secondary horseradish peroxidase conjugated affinity isolated goat anti-rabbit immunoglobulin (Dakopatts, A/S, Denmark) at a 1:5000 diution for 1 hour. Visualisation of the IGFBPs occurred following 3 seconds to 6 minutes exposure to X-ray film (Konica, Tokyo, Japan).

2.6 Results of WLB and WIB

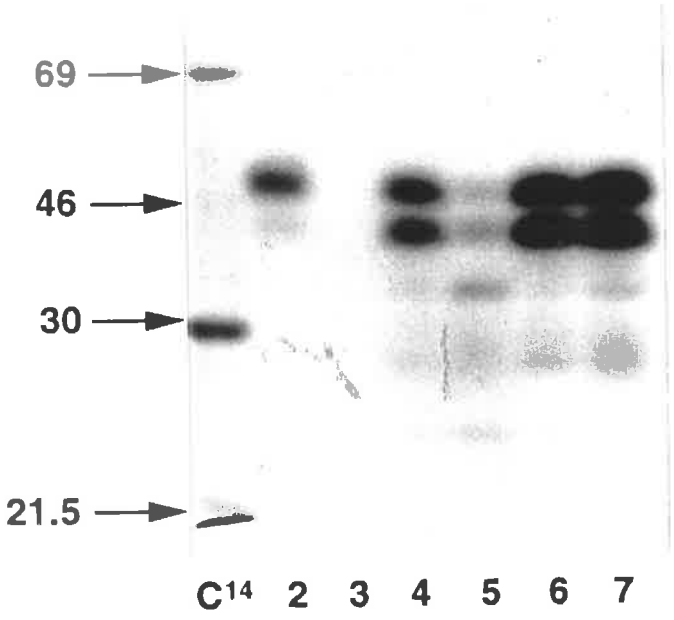
A ligand blot was performed, comparing prepubertal and pubertal CRF serum with that from normal prepubertal and pubertal boys respectively (see Figure 2.1). IGFBP-1 and -3 standards were included in this WLB to identify these proteins, initially by WLB and subsequently by immunoblotting with both anti-hIGFBP-1 and -3 antisera (see Figure 2.2). Additional WLBs were performed on individual CRF patient's sera and comparing CRF serum with age and sex-matched short and slowly growing children. The results of these WLB are discussed in the relevant chapters.

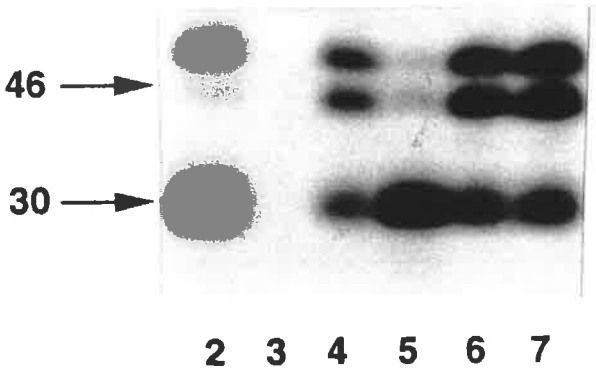
In Figure 2.1, the dominant protein bands identified by WLB in the serum was a doublet at 44-46 and 46-48 kDa. Further bands were identified at 34, 29 and 25 kDa on the ligand blot which were less intense. The IGFBP-3 standard (lane 2) appeared as a dominant band at 46-48 kDa, with a much fainter band at 44-46 kDa, whereas the IGFBP-1 standard (lane 3) did not appear to bind to the ligand at all. These findings are not dissimilar to those of Liu *et al*, where they report major bands at 41.5 and 38 kDa, with the fainter bands at 33, 28 and 23 kDa (Liu, Powell and Hintz, 1990).

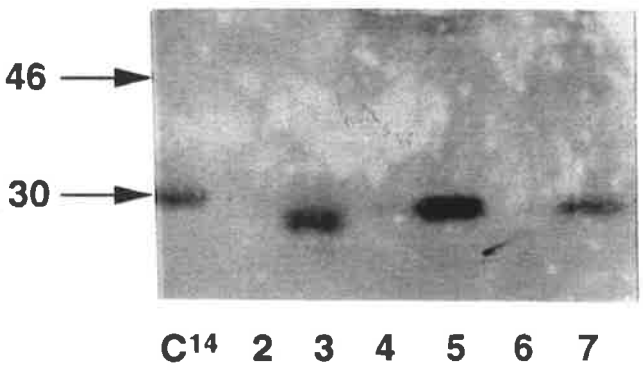
Figure 2.1 *Western ligand blot of IGFBP-3 and IGFBP-1 standard and normal and CRF prepubertal and pubertal serum.* Lane 2, IGFBP-3 standard; Lane 3, IGFBP-1 standard; Lane 4, prepubertal normal serum; Lane 5, prepubertal CRF serum; Lane 6 pubertal normal serum; Lane 7, pubertal CRF serum, ^{14}C molecular weight markers are shown in the left column.

Figure 2.2 *Western immunoblot of the Western ligand blot in Figure 2.1 with a specific anti-h IGFBP-3 antibody* (see Chapter 2.5). Lane 2, IGFBP-3 standard; Lane 3, IGFBP-1 standard; Lane 4, prepubertal normal serum; Lane 5, prepubertal CRF serum; Lane 6 pubertal normal serum; Lane 7, pubertal CRF serum, ^{14}C molecular weight markers are shown in the left column.

Figure 2.3 *Western immunoblot of the Western ligand blot in Figure 2.1 with a specific anti-h IGFBP-1 antibody* (see Chapter 2.5). Lane 2, IGFBP-3 standard; Lane 3, IGFBP-1 standard; Lane 4, prepubertal normal serum; Lane 5, prepubertal CRF serum; Lane 6 pubertal normal serum; Lane 7, pubertal CRF serum, ^{14}C molecular weight markers are shown in the left column.


69
46
30
21.5
C14 2 3 4 5 6 7

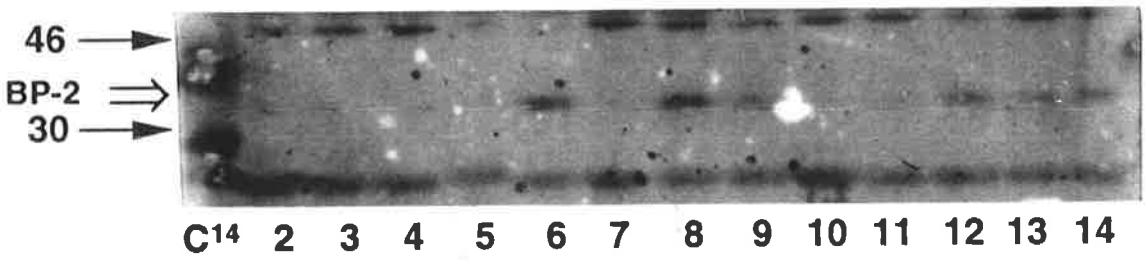

46
30
2 3 4 5 6 7

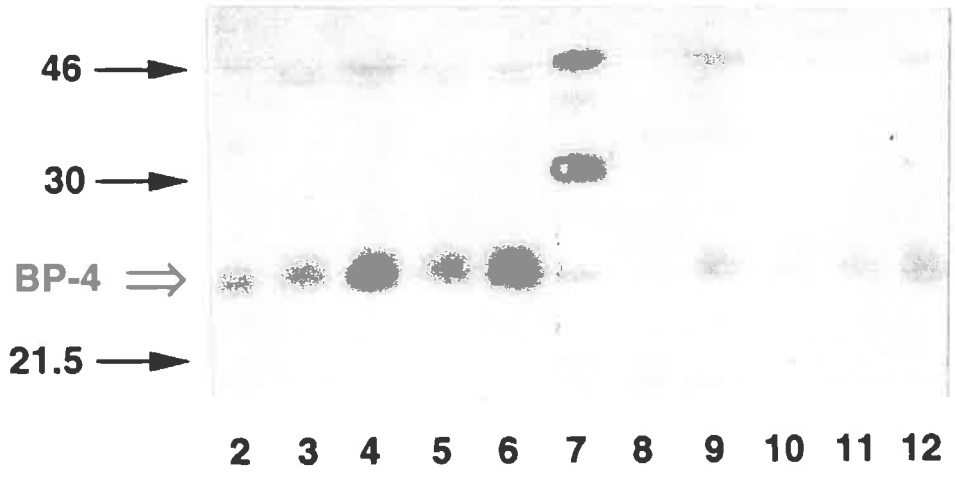

46
30
C14 2 3 4 5 6 7

The WLB shown in Figure 2.1 was immunoblotted with the IGFBP-3 antibody (Figure 2.2). The dominant band noted in the IGFBP-3 standard (Fig. 2, lane 2) was at 30 kDa, which did not appear at all on the ligand blot. Pure IGFBP-3 standard has been observed to be very unstable when stored at neutral pH (Baxter and Martin, 1986), reducing its activity in solution and this band is possibly due to some degradation of the IGFBP-3 standard resulting in a form which is immunoreactive but not able to bind to the ligand. A band at 46-48 kDa, and a fainter 44-46 kDa band, were also present in the IGFBP-3 standard on the WIB (lane 2), similar to the bands identified on the WLB (see Figure 2.1). The prepubertal and pubertal normal and CRF sera contained bands, identified by the IGFBP-3 antibody, at 44-46, 46-48 and 30 kDa. The 30 kDa IGFBP-3 form detected by WIB in all serum samples were not evident by ligand blotting (Fig. 2.1). This phenomenon has also been described by Kale *et al*, who found a 29 kDa IGFBP-3 band by WIB in normal and CRF sera which was not detected by ^{125}I-IGF-II ligand binding (Kale, Liu, Hintz, *et al*, 1996). The lane containing the IGFBP-1 standard (Fig. 2.2, lane 3) revealed no immunoreactive bands, confirming the specificity of the BP-3 antibody. However, when the IGFBP-1 antibody was used (see Figure 2.3), no bands were noted in lane 2 (IGFBP-3 standard), whereas a band at 28-29 kDa was present in the BP-1 standard lane (lane 3). The lanes containing normal prepubertal and pubertal serum revealed no immunoreactive protein bands with the IGFBP-1 antibody (lanes 4 and 6), whereas, the respective CRF serum did show a band each at 29 kDa (lanes 5 and 7). These WIBs confirm the specificity of the anti-hIGFBP-1 and -3 antibodies, as well as the differences between the ligand-binding IGFBP-3 doublet, and the immunoreactive 30 kDa BP-3 form. An immunoblot was also performed using bovine kidney cell (MDBK) antiserum (see Figure 2.4) which identified IGFBP-2 at 34 kDa . IGFBP-4 was identified at 25 kDa using an anti-ovine IGFBP-4 antiserum (see Figure 2.5) which revealed immunoreactive bands at 25 kDa.

Figure 2.4 *Western immunoblot of prepubertal and pubertal normal, CRF and transplant serum with an IGFBP-2 antibody* (see Chapter 2.5). Lane 2, prepubertal normal male; Lane 3, prepubertal normal female; Lane 4, pubertal normal male; Lane 5, pubertal normal female; Lane 6, prepubertal CRF male; Lane 7, prepubertal transplant male; Lane 8, prepubertal CRF female; Lane 9, prepubertal transplant female; Lane 10, pubertal CRF female; Lane 11, pubertal transplant female; Lane 12, pubertal CRF female; Lane 13, pubertal transplant female; normal adult female control. ^{14}C molecular weight markers are shown in the left column.

Figure 2.5 *Western immunoblot of prepubertal and pubertal normal, CRF and SSGC serum with a IGFBP-4 antibody* (see Chapter 3.2). Lane 2, prepubertal normal male; Lane 3, prepubertal SSGC male; Lane 4, prepubertal CRF male; Lane 5, prepubertal SSGC male on rhGH; Lane 6, prepubertal CRF male on rhGH; Lane 7, ovine IGFBP-3 QC; Lane 8, pubertal SSGC male; Lane 9, pubertal CRF male; Lane 10, pubertal SSGC male on rhGH; Lane 11, pubertal CRF male on rhGH; Lane 12, pubertal normal male.^{14}C molecular weight markers are shown in the left column.


46
BP-2
30
C14 2 3 4 5 6 7 8 9 10 11 12 13 14


46
30
BP-4
21.5
2 3 4 5 6 7 8 9 10 11 12

2.7 Fast protein liquid chromatography (FPLC)

Analytical gel filtration chromatography was used to separate different molecular size IGF-IGFBP complexes and the unbound growth factors. Serum (300 ml) was delipidated by extraction using an equal volume of Freon (1,1,2-trichloro-1,2,2-trifluoroethane; Du Pont, Bayswater, Victoria, Australia) and 250 ml of extracted serum was then incubated with ^{125}I-IGF-I (approx. 0.2ng) in 15 ml of FPLC buffer (see appendix A) at 4° C overnight or for 1 minute at 37° C. 200 ml of sample was then injected onto a Superose 12 10/30 FPLC size exclusion column (Pharmacia, North Ryde, NSW, Australia) under neutral conditions. The column was initially equilibrated with FPLC buffer, and molecular weight standards were run down the column before, and at the end of all the patient sample runs. Thyroglobulin (669 kDa), γ-globulin (150 kDa), bovine serum albumin (BSA) (69 kDa) and carbonic anhydrase (30 kDa) (Sigma Chemical Co., St. Louis, MO, U.S.A.), as well as ^{125}I-IGF-I (7.5 kDA), were used to calibrate the column in order to identify the molecular weight ranges of the various peaks resulting from the sample runs. An FPLC system (Pharmacia), consisting of a P-500 high precision pump, automatic injector, U-V monitor, fraction collector (Frac 100) and chart recorder (REC-482) was used. Fractions of 0.5 ml were collected (1-85) at a flow rate of 0.5ml/min. The radioactivity of each fraction was counted in a gamma-counter (LKB Wallc, 1261 Multigamma) to determine the elution profiles for the IGF-IGFBP complexes and the "free" or unbound ^{125}I-IGF-I. The fractions were then stored at -80°C for further analysis by WIB.

2.8 IGF-I and IGF-II Assays

2.8.1 Separation of the IGFs from IGFBPs

In order to quantify the concentration of the IGFs in CRF serum without the interference of the IGFBPs (Powell, Rosenfeld, Baker, *et al*, 1986), which, in earlier reports, had led to false underestimations of IGF-I (Enberg and Hall, 1984; Goldberg, Trivedi, Delmez, *et al*, 1982), we used size-exclusion liquid chromatography to separate the IGFBPs from the IGFs before assaying. This involved reducing the pH of the sample to 2.8 to dissociate the IGF-IGFBP complexes before separation of the components by column chromatography (Owens, Johnson, Campbell, *et al*, 1990).

For each sample, 40 µl of serum was mixed with 260 µl of MilliQ water, and mixed with 100 µl 4x Mobile Phase (0.8 M acetic acid, 0.2 M trimethylamine, 0.4% (v/v) Tween-20, adjusted to pH 2.8 and filtered through a 0.45 µm GV filter membrane (Millipore Corp., Bedford, MA, USA) and degassed overnight). In order to inhibit blocking of the column by serum lipids, the samples were defatted by the addition of 400 µl of Freon, vortexed and then centrifuged at 1300 rpm for 10 minutes at 4°C. 300 µl of the aqueous phase was then placed into an injection vial. The samples were injected onto a Protein Pak 125 HPLC column (Waters Associates Inc., Milford, MA, USA) using a WISP 710 B autosampler (Waters Associates Inc., Milford MA, USA). A tracer run of ^{125}I-IGF-I was performed before applying the samples to ensure adequate recovery of the protein from the column. The samples were fractionated in 1x Mobile Phase which had been filtered and degassed overnight. The samples eluted at 0.5 ml/min. Four separate pools were collected, in order: IGFBP region, intermediate fraction, IGF region and

tail pools. As the column was used continually in the laboratory for the separation of the IGFs from the IGF-IGFBP complexes prior to IGF assay, it had been previously determined that IGF-I eluted at 8-8.5 ml and this pool was used in the IGF-I radioimmunoassay and IGF-II radioreceptorassay.

2.8.2 Radioimmunoassay for IGF-I (RIA)

A double antibody radioimmunoassay (RIA) (Owens, Johnson, Campbell, *et al*, 1990) was used to determine IGF-I immunoreactivity in patient and control sera extracted as described in 2.7.1. All samples were measured in triplicate. Samples (100 µl) were neutralised by the addition of 60 µl 0.4 M Tris base, then mixed with 200 µl of IGF-RIA buffer (see Appendix A). Known concentrations of IGF-I (2.5- 1300 pg in 200 µl of IGF-RIA buffer) were prepared with 60 µl 0.4 M Tris base and 100 µl of the acid column Mobile Phase as standards. A rabbit polyclonal antiserum prepared against hIGF-I (Conlon 89/1, courtesy of Dr. P.C. Owens) was added in 50 µl of IGF-RIA buffer resulting in a final concentration of 1:20. 'Blank' tubes were included to assess non-specific binding, with IGF-RIA buffer replacing the antibody. 'Reference' tubes to determine the value for maximum uncompeted radioligand binding in the assay, contained IGF-RIA buffer as zero standard and 'total' tubes contained only tracer. Radiolabelled IGF-I, (20,000 cpm in 50 µl of IGF-RIA buffer) was added to all tubes and incubated overnight. The following day, primary immune complexes were precipitated with sheep anti-rabbit immunoglobulin (50 µl of 1/200 concentration; Silenus Laboratories Pty. Ltd., Hawthorn, Victoria, Australia) and rabbit immunoglobulin (10 µl of 1/200 concentration; Silenus Laboratories Pty. Ltd., Hawthorn, Victoria, Australia). All tubes were vortexed and incubated at room temperature for 30 minutes. 1 ml of 6.6% polyethylene

glycol (PEG) solution was added, the tubes vortexed again and centrifuged at 4,000 rpm for 20 minutes at 4° C. The supernatant was aspirated and the pellet counted in a gamma counter. IGF-I concentrations in the unknown samples were measured by comparison with the standards using RiaCalc (version 2.57; Pharmacia / Wallac Oy, Turku, Finland). Samples of known IGF-I concentration were included as quality controls in each assay.

2.8.3 Radioreceptor assay for IGF-II (RRA)

A radioreceptor assay (Owens, Kind, Carbone, *et al,* 1994) using Ovine Placental Microsomes (OPM) (courtesy of Dr. P.C. Owens, Co-operative Research Centre for Tissue Growth and Repair, CSIRO Division of Human Nutrition, Adelaide, S.A., Australia) as receptor, was employed to measure the IGF-II immunoreactivity in patient and control sera. The OPM was reconstituted by adding 0.5 ml radioreceptor assay (RRA) buffer (see Appendix A) and incubating on ice. The method for this assay is similar to the IGF-I RIA, exchanging the Conlon anti-hIGF-I antibody for the OPM which was used in a final concentration of 1/300. rhIGF-II (Lilly Research Laboratories, Indianapolis, IN, U.S.A.) was used as standard and radioligand. RRA buffer replaced the IGF-RIA buffer used in the RIA. After overnight incubation of the samples and standards with the receptor (OPM) and tracer, 1 ml of RRA buffer was added to all tubes except the totals, which contained only tracer. The tubes were then spun at 4,000 rpm for 30 minutes at 4° C. The supernatant was aspirated and the pellet counted in a gamma counter.

2.9 Radioimmunoassay for IGFBP-3, IGFBP-1 and the acid labile subunit (ALS)

Serum levels of IGFBP-3 (Baxter and Martin, 1986), ALS (Baxter, 1990) and IGFBP-1 (Baxter, Martin and Wood, 1987) were measured by specific RIA in the laboratories of Professor Robert C. Baxter (Department of Endocrinology, Kolling Institute for Medical Research, St. Leonards, N.S.W., Australia).

2.10 Acknowledgements

I must thank Sabine Harrer for her invaluable work in setting up and calibrating the FPLC equipment and for performing the early FPLC chromatography, as well as the IGF-I and IGF-II assays.

CHAPTER 3

IGFS AND IGFBPS IN CHILDREN WITH CHRONIC RENAL FAILURE TREATED WITH RECOMBINANT HUMAN GROWTH HORMONE

3.1 Introduction

Growth retardation is a common consequence of chronic renal failure (CRF) in children and can be associated with severe psychological disturbances (Rigden, Rees and Chantler, 1990). Although the possible causes of this growth failure have been extensively studied, attempts to identify a specific underlying mechanism have been disappointing (French and Genel, 1984). The aetiology is multifactorial, with the age of onset of the renal disease (Betts and Magrath, 1974), metabolic acidosis, renal osteodystrophy, poor calorie intake and disturbances of various growth factors having been implicated (Fine, 1992). The age of onset is critical, as is the severity of the disease, with a reduction in growth velocity being reported once the GFR falls below 25 ml/min/1.73m^2 (French and Genel, 1984). Correction of acidosis, maintenance of salt and water balance, prevention of renal osteodystrophy, and improved nutrition have been proposed to improve growth in these children (French and Genel, 1984). We have previously shown that aggressive dietary management, by means of protein and phosphate restriction, together with improved calorie intake, can improve growth in association with slowed deterioration of renal failure and control of secondary hyperparathyroidism (Jureidini, Hogg, van Renen, *et al*, 1990). However, only minimal "catch-up" growth in significantly growth retarded children was achieved. Despite some improvement in linear growth which has been reported with conservative methods (Jureidini, Hogg, van Renen, *et al*, 1990; Polito, La Manna and Iovene, 1995), approximately 50% of patients studied by the European Dialysis and Transplant Association had reached a final height of less than the third percentile of the normal population (Mehls, Scheafer and Tonshoff, 1991). “Catch-up” growth is rarely reported after successful renal transplantation, due both to a degree of reduction in renal function and to steroid treatment, required as part of the immunosuppresive regimen (Fine,

Stablein and Tejani, 1995). Short stature also contributes to the problems associated with rehabilitation after transplantation, which include impaired psychosocial adaptation (Henning, Tomlinson and Rigden, 1988; Rosenkranz, Bonzel, Bulla, *et al*, 1992).

Abnormalities of the GH - IGF axis in CRF have been well described (Blum, 1991; Blum, Ranke, Kietzmann, *et al*, 1991; Goldberg, Trivedi, Delmez, *et al*, 1982; Powell, Liu, Baker, *et al*, 1996; Powell, Rosenfeld, Baker, *et al*, 1986). Elevated growth hormone levels have been reported in both adults (Samaan and Freeman, 1970) and children (El-Bishti, Counahan, Bloom, *et al*, 1978) with CRF. The anabolic actions of growth hormone are mediated in the body by the insulin-like growth factors, IGF-I and IGF-II, produced primarily in the liver (Cohick and Clemmons, 1993c), but also locally in other target tissues (Lowe, 1991). Serum IGF levels in CRF have been reported as elevated (Enberg and Hall, 1984), normal (Powell, Rosenfeld, Sperry, *et al*, 1987) or low (Goldberg, Trivedi, Delmez, *et al*, 1982; Rees, Rigden, Ward, *et al*, 1990) and IGF-II levels as 1.5 times greater than normal (Powell, Rosenfeld, Sperry, *et al*, 1987). The discrepancy in these results may be explained by the interference of the insulin-like growth factor binding proteins (IGFBP-1 to -6) in the assays (Powell, Rosenfeld, Baker, *et al*, 1986). Once the IGFBPs were adequately removed from the serum by acid chromatography, normal levels of IGF-I and normal or slightly elevated levels of IGF-II were found (Blum, 1991; Powell, Rosenfeld, Sperry, *et al*, 1987). However, reduced IGF bioactivity has been demonstrated in CRF despite normal serum concentrations of the growth factors (Goldberg, Trivedi, Delmez, *et al*, 1982; Phillips and Kopple, 1981; Tonshoff, Mehls, Heinrich, *et al*, 1990) and this may be a consequence of the increased binding of the IGFs to their binding proteins. Elevated concentrations of low molecular weight IGF binding proteins, possibly due to reduced renal clearance, have been implicated (Blum, Ranke, Kietzmann, *et al*,

1991; Goldberg, Trivedi, Delmez, *et al*, 1982; Lee, Hintz, Sperry, *et al*, 1989) and may therefore contribute to the growth retardation.

Animal trials using recombinant human growth hormone (rhGH) have demonstrated improvement in linear growth and in food utilization in CRF (Mehls, Ritz, Hunziker, *et al*, 1988). These findings provided a good rationale for the use of rhGH in growth retarded children with CRF, and several clinical trials involving small groups of children have been performed, each demonstrating accelerated growth responses (Koch, Lippe, Nelson, *et al*, 1989; Lippe, Fine, Koch, *et al*, 1988; Rees, Rigden, Ward, *et al*, 1990; Tonshoff, Mehls, Heinrich, *et al*, 1990; Tonshoff, Mehls, Schauer, *et al*, 1989; van Renen, Hogg, Sweeney, *et al*, 1992). Our group, using combined strict dietary therapy and rhGH, showed a significant improvement in growth velocity in 9 boys (van Renen, Hogg, Sweeney, *et al*, 1992). It has been hypothesized that the efficacy of rhGH in overcoming the growth retardation of CRF is due to an elevation of serum IGF-I concentrations, thus overcoming the inhibitory effects of accumulated IGFBPs (Blum, Ranke, Kietzmann, *et al*, 1991; Tonshoff, Tonshoff, Mehls, *et al*, 1992).

In this study, we proposed to identify changes in the serum concentrations of IGF-I and -II, and the IGFBPs-1, -2, -3 and -4, and the acid-labile subunit (ALS) which could be attributed to rhGH treatment, by comparing pretreatment serum concentrations with those found during therapy. The study of these changes might help to increase our understanding of the mechanism of action of this highly successful growth-promoting agent and provide further insight into the growth "resistance" of CRF.

3.2 Patients and Methods

10 boys, with moderate to severe CRF and associated short stature, were treated for 2 years with daily subcutaneous recombinant human growth hormone (rhGH - Genotropin, Kabi Pharmacia, Stockholm, Sweden) at a dose of 30 IU/m^2/week. Data on 9 of these children (patients \1-9), involved in a clinical trial, have already been reported (van Renen, Hogg, Sweeney, *et al*, 1992). All children met the inclusion criteria for the clinical trial which included : (1) CRF not treated with dialysis; (2) GFR < 60 ml/min/1.73m^2 body surface area; (3) height less than the 3rd normal percentile and/or a height velocity < 50th percentile for bone age; (4) aged between 3 and 15 years; (5) pubertal development ≤ to Tanner stage 2; (6) no other illnesses or disease likely to interfere with growth, especially systemic illnesses, chromosomal abnormality, endocrine disease or recognisable syndrome; (7) written informed parental consent and (8) a history of strict dietary management and full auxological assessment by our Renal Unit for at least 2 years before entry into the trial.

An additional child (patient 10) entered the trial 6 months after it had commenced, and completed the study 6 months after the other children. This boy did not meet the initial entry criteria for the study with regard to his age (2.6 years), therefore his results were not included in the previous publication (van Renen, Hogg, Sweeney, *et al*, 1992). In the present study, in order to account for the complicating effect of the endogenous growth spurt found during puberty, which is thought to coincide with a testicular volume of approximately 8-10 ml (Marshall, 1981), we elected to divide the children into two groups *viz* prepubertal and pubertal, as assessed by a testicular volume of less than or more than 4 ml respectively at the end of the 2 year study period. By these criteria five of the children were prepubertal and five

were pubertal, and the data were analysed according to these two groups. In the prepubertal group, ages at the commencement of the 2 years treatment, ranged between 2.6-10.0 years (mean 6.1 SD± 2.1) with bone ages between 1.3-8.3 years (mean 4.8 ± 2.5). GFRs in this group ranged from 11-45 ml/min/1.73m^2 (mean 26.0 ± 14.4). In the pubertal boys, ages ranged from 11.4-15.7 years (mean 13.1 ± 1.7) with bone ages between 9.6-13.0 years (mean 10.2 ± 1.8) and GFRs between 12-62 ml/min/1.73m^2 (mean 32.0 ± 19.8).

Serum collected before and during rhGH treatment was analysed retrospectively for: IGF-I and IGF-II by specific RIA and RRA, respectively, following extraction by high performance liquid chromatography under acidic conditions (see Chapter 2.7.1), and IGFBP-3, ALS and IGFBP-1 in unextracted serum (see Chapter 2.7.4). Serum IGFBPs were characterised by Western ligand blotting (see Chapter 2.4) in each child, from sequential serum samples from at least 2-5 years before, and then during rhGH treatment. Western immunoblotting, employing specific antibodies was performed to identify the molecular weights of the individual binding proteins (see Chapter 2.5) and thereby determine their respective positions on the WLBs. A pool of normal prepubertal male serum was used as the prepubertal control, the average age of the children included in this pool being 6.3 years, and serum from a normal pubertal male aged 13 years (Tanner stage 2) was used as the pubertal control. Normal adult male control serum was included on the WLB of the oldest pubertal boy (patient 9) for comparison. In addition, we were able to compare IGFBP-1 and -3 profiles by WLB and RIA, in a prepubertal and a pubertal boy with CRF, with an age and sex matched short and slowly growing boy respectively, before and on rhGH treatment (see Figure 3.12). Blood was obtained from short and slowly growing children who were followed by the Department of Endocrinology, Women's and Children's Hospital, Adelaide. These children had no known medical cause for

their poor growth and were treated with rhGH during a clinical trial. These children were divided into prepubertal and pubertal groups by the same criteria used for the CRF patients. Serum taken at the time of routine venepuncture had been stored at -70° C and an aliqout of this sample was generously donated for inclusion in this present study by Dr. Jenny Couper. IGFBP-4, was identified at 25 kDa by WIB, using an anti-ovine IGFBP-4 antibody, from the nitrocellulose filter from this WLB (see Chapter 2.6, Figure 2.5).

The study was approved by the Ethics Committee of the Women's and Children's Hospital. The recruitment of patients to clinical trials is a time-consuming and responsible task for clinicians, particularly when, as is the case with rhGH treatment, the therapy (daily injections) is intrusive and expensive (Postlethwaite, Reynolds, Wood, *et al*, 1995). As is often the case in clinical studies involving treatment, patient compliance can be an issue and requires regular monitoring, as well as education of both doctors and patients (Meyers, Weiland and Thomson, 1995). Hippocrates, in the fifth century BC, said, "It is not enough for the physician to do what is necessary, but the patient and the attendants must do their part as well, and the circumstances must be favourable" (Chadwick and Nann, 1950). Two patients (patients 10 and 9) reported a lack of compliance at approximately 21 months, due to disenchantment with daily injections, and their 24 month values, although included in the Figures for interest, were not included in the data analyses. Patients 3 and 4 underwent renal transplantation at approximately the 18 month time-point of the study and their results are discussed in further detail in Chapter 5. Because of the possible effects of renal transplantation on the results, the relevant values from these children after transplantation were also excluded from the data analyses but are shown in the Figures for interest. Due to the low numbers of children in the two groups, I have elected not to attempt to analyse the changes in the IGF and IGFBP assay values statistically, but rather to discuss the trends observed with the addition of rhGH therapy.

Patient results are shown in Figures 3.1 - 3.7 in order of increasing chronological age, from the youngest, Patient 10, to the oldest, Patient 9. Group data are expressed as mean (± SD).

3.3 Results

3.3.1 Height velocity

All the children responded to rhGH therapy with improved height velocities, particularly in the first 12 months on treatment. Height velocity in the 5 prepubertal boys rose from 5.6 (± 1.8) cm/year before therapy to 9.7 (± 1.0) cm/year after 12 months on treatment, but slowed to 6.6 (± 1.6) cm/year by two years. In the pubertal group, height velocity increased from 5.0 (± 1.6) cm/year without rhGH to 9.8 (± 2.1) cm/year by 12 months on treatment, and slowed to 8.5 (± 2.8) cm/year at 2 years.

3.3.2 IGF-I Data

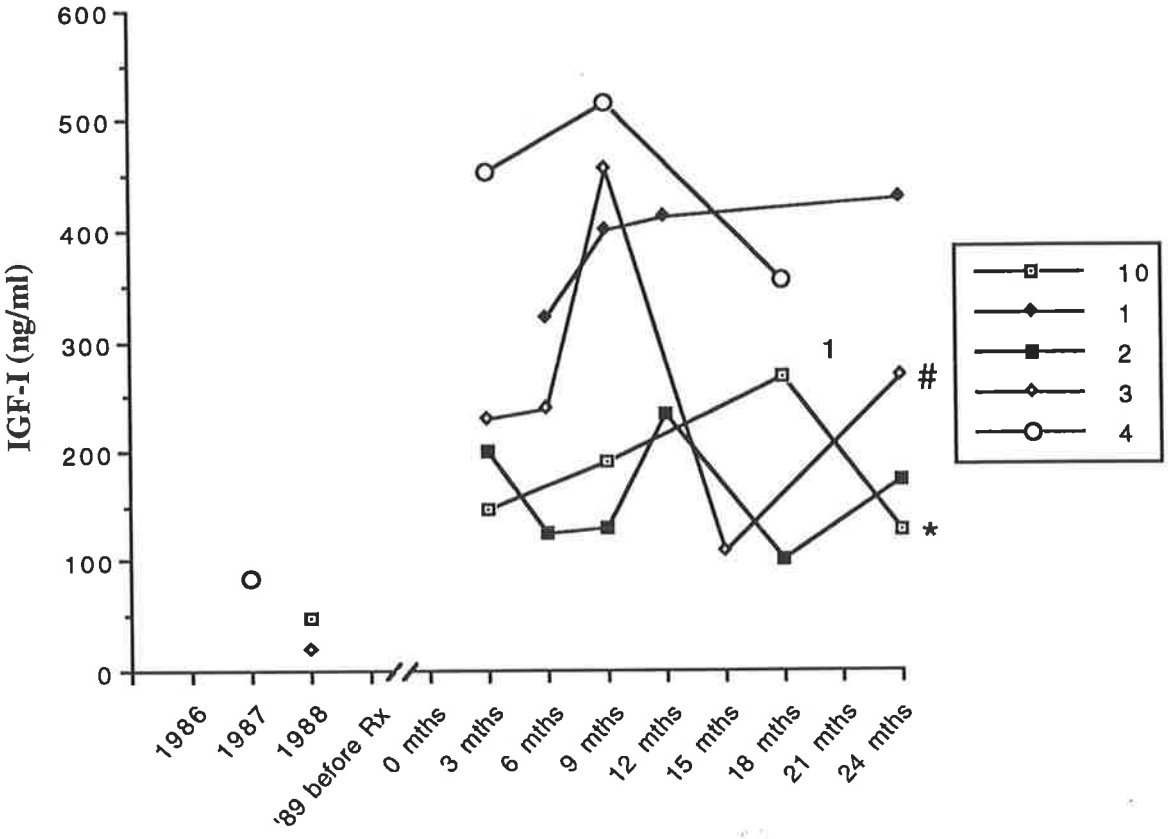
Serum IGF-I concentrations increased approximately 5 fold compared with pretreatment levels in three of the five children in the prepubertal group during rhGH therapy (see Figure 3.1a). Pre-rhGH treatment serum samples were not available from 2 of the children. The increase ranged from 4.1 to 6.3 times with a mean rise of 5.2 (± 1.1) fold (n=3). Mean IGF-I levels before treatment were 58.6 (± 23.6) ng/ml (n=3) increasing to a mean of 290.8 (± 121.2) ng/ml (n=5) during the whole treatment period with a mean peak at 9-12 months of 338.2 (± 169.5)

Fig 3.1 *Changes in serum IGF-I concentrations in 5 prepubertal boys (a) and 5 pubertal boys (b) with CRF treated with rhGH for 2 years.* Serum IGF-I concentrations were measured retrospectively in serum samples collected before, and at intervals during treatment.

* lack of compliance with treatment at this time point .

\# patient had undergone renal transplantation before this sample was collected.

a) Serum IGF-I concentrations in prepubertal boys on rhGH



b) Serum IGF-I concentrations in pubertal boys on rhGH

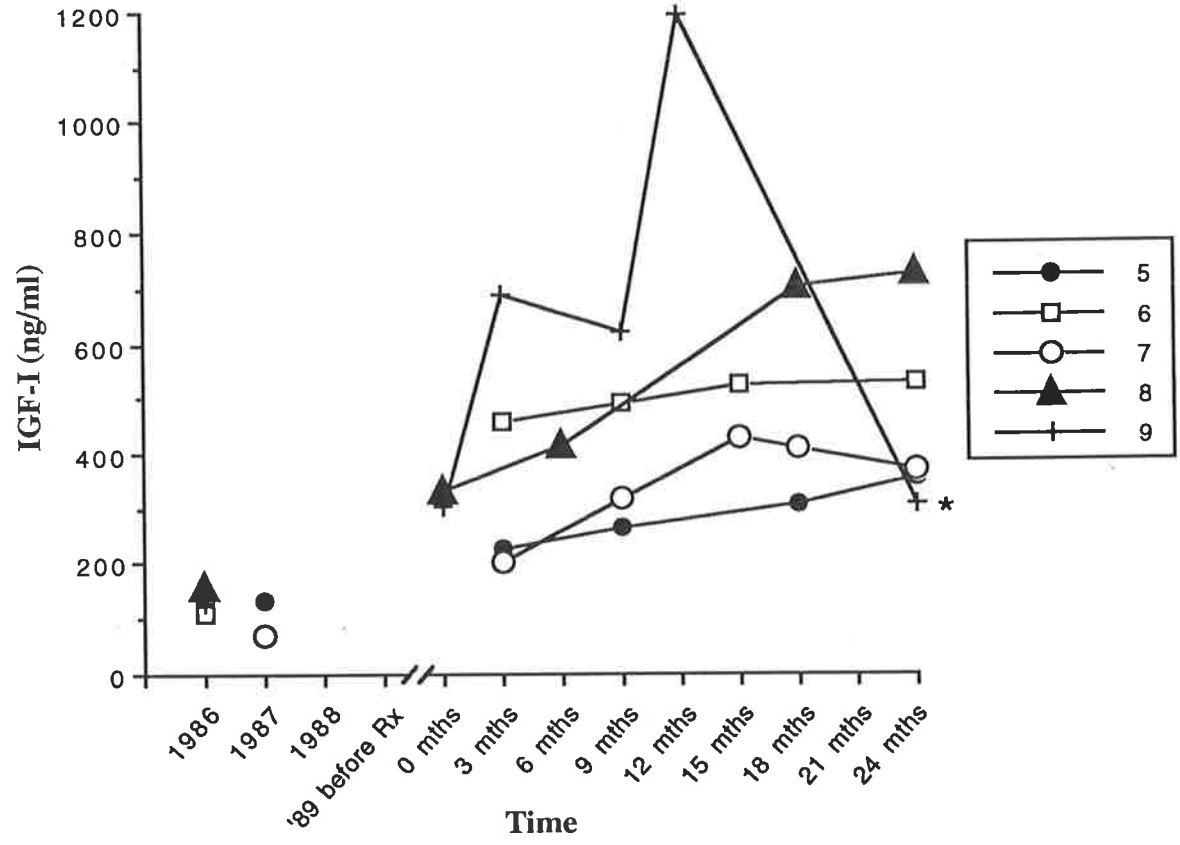
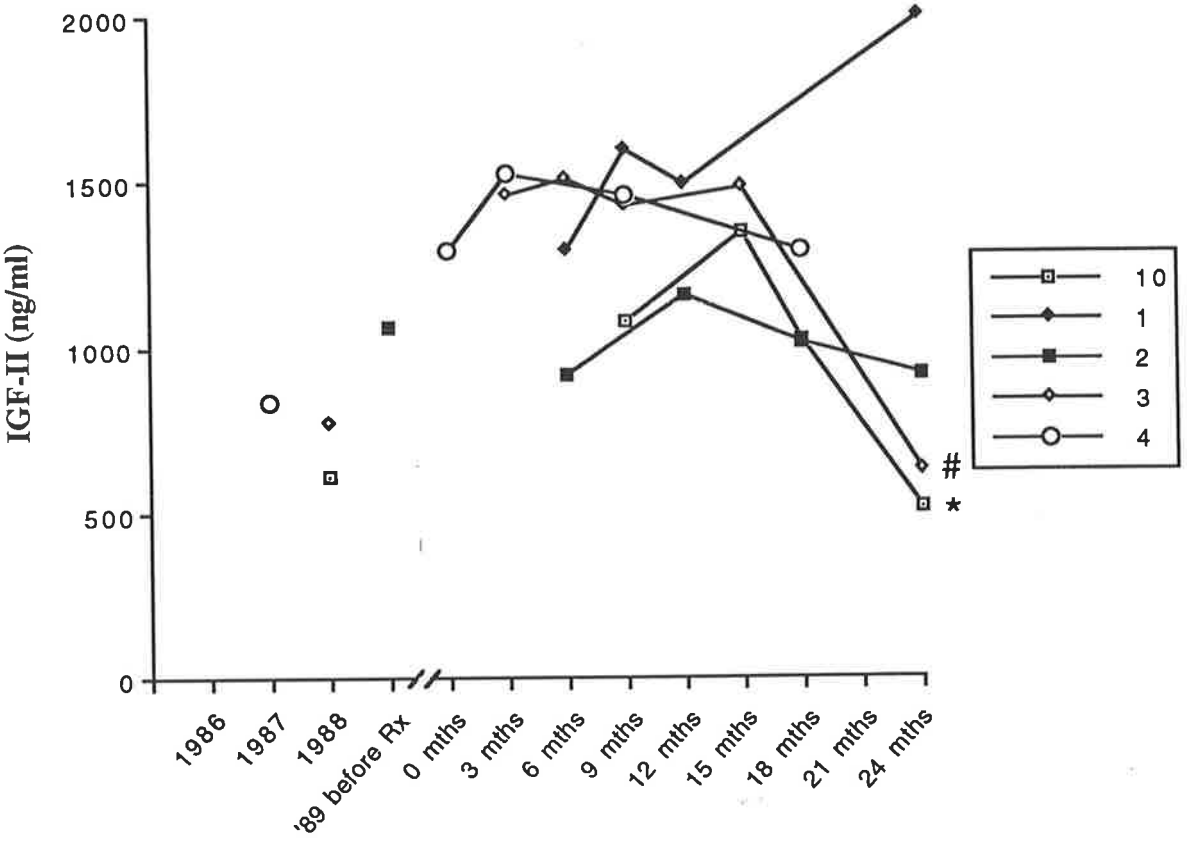


Fig 3.2 *Changes in serum IGF-II concentrations in 5 prepubertal boys (a) and 5 pubertal boys (b) with CRF treated with rhGH for 2 years.* Serum IGF-II concentrations were measured retrospectively in serum samples collected before, and at intervals during treatment.

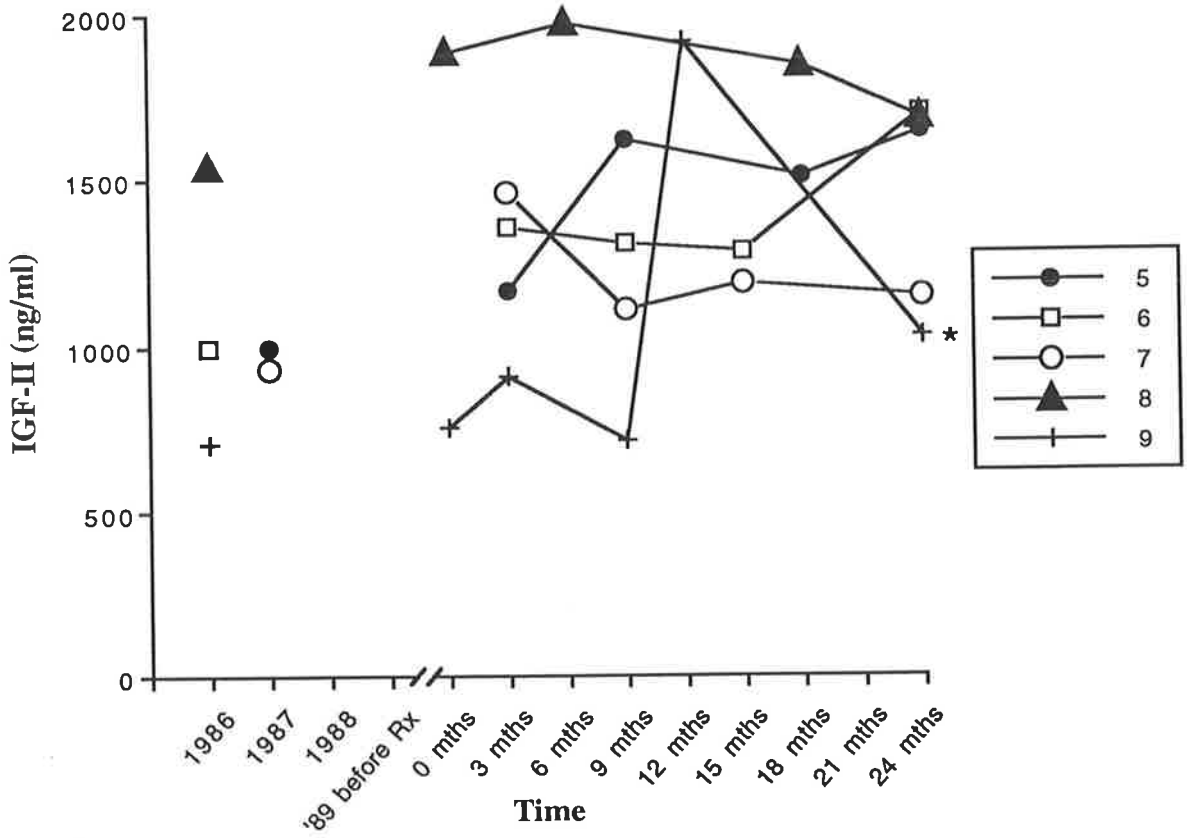
* lack of compliance with treatment at this time point .

\# patient had undergone renal transplantation before this sample was collected.

a) **Serum IGF-II concentrations in prepubertal boys on rhGH**



b) **Serum IGF-II concentrations in pubertal boys on rhGH**



ng/ml (n=5). Patient 3 showed an 11 fold rise in IGF-I values at 9 months, which fell dramatically at 15 months, possibly in relation to a lack of compliance at the time of preparation for renal transplantation at approximately 18 months. In the pubertal group, serum IGF-I concentrations rose approximately 3 fold during rhGH therapy (see Figure 3.1b), with a range of between 2.2 and 4.7 fold increase over the pretreatment levels with a mean increase of 3.2 (± 1.3) times (n=5). Mean IGF-I values before treatment were higher than in the prepubertal group, at 191.0 (± 118.5) ng/ml, increasing to a mean of 517.5 (± 219.3) ng/ml over the 2 year period (n=5). There was a consistent rise in IGF-I levels over the 24 months in these children with a peak at 12 months observed in only one boy, *viz* Patient 9, who subsequently became non-compliant by 24 months, resulting in a dramatic fall in IGF-I levels at this time point. We found no correlation between height velocity and IGF-I values in our children. We noted a very slight increase in the molar ratio between the sum of IGF-I and -II and IGFBP-3 at 9-12 months on rhGH (0.0526) compared with before therapy (0.0522), which was not sustained at 24 months.

3.3.3 IGF-II Data

During rhGH therapy, the serum IGF-II concentrations in the prepubertal boys rose, on average, 1.5 fold over the pretherapy values, compared to the average 5 fold elevation seen in the IGF-I concentrations. The range of increases was between 0.9 to 1.9 times (see Figure 3.2a) (n=4). Mean IGF-II levels before treatment were 935 (± 303) ng/ml (n=4), increasing to a mean of 1328 (± 247) ng/ml (n=5) over the 24 months on rhGH. In the pubertal group, the increase in serum IGF-II concentrations observed with rhGH was similar to that noted in the

prepubertal group, showing an average 1.4 fold rise compared to pretreatment values, the range being 1.0-1.6 fold (n=5) (see Figure 3.2b). The response to rhGH, reflected in the change in IGF-II concentrations from before treatment, was less than that of IGF-I. The mean pre-rhGH levels were 1,114 (± 445) ng/ml (n=5) increasing to a mean of 1,429 (± 261) ng/ml (n=5) during the treatment period. The peak at 12 months and the fall at 24 months in IGF-II values in Patient 9 coincided with that observed in the IGF-I levels.

3.3.4 IGFBP-3 Data

rhGH therapy in the prepubertal boys resulted in an average increase of serum IGFBP-3 levels of 1.7 times over the baseline levels, with a range of between 1.3-1.9 times (n=4). The mean pretreatment level was 3.2 (± 0.6) μg/ml and this rose to a mean of 5.5 (± 0.9) μg/ml (n=4) during therapy, with a peak in levels of 6.2 (± 0.6) μg/ml (n=4) at 9-12 months (see Figure 3.3a). The values found on rhGH were above the normal laboratory reference range for children aged 2-10 years which is 1.1-4.0 μg/ml (Baxter and Martin, 1986), although the pretreatment concentrations were within the normal range. In the pubertal group, serum IGFBP-3 levels during therapy showed a similar increase to the prepubertal boys, of approximately 1.6 times above pretreatment values, with a range of between 1.2-2.0 fold increase (n=5). The mean pre-rhGH level was 3.9 (± 1.1) μg/ml, which was within the normal range, rising during treatment to 6.1 (± 1.3) μg/ml (see Figure 3.3b) which is at the upper limit of the normal reference range for children aged 10-18 years, which is 2.0-5.0 μg/ml (Baxter and Martin, 1986). As noted in the prepubertal group, there was a peak at 9-12 months on treatment, of 6.5 (± 2.5) μg/ml (n=5). There was a significant inverse correlation between IGFBP-3 and GFR at the 6 month time point of the study ($r = -0.88$, $p<0.05$) which was not

evident before treatment, and a weaker, non-significant correlation was noted at 12 months (r= -0.78).

3.3.5 ALS Data

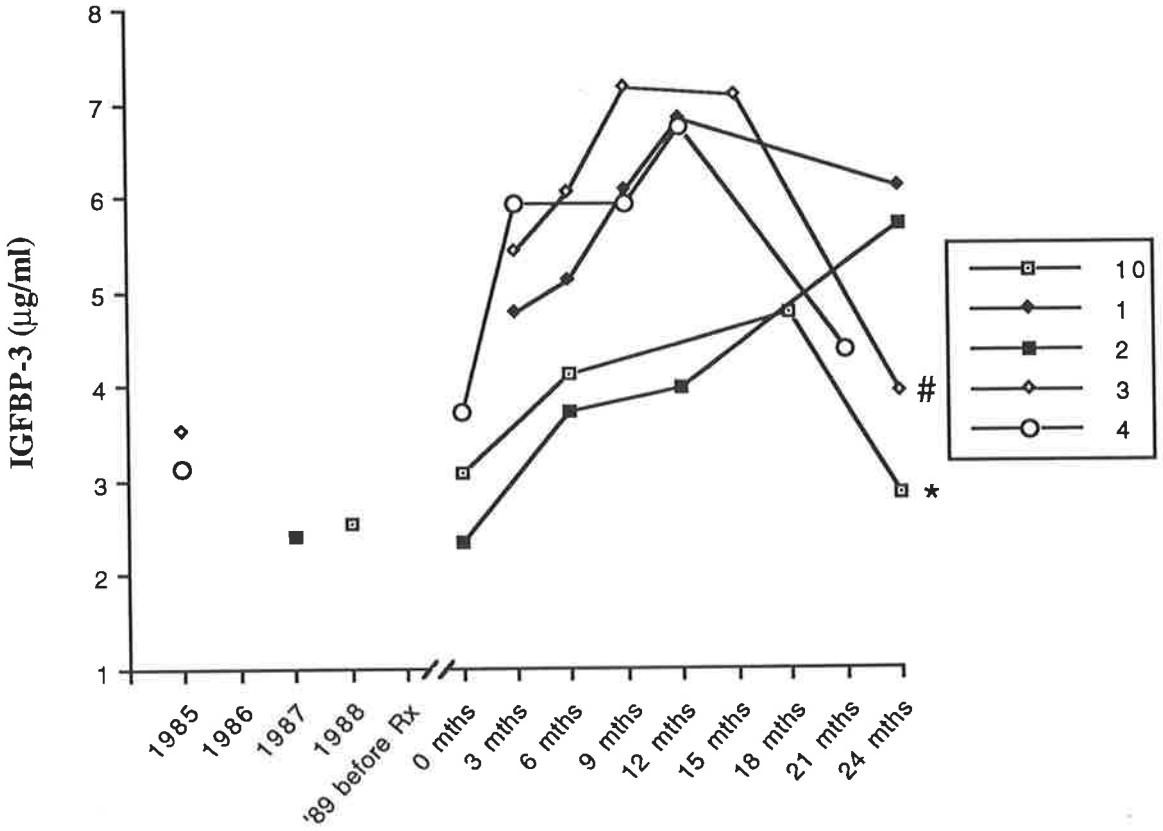
Serum levels of the acid-labile subunit (ALS) rose in parallel to serum IGFBP-3 levels with rhGH. The level in the prepubertal boys increased an average 1.9 fold over baseline levels, with a range of elevation of between 1.5-2.5 times (n=4) (see Figure 3.4a). The mean pre-rhGH ALS level was 22.3 (± 6.5) μg/ml (n=4) which rose to 40.9 (± 7.1) μg/ml (n=5) during therapy (normal reference range for children 2-10 years = 4-28 μg/ml (Baxter, 1990)). As seen in the IGFBP-3 values, there was a peak in ALS concentration during treatment at 9-12 months of 49.9 (± 13.4) μg/ml (n=4). In the pubertal group, we observed an approximately 1.7 fold rise in serum levels during treatment, with between 1.4 and 2.0 fold increases (n=5) (see Figure 3.4b). The mean pre-therapy value, which fell within the normal range, was 29.6 (± 6.5) μg/ml (n=5), (normal reference range for children 10-18 years = 12-35 μg/ml (Baxter, 1990)), increased to a peak at 9-12 months of 50.9 (± 15.3) μg/ml (n=5), with a mean of 50.1 (± 8.6) μg/ml (n=5) found during the 24 months on therapy. We noted a statistically significant correlation between IGFBP-3 and ALS before treatment ($r = 0.92$, $p<0.001$), at 12 months on therapy ($r = 0.96$, $p<0.01$) and at 24 months ($r = 0.86$, $p<0.01$).

Fig 3.3 *Changes in serum IGFBP-3 concentrations in 5 prepubertal boys (a) and 5 pubertal boys (b) with CRF treated with rhGH for 2 years.* Serum IGFBP-3 concentrations were measured retrospectively in serum samples collected before, and at intervals during treatment

* lack of compliance with treatment at this time point .

\# patient had undergone renal transplantation before this sample was collected.

a) Serum IGFBP-3 concentrations in prepubertal boys on rhGH



b) Serum IGFBP-3 concentrations in pubertal boys on rhGH

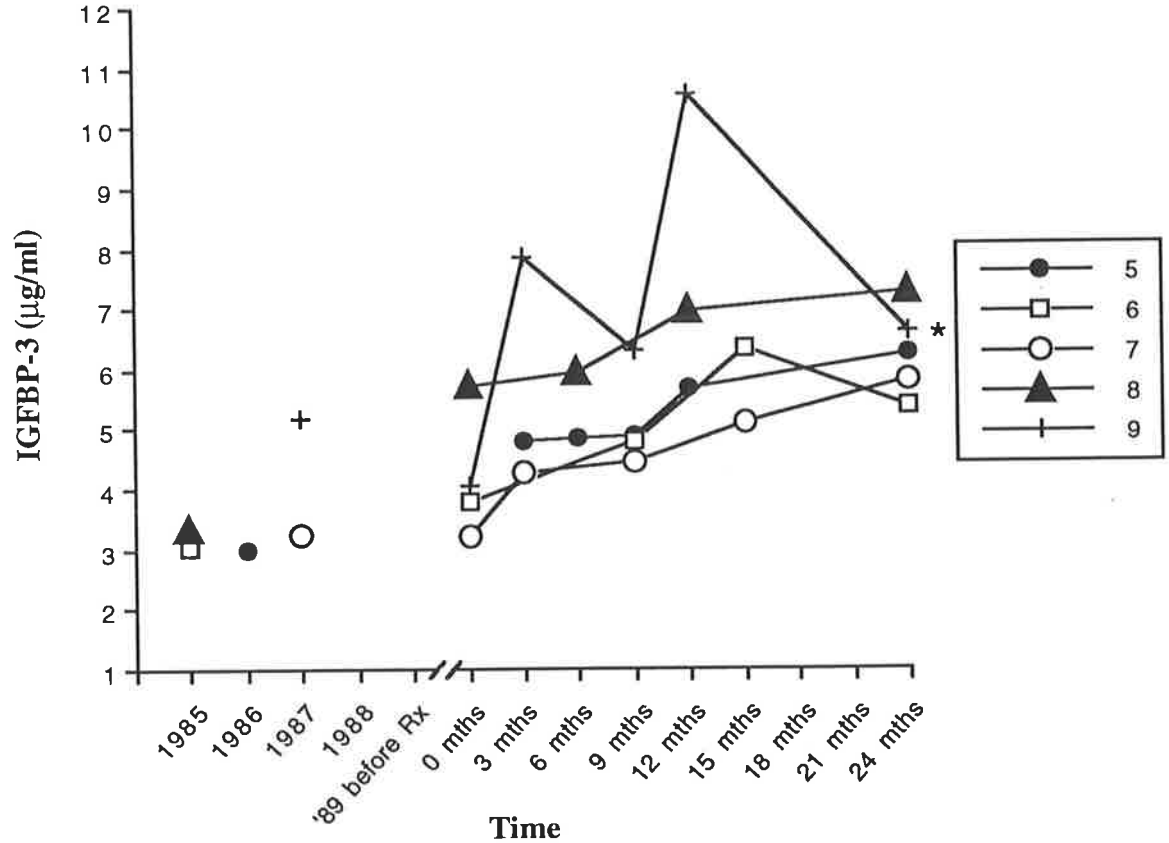
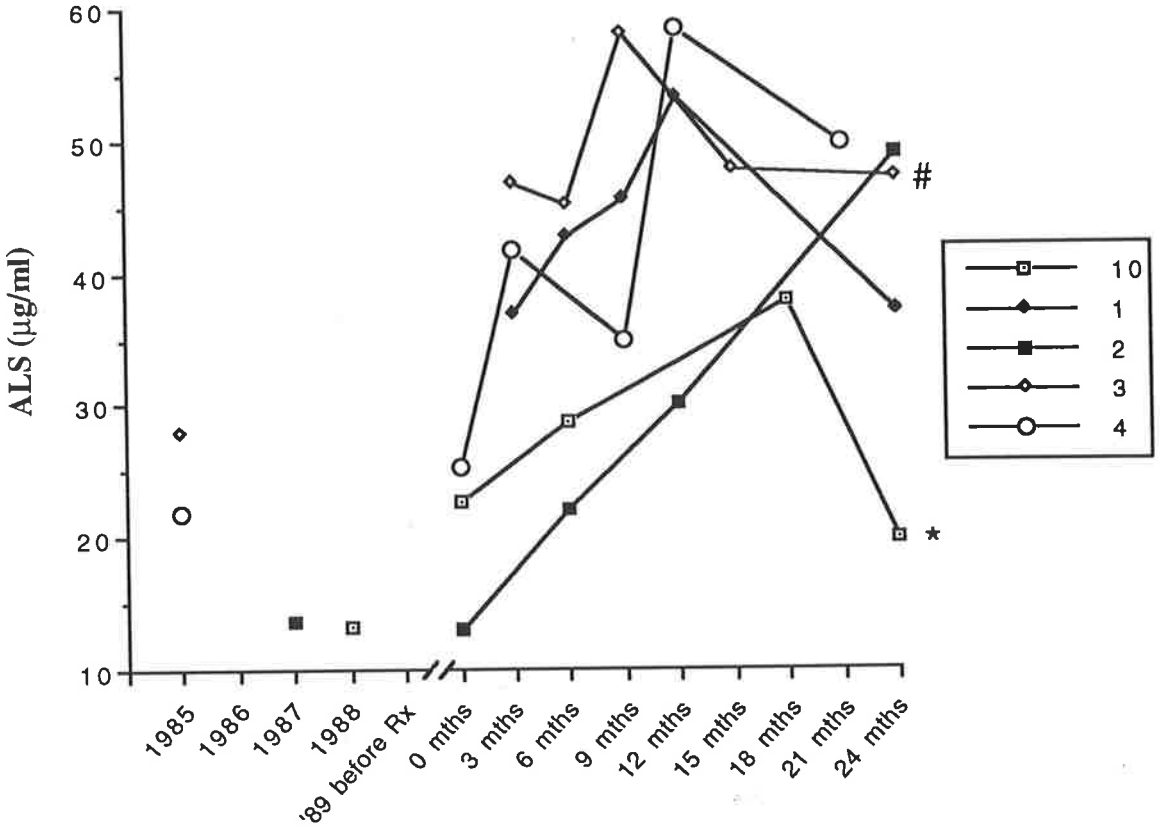


Fig 3.4 *Changes in serum ALS concentrations in 5 prepubertal boys (a) and 5 pubertal boys (b) with CRF treated with rhGH for 2 years.* Serum ALS concentrations were measured retrospectively in serum samples collected before, and at intervals during treatment

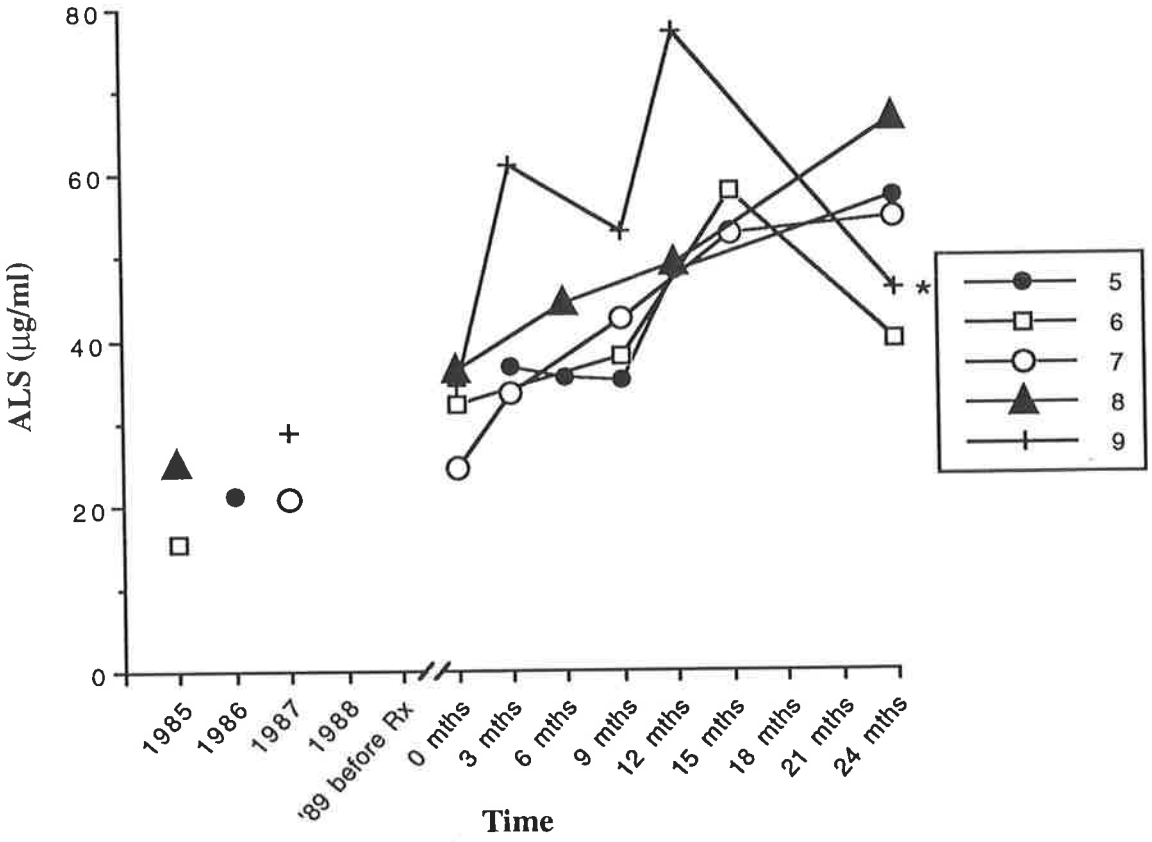
* lack of compliance with treatment at this time point .

\# patient had undergone renal transplantation before this sample was collected.

a) Serum ALS concentrations in prepubertal boys on rhGH



b) Serum ALS concentrations in pubertal boys on rhGH



3.3.6 IGFBP-1 Data

On average, fasting IGFBP-1 levels fell during rhGH therapy in the prepubertal group. The mean serum concentration before treatment was 559.2 (± 326.5) ng/ml (n=4), decreasing to a mean of 442.7 (± 228.7) ng/ml (n=5) on rhGH (see Figure 3.5a). This pattern was similar in the pubertal group, where the mean fasting IGFBP-1 level before treatment was 458.8 (± 142.0) ng/ml (n=5), falling to a mean of 290.6 (± 73.0) ng/ml (n=5) during therapy (see Figure 3.5b). Serum IGFBP-1 concentrations were on average higher in the prepubertal boys compared with the pubertal group. As we have previously reported, serum insulin levels rose from normal to slightly above the normal fasting reference range with rhGH treatment (van Renen, Hogg, Sweeney, *et al*, 1992). In Fig 3.6 a and b, and Fig 3.7 a and b, we compared the changes in serum IGFBP-1 levels with the changes in serum insulin levels over the 2 years of rhGH therapy in both groups of children. An inverse relationship was observed between IGFBP-1 and insulin concentrations, which was particularly noticeble in patients 10 and 4 in the prepubertal boys and 7, 8 and 9 in the pubertal group in the Figures, however we did not find a statistically significant correlation between IGFBP-1 and insulin levels before or during rhGH treatment.

3.3.7 Western Ligand and Immunoblots

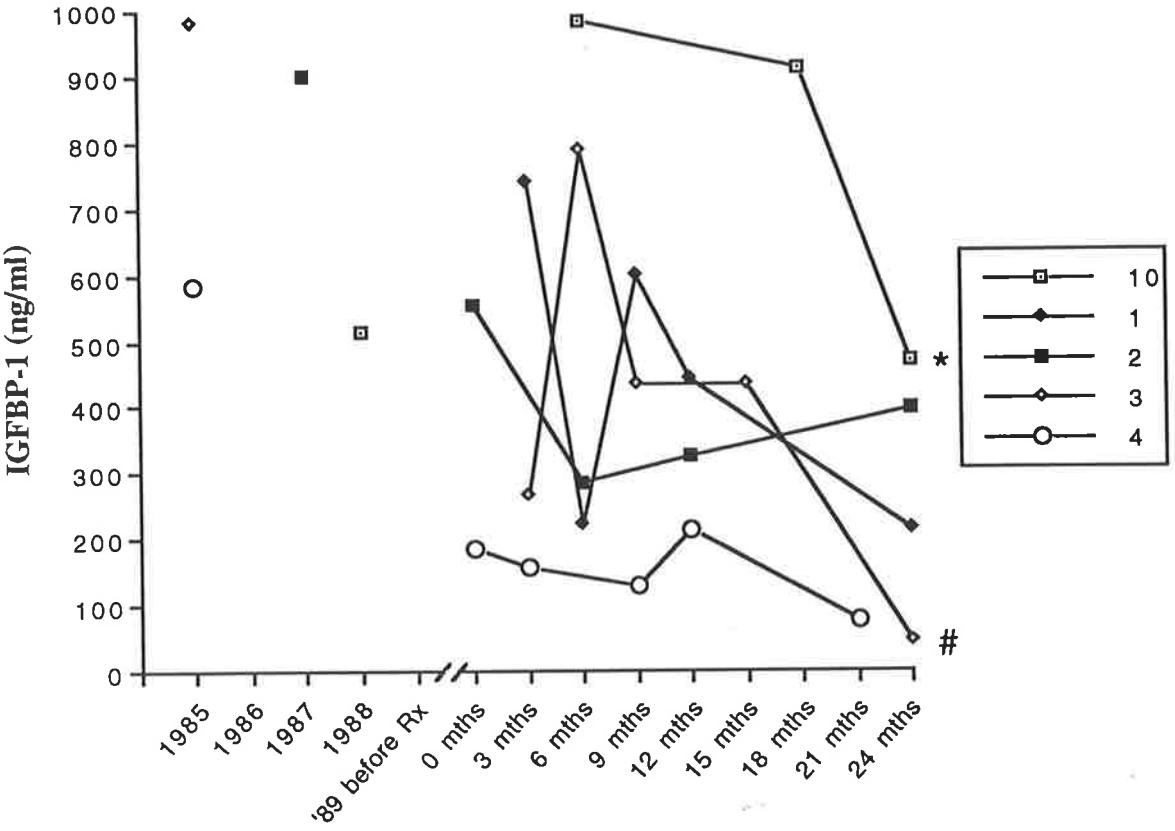
Although each individual child in this study had his serum IGFBPs characterised by WLB, I have elected to discuss the changes seen on the ligand blots associated with rhGH treatment

Fig 3.5 *Changes in serum IGFBP-1 concentrations in 5 prepubertal boys (a) and 5 pubertal boys (b) with CRF treated with rhGH for 2 years.* Serum IGFBP-1 concentrations were measured retrospectively in serum samples collected before, and at intervals during treatment

* lack of compliance with treatment at this time point .

\# patient had undergone renal transplantation before this sample was collected.

a) Serum IGFBP-1 concentrations in prepubertal boys on rhGH



b) Serum IGFBP-1 concentrations in pubertal boys on rhGH

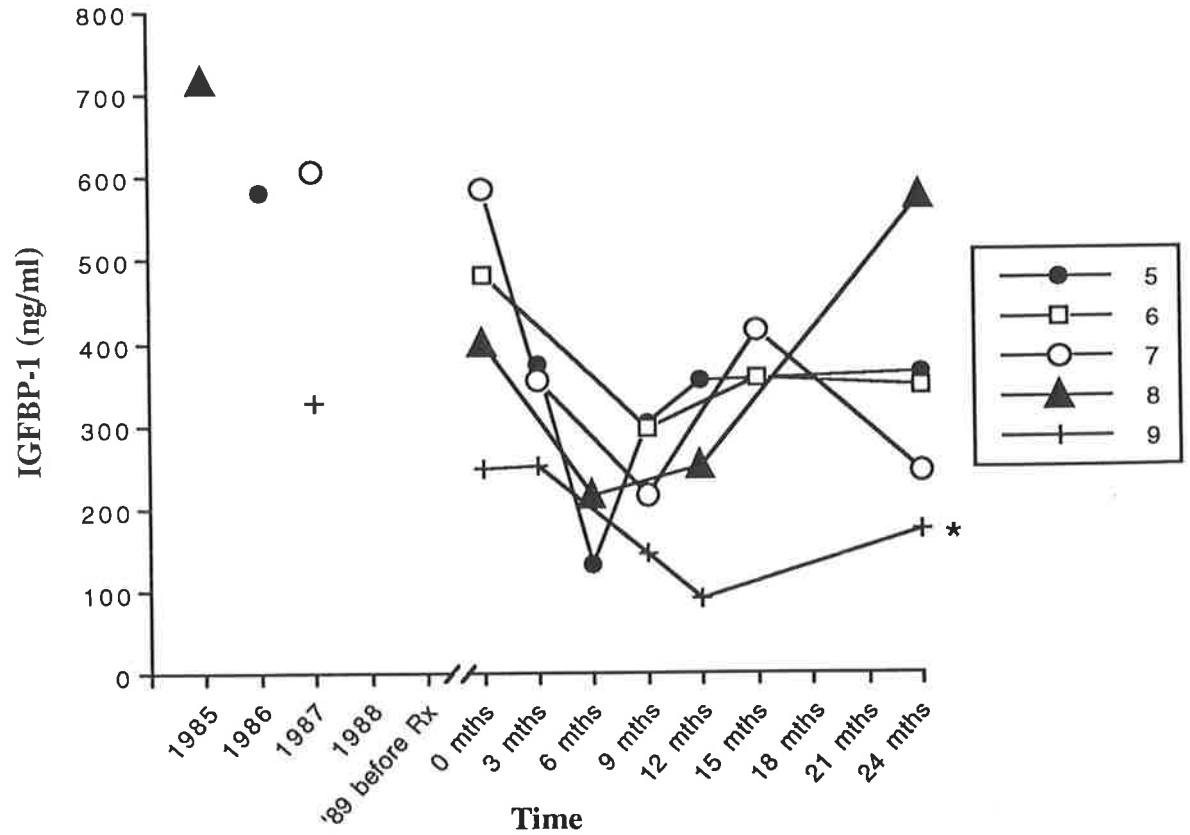
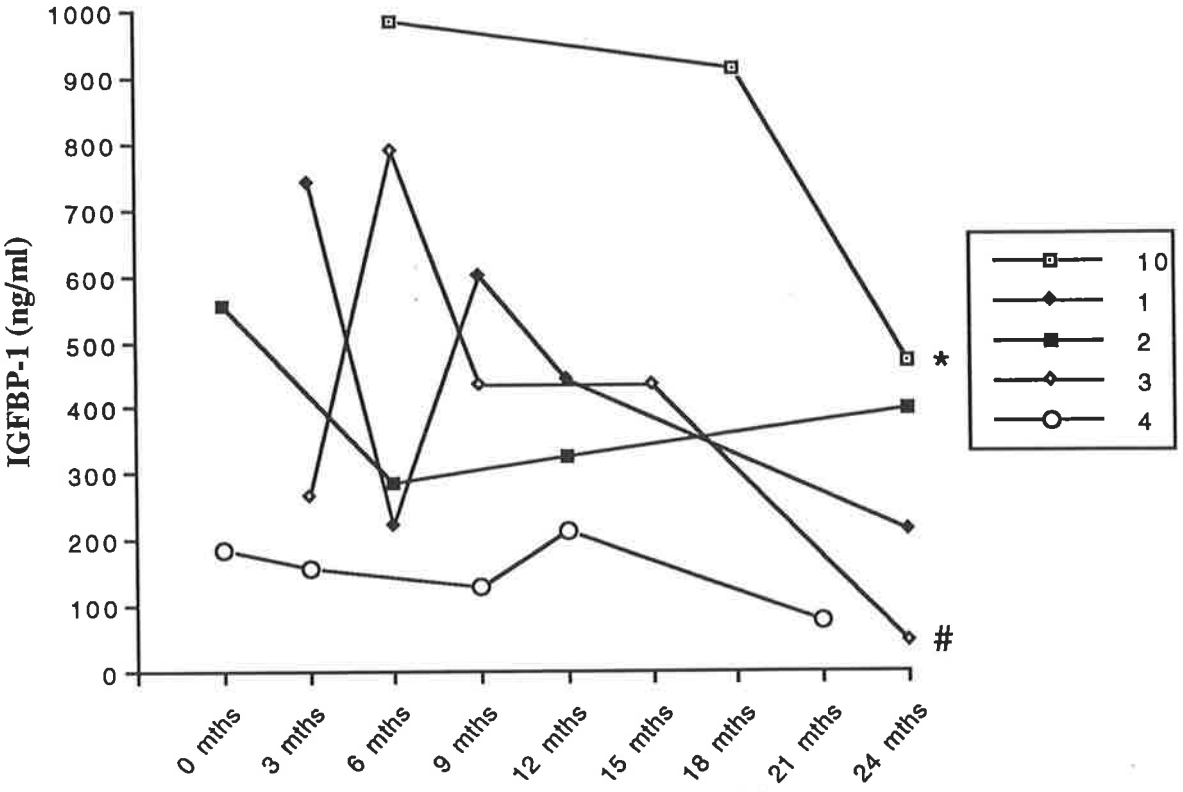


Fig 3.6 *Changes in serum IGFBP-1 (a) and fasting serum insulin (b) concentrations in 5 prepubertal boys with CRF treated with rhGH for 2 years.* Serum IGFBP-1 concentrations were measured retrospectively, and fasting insulin levels were measured in serum samples collected before and at intervals during treatment.

* lack of compliance with treatment at this time point .

\# patient had undergone renal transplantation before this sample was collected.

a) Serum IGFBP-1 levels in prepubertal boys on rhGH



b) Serum Insulin levels in prepubertal boys on rhGH

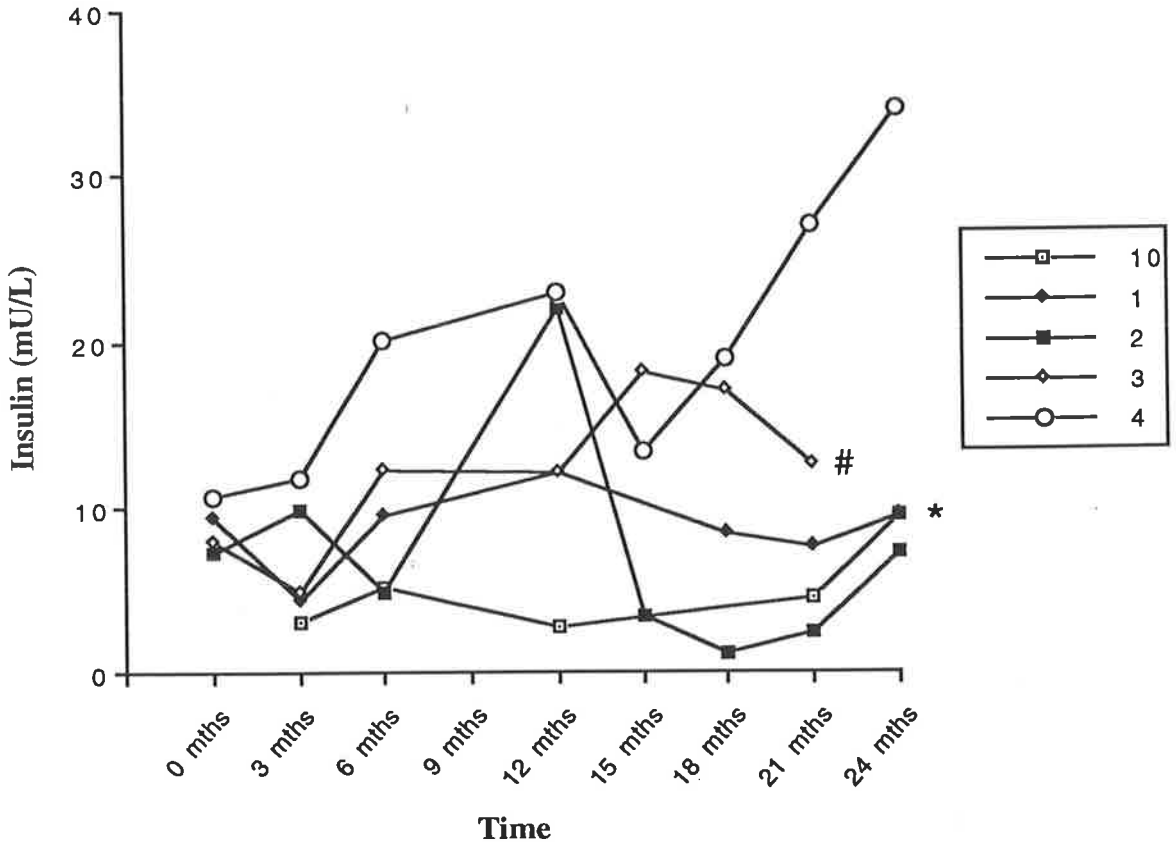
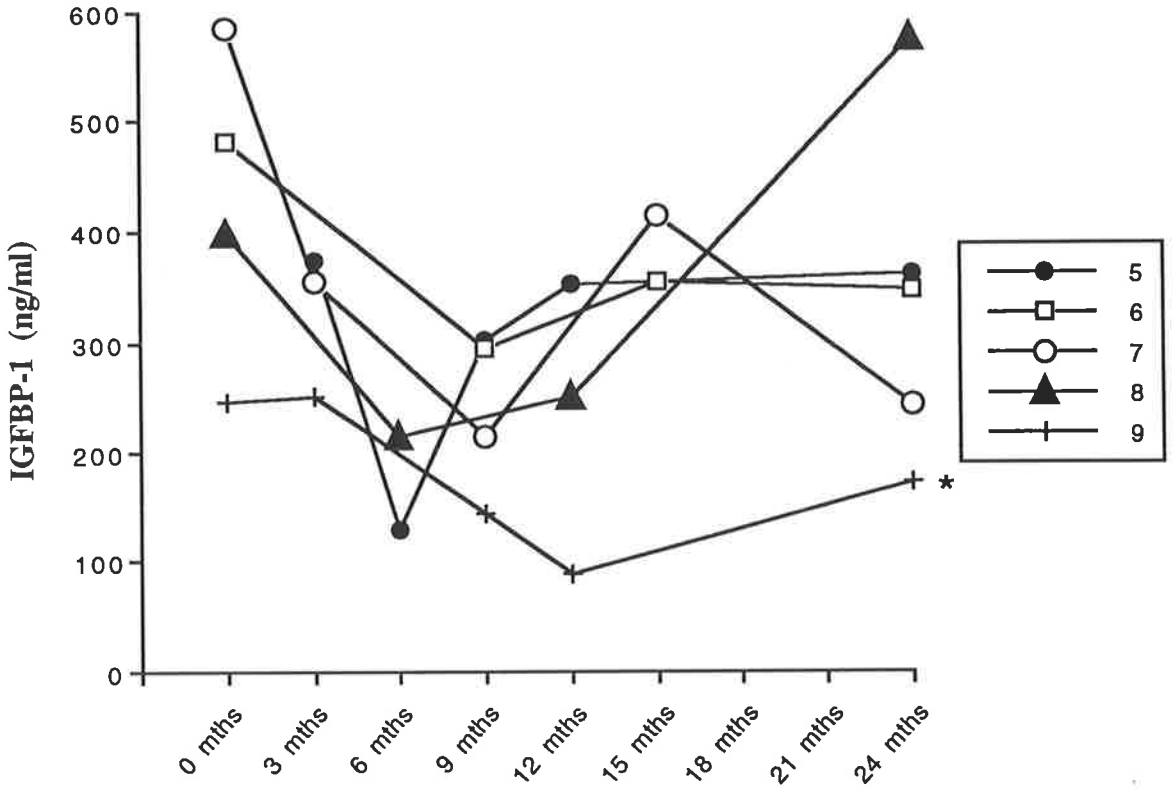


Fig 3.7 *Changes in serum IGFBP-1 (a) and fasting serum insulin (b) concentrations in 5 pubertal boys with CRF treated with rhGH for 2 years.* Serum IGFBP-1 concentrations were measured retrospectively, and fasting insulin levels were measured in serum samples collected before and at intervals during treatment.

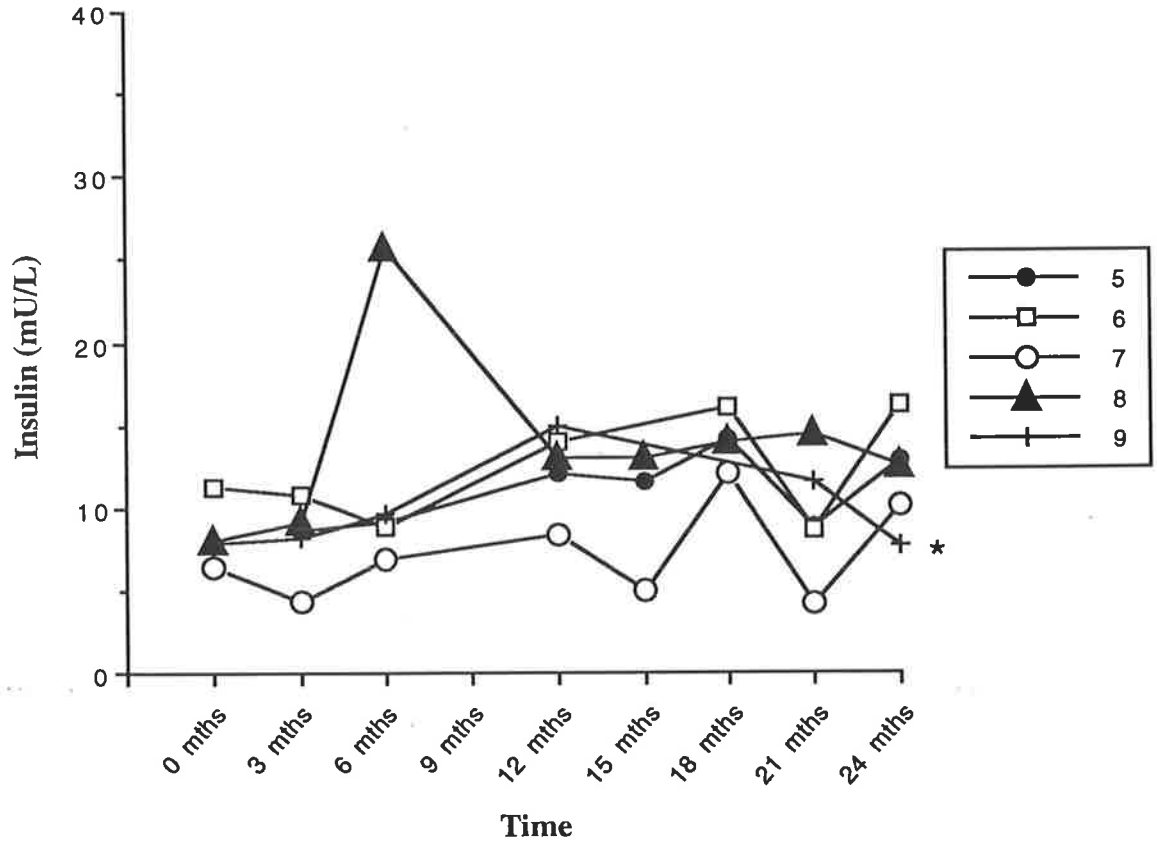
* lack of compliance with treatment at this time point .

\# patient had undergone renal transplantation before this sample was collected.

a) Serum IGFBP-1 levels in pubertal boys on rhGH



b) Serum Insulin levels in pubertal boys on rhGH

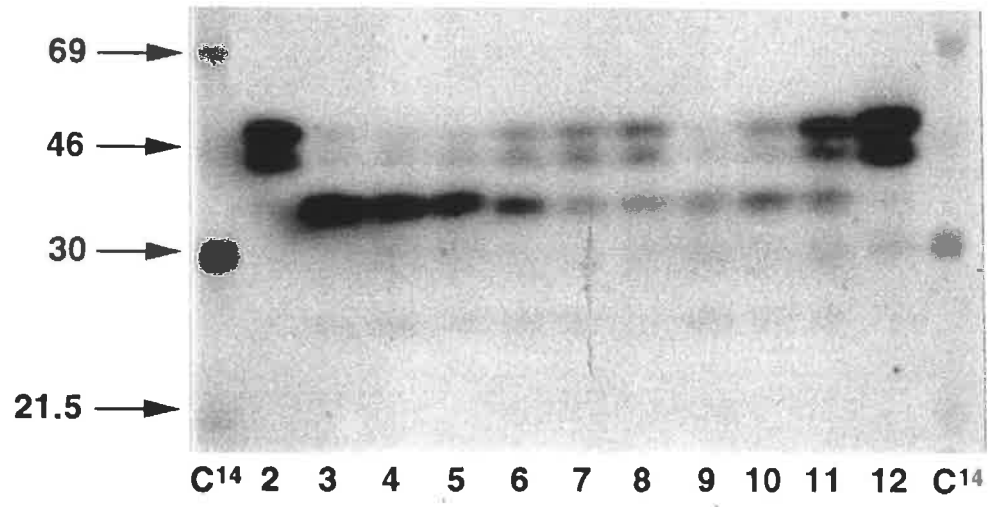


from only two boys from each of the prepubertal and pubertal groups, and to show the ligand blots from the other children in the two groups in Appendix B. In the prepubertal group, Patients 3 and 4, received renal transplants, and their WLBs and WIBs are discussed in detail in Chapter 5. I have elected to discuss in particular, Patient 10, the youngest of the children and Patient 9, the oldest boy, to describe the age-related changes seen on WLB before, and in response to rhGH treatment. Changes observed on WLB in Patients 2 and 7, from the prepubertal and pubertal groups respectively, are also described.

Five proteins bands which bound to the ligands, *viz.* ^{125} I-IGF-I or ^{125} I-IGF-II, were identified in each child (See Figures 3.8-3.11). The dominant band in Patient 10 (Figure 3.8), the youngest child, was seen at 34 kDa on the WLB. This band was most dense at age 1.25 years, before rhGH therapy, and declined progressively with age until approximately 3.0 years of age (lanes 3-6), after which the density appeared to remain relatively constant (lanes 4-11). This band was not clearly visible in either of the control sera (lanes 2 and 12). This band was identified by WIB as IGFBP-2 (see Chapter 2, Figure 2.4). In contrast, two protein bands, seen at 44-46 and 46-48 kDa, increased with age, showing a clear inverse relationship with the declining intensity of the IGFBP-2 band (lanes 3-7). These bands, at age 5.5 years in the patient, were considerably less dense than that of the normal prepubertal control pooled sample in which the average age of the subjects was 6.3 years. This doublet increased in intensity both with increasing age and on rhGH therapy, although the isolated reduced intensity of the doublet in lane 9 is possibly due to a technical problem. This doublet, was identified by WIB as IGFBP-3 (Chapter 2, Figure 2.2). A band, faintly visible at approximately 29-30 kDa on the WLB did not appear to be altered by increasing age or rhGH therapy. It was identified by specific antibody on WIB as IGFBP-1 (Chapter 2, Figure 2.3). The fifth band identified, was

Figure 3.8 *Western ligand blot of sequential serum samples from Patient 10.* (see Chapter 3.2). The ligand used was ^{125}I-IGF-II. ^{14}C molecular weight markers are shown in the left and right columns. Lane 2, prepubertal normal male; Lanes 3-4, Patient 10 aged 1.3-2.6 years, no rhGH, GFR of 25-21 ml/min/1.73m^2; Lanes 5-9, aged 2.8-4.0 years, on rhGH, stable GFR; Lanes 10-11, aged 4.5-5.5 years no rhGH, stable GFR; Lane 12, pubertal normal male.

Figure 3.9 *Western ligand blot of sequential serum samples from Patient 2.* (see Chapter 3.2). The ligand used was ^{125}I-IGF-II. ^{14}C molecular weight markers are shown in the left and right columns. Lane 2, prepubertal normal male; Lanes 3-6, Patient 2 aged 3.0-5.5 years, no rhGH, GFR of 65-45 ml/min/1.73m^2; Lanes 7-10, aged 6.0-7.5 years, on rhGH, GFR of 47-43 ml/min/1.73m^2; Lane 11, aged 8.3 years no rhGH, GFR of 39 ml/min/1.73m^2; Lane 12, pubertal normal male.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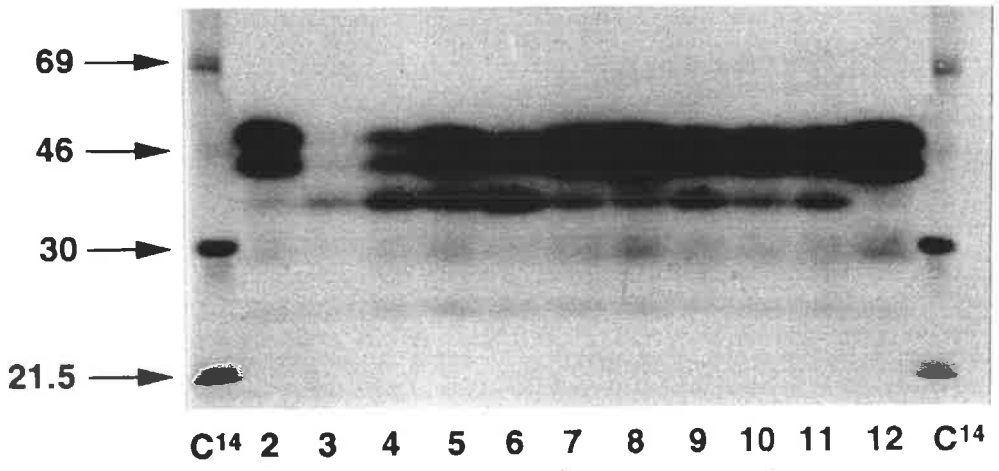
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faintly visible at 24 kDa, and was recognised by the anti-IGFBP-4 antibody on WIB (Chapter 2, Figure 2.5). The GFR in this child remained stable throughout the treatment period and therefore would have had little effect on the changes noted in the IGFBPs on WLB.

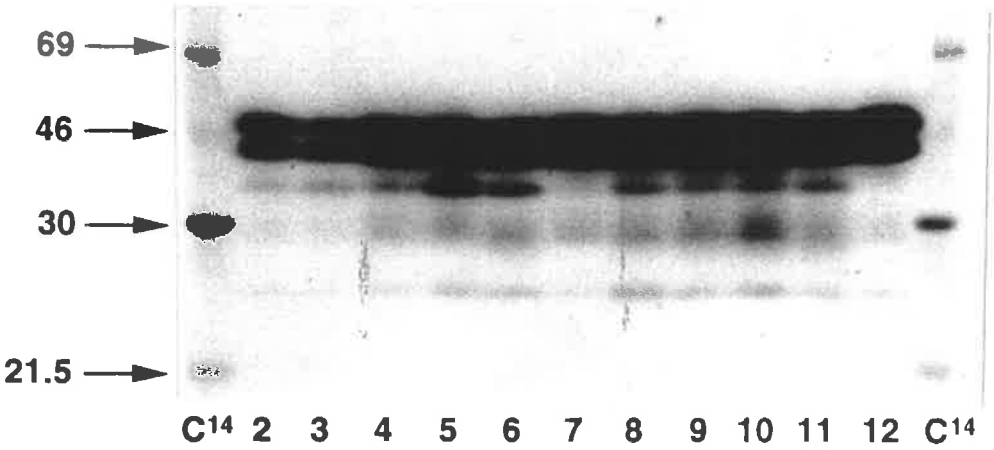
In prepubertal Patient 2 (see Figure 3.9), the IGFBP-2 band (34 kDa) was also the dominant band seen at the youngest (2.9 years (lane 3)). This band remained dense in this patient, not showing the inverse relationship with the IGFBP-3 doublet so clearly noted in Patient 10, although the intensity did appear to decrease slightly with increasing age and rhGH therapy (lanes 7-8). As with patient 10 (Figure 3.8), the IGFBP-3 doublet became increasingly dominant with increasing age (lanes 4-6) and rhGH treatment (lanes 7-10). IGFBP-1 (29-30 kDa) and -4 (24 kDa) did not appear to be altered by therapy. Renal function altered very little during rhGH treatment in this boy thereby presumably having little effect on the IGFBPs.

The dominant IGFBP identified by WLB in pubertal Patient 9 (see Figure 3.10) was the IGFBP-3 doublet (44-48 kDa) which demonstrated the characteristic increase in intensity with age (lanes 3-6) and rhGH therapy (lanes 8-10). IGFBP-2, IGFBP-1 and IGFBP-4 appeared increased in the patient's serum, compared with the controls (lanes 2, 7 and 12), and also in association with declining renal function (lanes 5-6 and 8-11). These bands did not seem to be altered by growth hormone therapy (lanes 8-10).

The IGFBP-3 doublet was also the predominant feature on WLB in pubertal Patient 7 (see Figure 3.11) and appeared increased with age (lanes 3-7) and rhGH therapy (lanes 8-11). IGFBP-1, -2 and -4 bands were again more intense in the CRF boy than in the normal pubertal

Figure 3.10 *Western ligand blot of sequential serum samples from Patient 9* (see Chapter 3.2). The ligand used was ^{125}I-IGF-I. ^{14}C molecular weight markers are shown in the left and right columns. Lane 2, prepubertal normal male; Lanes 3-6, Patient 9 aged 12.8-15.8 years, no rhGH, GFR of 37-13 ml/min/1.73m^2; Lanes7, pubertal normal male; Lanes 8-10, Patient 9 aged 15.8-16.6 years on rhGH, GFR of 14 ml/min/1.73m^2 -haemodialysis; Lane 11, Patient 9, aged 17.8 years, no rhGH; Lane 12, normal adult male.

Figure 3.11 *Western ligand blot of sequential serum samples from Patient 7* (see Chapter 3.2). The ligand used was ^{125}I-IGF-I. ^{14}C molecular weight markers are shown in the left and right columns. Lane 2, prepubertal normal male; Lanes 3-7, Patient 7 aged 7.4-12.1 years, no rhGH, GFR of 24-17 ml/min/1.73m^2; Lanes 8-11, aged 12.8-15.0 years, on rhGH, GFR of 17 ml/min/1.73m^2 -haemodialysis; Lane 12, pubertal normal male.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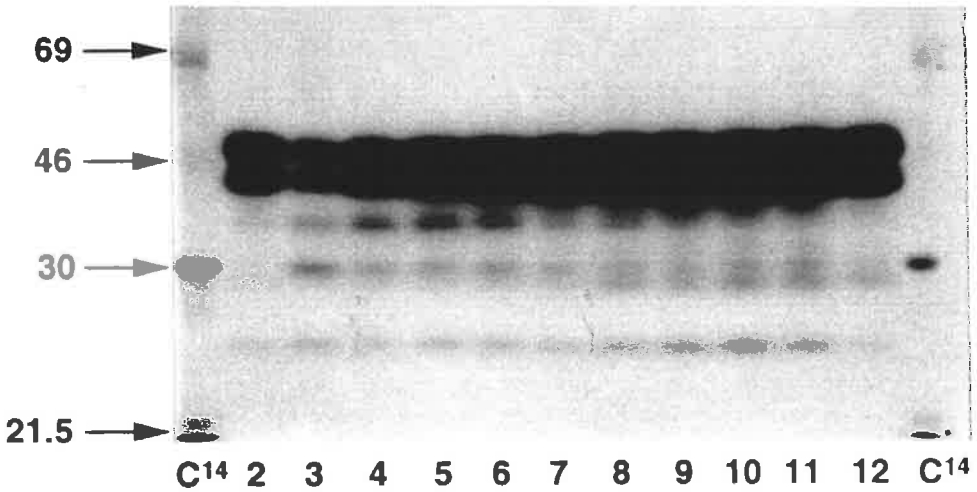
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control and did not appear to be altered by rhGH, however the IGFBP-4 band appeared to increase in association with the decline in renal function (lanes 7-11).

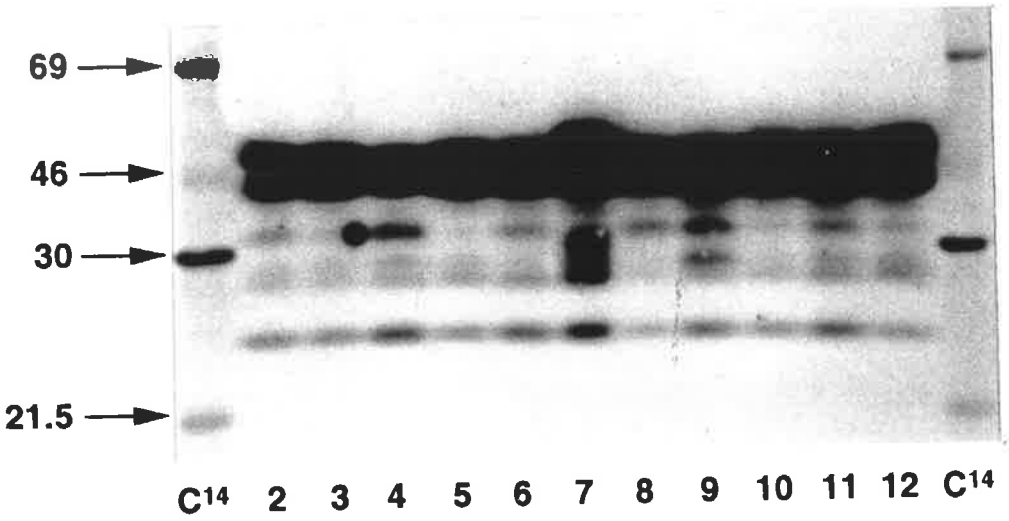
Figure 3.12 demonstrates the comparison between individual CRF and SSGC children. Increased ligand binding was observed in both prepubertal and pubertal CRF sera compared with the respective SSGC children, both before and after the introduction of rhGH. This is evident in lanes 4 and 6, and lanes 9 and 11, where IGFBP-2 and IGFBP-4 bands in the CRF patients were more intense compared to the SSGC boys, lanes 3 and 5, and 8 and 10 respectively. These particular IGFBP bands were also more intense in the CRF patients before rhGH therapy (lanes 4 and 9) than their respective normal controls (lanes 2 and 12). BP-1 also appeared to be increased in the pubertal CRF child before rhGH therapy (lane 9). The IGFBP-3 doublet did appear to increase in density with rhGH treatment in the prepubertal CRF boy, however, little effect due to rhGH was noted in the prepubertal SSGC, and both the pubertal SSGC and CRF boys on the WLB. Table 3.1 compares the IGFBP-3 and -1 RIA data between the same prepubertal and pubertal CRF, SSGC and normal control boys whose sera were analysed by WLB in Figure 3.12. These data confirm the increase in IGFBP-3 noted on WLB in the prepubertal CRF child during rhGH therapy, as well as the marked elevated BP-1 concentration in the pubertal CRF boy before commencing rhGH treatment. Serum IGFBP-3 levels in the CRF children showed a greater increase in response to rhGH therapy than the respective SSGC boys (Table 3.1), with the greatest response being in the prepubertal CRF male. IGFBP-1 levels decreased in the CRF children during therapy, whereas levels in the SSGC males remained virtually unchanged.

Table 3.1 Comparison between IGFBP-3 and -1 RIA values in CRF and SSGC children before and during rhGH treatment

Patient	prepubertal CRF	prepubertal SSGC	prepubertal normal	Pubertal CRF	Pubertal SSGC	Pubertal normal
Age at sampling (years)	10.0	10.3	6.3	12.1	12.3	13.3
Bone age (years)	8.0	10.0	N/A	8.0	11.0	N/A
Before rhGH						
IGFBP-3 (µg/ml)	3.7	3.6	3.2	3.8	2.9	5.5
IGFBP-1(µg/ml)	185.5	133.9	40.9	481.9	25.8	24.6
During rhGH						
IGFBP-3 (µg/ml)	8.4	5.5	N/A	6.4	4.3	N/A
IGFBP-1(µg/ml)	117.0	148.0	N/A	355.3	24.7	N/A
Duration of rhGH treatm	18 months	16 months		14 months	12 months	

N/A : not available

Figure 3.12 *Western ligand blot of prepubertal and pubertal normal, CRF and SSGC serum.* (see Chapter 3.2).The ligand used was ^{125}I-IGF-I. Lane 2, prepubertal normal male; Lane 3, prepubertal SSGC male; Lane 4, prepubertal CRF male; Lane 5, prepubertal SSGC male on rhGH; Lane 6, prepubertal CRF male on rhGH; Lane 7, ovine IGFBP-3 QC; Lane 8, pubertal SSGC male; Lane 9, pubertal CRF male; Lane 10, pubertal SSGC male on rhGH; Lane 11, pubertal CRF male on rhGH; Lane 12, pubertal normal male.^{14}C molecular weight markers are shown in the left and right columns.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3.4 Discussion

The improvement in height velocity seen in our patients on rhGH treatment is similar to that reported in several other studies involving children with CRF (Hokken-Koelega, Stijnen, De Muinck Keizer-Schrama, *et al*, 1991; Lippe, Fine, Koch, *et al*, 1988; Rees, Rigden, Ward, *et al*, 1990; Tonshoff, Mehls, Heinrich, *et al*, 1990; Tonshoff, Mehls, Schauer, *et al*, 1989). It is notable that the greatest change in height velocity in our children was seen in the first 12 months of therapy, followed by a decline in the subsequent year, although the growth velocity in the second year was still in excess of pretreatment values. Our patients were all in preterminal renal failure during the first year of therapy, corresponding to the greatest improvement in growth velocity. During the second year, however, when the mean velocity did slow, two boys required dialysis and one of these was subsequently transplanted, while another child also received a renal graft. An rhGH dose of 4 IU/m^2/day, similar to the dose of 30 IU/ m^2/week used in our trial (van Renen, Hogg, Sweeney, *et al*, 1992), was reported by Hokken-Koelega *et al* to result in a growth response, in the first 12 months, which matched our findings, and also decreased during the second year of treatment (Hokken-Koelega, Stijnen, de Jong, *et al*, 1994). Eight of their patients were managed conservatively, whereas 15 were receiving dialysis therapy for end stage renal failure. Tönshoff *et al* report an increase in height velocity from a median baseline 4.3 to 10.0 cm/year in the first year of rhGH treatment in 17 prepubertal preterminal CRF children, falling to 6.6 cm/year in 9 patients in the second year. The dose of rhGH used was 30 IU/ m^2/week (Tonshoff, Dietz, Haffner, *et al*, 1991). These results are very similar to our own, except that our children displayed a slightly better pretreatment mean growth velocity than did their subjects, which may be related to our strict

dietary management (Jureidini, Hogg, van Renen, *et al*, 1990). They noted that the growth response after the first year on therapy was significantly higher in the children with preterminal CRF than those with end stage renal failure. A further study has reported age to be the most important predictor of response to rhGH therapy in 23 prepubertal children with CRF, with the best response observed in the youngest, conservatively managed children (Rees and Maxwell, 1996).

The most significant biochemical response to rhGH treatment in our children was the increase in serum IGF-I concentrations above baseline levels. This was more impressive in the prepubertal boys compared to the pubertal group. The pretreatment IGF-I levels in our patients were in the low normal range and were not dissimilar to those reported by Hokken-Koelega *et al* and Tönshoff *et al* in their studies (Hokken-Koelega, Stijnen, de Jong, *et al*, 1994; Tonshoff, Tonshoff, Mehls, *et al*, 1992). Their RIAs were also performed after acid-chromatography extraction of serum to remove the effect of interference of the IGFBP's in the assay (Powell, Rosenfeld, Baker, *et al*, 1986). The lack of correlation between IGF-I values and height velocity during rhGH treatment in our children, is supported by the studies of Hokken-Koelega *et al,* who also reported a lack of correlation between the IGF-I standard deviation score (SDS) and height velocity SDS, both before, and during GH therapy (Hokken-Koelega, Stijnen, de Jong, *et al*, 1994). A further study in CRF children, without rhGH treatment, also found no association between circulating levels of IGF-I and growth parameters (Hodson, Brown, Roy, *et al*, 1992) and, furthermore, no correlation between plasma IGF-I levels and growth velocity was observed during a study of short-term treatment of GH-deficient children treated with rhGH (Lopes, Theintz, Torresani, *et al*, 1992). The lack of association between growth and IGF-I concentrations in these different groups of children

suggests the possible involvement of additional factors, which need to be taken into account when attempting to understand the complex mechanism of growth.

IGF-II concentrations before rhGH therapy in our prepubertal boys were in agreement with those reported by Hokken-Koelega *et al*, and higher, on average, than those reported by Tönshoff *et al* before treatment. rhGH treatment in our patients resulted in a greater elevation of this growth factor than that reported in these two studies (Hokken-Koelega, Stijnen, de Jong, *et al*, 1994; Tonshoff, Tonshoff, Mehls, *et al*, 1992), however despite this, the rise in IGF-II concentrations was considerably less than the rise in IGF-I levels in both prepubertal and pubertal groups. The relative increase, however, in IGF-II values on rhGH was similar in both groups. In 1993, Argente and co-workers studied a large cohort of healthy Spanish children to develop normative data for the IGFs and IGFBPs, and to relate these results to age and sex-related changes (Argente, Barrios, Pozo, *et al*, 1993). The results showed a slow but steady increase in serum IGF-I levels during early to mid-puberty (Tanner stages I-III), with no significant differences found between Tanner stages III and IV, followed by a significant decline in IGF-I levels between stages IV and V. In contrast, IGF-II concentrations did not alter significantly between Tanner stages I and IV, but showed a similar decline in levels between Tanner stages IV and V. This normal physiological difference between IGF-I and -II concentrations during growth and development may account for the observed differences in the responses of these growth factors to rhGH treatment in our two groups of children.

Serum levels of IGFBP-3 and ALS in our children, which were within the range for normal healthy children before rhGH, increased in response to therapy to a similar degree, resulting in the conservation of the significant correlation between IGFBP-3 and ALS throughout the treatment period. Our baseline IGFBP-3 results were similar to those from another study in

which the same RIA was used to measure serum concentrations (Hodson, Brown, Roy, *et al*, 1992). Our data are similar to those reported by Tönshoff *et al* (Tonshoff, Tonshoff, Mehls, *et al*, 1992), but lower than those obtained by Hokken-Koelega and co-workers (Hokken-Koelega, Stijnen, de Jong, *et al*, 1994). Differences in the immunoreactivity of the different antibodies employed in the various IGFBP-3 RIAs might account for the variation in reported serum concentrations. IGFBP-3 levels have been reported as being elevated in CRF by several workers (Blum, Ranke, Kietzmann, *et al*, 1991; Goldberg, Trivedi, Delmez, *et al*, 1982; Powell, Liu, Baker, *et al*, 1996; Tonshoff, Blum, Wingen, *et al*, 1995) due to an abundance of IGFBP-3 fragments in the 20-60 kDa serum fraction on size-exclusion chromatography (Blum, Ranke, Kietzmann, *et al*, 1991) and it is possible that these may not be recognised by certain antisera specific for the intact IGFBP-3 molecule. A molar excess of IGFBP-3 compared with the sum of IGF-I and IGF-II has been reported in CRF (Tonshoff, Blum, Wingen, *et al*, 1995). The very slight increase in the ratio of IGF-I + -II: IGFBP-3 noted in our study at 9-12 months was not statistically significant in our small groups of patients, however it does correspond to the period of greatest gain in height velocity and suggests that the supposed inhibitory effects of high molar levels of IGFBP-3 were counteracted by the significantly elevated IGF-I and moderately elevated IGF-II concentrations induced by rhGH treatment. A significant inverse relationship between IGFBP-3 RIA levels and GFR has been reported by Tönshoff and co-workers in 1995 suggesting that the serum concentrations of IGFBP-3 increase with declining renal function (Tonshoff, Blum, Wingen, *et al*, 1995). This was not evident before rhGH treatment in our study and was noted only at the 6 month time point, associated with rising IGFBP-3 levels in response to therapy.

The fasting serum IGFBP-1 levels in our CRF patients were highly elevated before commencing rhGH therapy, a finding which agrees with that of several other workers (Blum,

Ranke, Kietzmann, *et al*, 1991; Hokken-Koelega, Stijnen, de Jong, *et al*, 1994; Lee, Hintz, Sperry, *et al*, 1989; Tonshoff, Tonshoff, Mehls, *et al*, 1992) and this concentration may contribute significantly to the overall increase in IGF binding capacity found in CRF (Blum, Ranke, Kietzmann, *et al*, 1991; Lee, Hintz, Sperry, *et al*, 1989). GH treatment resulted in a decrease in the IGFBP-1 concentrations in both our groups of children, a feature which has been reported in two controlled rhGH trials in children with CRF (Hokken-Koelega, Stijnen, de Muinck Keizer-Schrama, *et al*, 1991; Powell, Attie, Hintz, *et al*, 1995), although this was shown to be dose-dependent and not sustained beyond the first 12 months in one study (Hokken-Koelega, Stijnen, de Muinck Keizer-Schrama, *et al*, 1991). IGFBP-1 may play a significant role in modulating the insulin-like activity of the circulating IGFs, possibly by blocking IGF actions at the tissue level (Lewitt, 1994). Serum IGFBP-1 levels in humans have been shown to fluctuate more than 10-fold over a few hours (Baxter and Martin, 1989) and both in vivo and in vitro studies have indicated that these fluctuations are largely due to regulation of IGFBP-1 transcription by insulin (Holly, 1991; Lee, Conover and Powell, 1993; Lewitt, Denyer, Cooney, *et al*, 1991). We have previously reported that fasting serum insulin levels rise during rhGH therapy (van Renen, Hogg, Sweeney, *et al*, 1992), and a recent study, monitoring carbohydrate metabolism in CRF children on GH therapy for 5 years, showed a significant increase in serum insulin levels over this time, although levels remained within the normal range (Saenger, Attie, DiMartino-Nardi, *et al*, 1996). The reduction in serum concentrations of IGFBP-1 noted in our study and the inverse relationship between insulin and IGFBP-1 observed in our patients, has also recently been observed by Powell *et al* (Powell, Liu, Baker, *et al*, 1996) and further confirms insulin as a potent regulator of IGFBP-1.

Characterisation of IGFBPs -1, -2, -3 and -4 by WLB and WIB in our CRF children revealed that the normal inverse correlation between IGFBP-2 and IGFBP-3 with increasing age is

maintained in CRF. Ligand blotting clearly showed an increase in IGFBP-2 and -4 in CRF children compared with SSGC and normal children, while rhGH treatment possibly resulted in a slight decrease in IGFBP-2, although this finding would best be confirmed by IGFBP-2 RIA. The IGFBP-3 doublet did not appear to be altered by GH therapy on the WLB, whereas the IGFBP-3 RIA indicated a substantial increase in the CRF serum concentrations during treatment. We have identified a 30 kDa IGFBP-3 form in normal and CRF children by WIB which does not bind to the ligand in WLB (see Fig.2.2), also Powell and co-workers have shown 14 and 19 kDa IGFBP-3 fragments in CRF serum by immunoprecipitation which do not appear on WLB (Powell, Liu, Baker, *et al*, 1993). It would thus appear that some immunoreactive forms of IGFBP-3 cannot bind the iodinated ligand. Whether these forms of IGFBP-3 also do not bind endogenous ligand *in vivo* is open to speculation (Chapter 4.4). This discrepancy between ligand blotting and radioimmunoassay was initially reported in 1992, when Gargosky *et al* emphasized the need to characterize IGFBPs by both ligand binding and immunological methods (Gargosky, Pham, Wilson, *et al*, 1992).

3.5 Conclusions

Elevated serum IGFBP-1, -2, -4 levels identified in CRF patients compared with normal and SSGC children, together with low-normal IGF-I concentrations, may result in an increased IGF binding capacity with a consequent reduction in IGF bioactivity leading to growth retardation. Growth hormone treatment results in a significant increase in linear growth in children with CRF which is greatest during the first 12 months of therapy, in association with a significant increase in serum IGF-I concentrations, a moderate increase in IGF-II, IGFBP-3 and ALS levels and a fall in IGFBP-1 and possibly also IGFBP-2 concentrations.

3.6 Acknowledgements

My sincere thanks go to Sabine Harrer for performing the IGF assays and to Callum Gillespie for his patience in teaching me the intricacies of Western ligand and immunoblotting.

CHAPTER 4

DISTRIBUTION OF IGF-IGFBP COMPLEXES IN SERUM OF CHILDREN WITH CHRONIC RENAL FAILURE AND TRANSPLANTATION BEFORE AND AFTER GH TREATMENT

4.1 Introduction

The complex pathogenesis of the growth retardation in CRF has resulted in much research being carried out in an attempt to identify a predominant cause for this lack of linear growth despite optimal medical and dietary management (van Renen, Hogg, Sweeney, *et al*, 1992) and normal serum growth hormone and IGF levels (Hodson, Brown, Roy, *et al*, 1992; Powell, Rosenfeld, Sperry, *et al*, 1987). Recently, the focus of this research has concentrated on the IGFBPs, reportedly elevated in CRF (Blum, Ranke, Kietzmann, *et al*, 1991), and their association with the IGFs in the ternary and binary complexes found in serum (Baxter, 1994). The IGFs and their specific binding proteins form complexes of different molecular weights in the serum and at any one time there is a very small amount of unbound or "free" IGF present in the serum (Baxter, Holman, Corbould, *et al*, 1995). The complexes appear to have different functions (Baxter, 1994).

The larger IGF-IGFBP complex is formed when IGF-I or -II, IGFBP-3, and an acid-labile sub-unit (ALS) combine to form a complex with a molecular weight of approximately 150 kDa (Baxter, Martin and Beniac, 1989). This complex is found only in the circulation, as it is too large to cross the endothelial barrier, and it's major function appears to be as a reservoir for the growth factors by increasing their half-life in the blood stream (Baxter, 1991; Martin and Baxter, 1992). It also plays an important part in glucoregulation as the IGFs bound in the ternary complex do not exert insulin-like activity (Baxter, Holman, Corbould, *et al*, 1995). The smaller molecular weight IGF-IGFBP complexes are formed when the growth factors bind to any one of the IGFBPs, without combining with ALS (Jones and Clemmons, 1995). These protein complexes are small enough to cross from the circulation into the interstitial tissues, where they may serve to transport the growth factors to specific tissue binding sites and may enhance or inhibit binding to these sites (Clemmons, 1992).

The aim of this study was to compare and contrast the IGF-I binding profiles in the serum of children with CRF with those of normal children, and short and slowly growing children (SSGC), to ascertain whether there were marked discrepancies between these groups which might possibly enhance our understanding of the growth failure of CRF. We also proposed to identify the different IGFBPs present in the large (150 kDa) and small (30-69 kDa) molecular weight complexes in the various groups. The effects of rhGH treatment, dialysis, and renal transplantation on these binding profiles in the CRF children were also examined to establish any notable differences. The improved growth, as a result of rhGH therapy, has been discussed in Chapter 3, and possible changes in the IGF-I binding patterns seen in the treated CRF children could further contribute to the understanding of how this hormone brings about the improved growth response. Both prepubertal and pubertal groups from both sexes were compared to assess any alterations in binding pattern which may possibly be attributed to secondary sexual development.

4.2 Materials and Methods

4.2.1 Patients

Samples from the normal control children were obtained at the time of venepuncture for another reason, after written patient and/or parental consent had been given. Blood from the SSGC group was obtained with consent during routine venepucture at an out-patient review visit. A small volume of additional blood was collected during routine biochemical tests from children with CRF, children receiving dialysis therapy for end-stage renal disease and those children who had undergone renal transplantation, before and during therapy with rhGH. In the

SSGC and renal patient groups we obtained blood from prepubertal (testicular volume < 4 ml for males and Tanner breast stage 1 for females), as well as pubertal subjects (testicular volume > 4 ml in males and Tanner breast stage > 1 for girls). The normal prepubertal children were arbitrarily designated to this category according to their age (</= 10 years) at the time of venepuncture, as no pubertal staging was available. The normal pubertal males and females, however, were categorised according to secondary sexual characteristics in the same way as the CRF and SSGC children. Serum samples from both prepubertal and pubertal males and females were pooled, if more than one sample was available, in the categories which we proposed to compare, *viz* normal; SSGC; CRF; CRF treated with rhGH; dialysis; dialysis treated with rhGH; transplanted; transplanted on rhGH. The children were grouped into four groups on the basis of pubertal status and gender (Table 1). In the dialysis subgroup only male samples were available. In the case of the prepubertal transplanted male and female, and the prepubertal and pubertal slowly-growing female children, we were not able to form pools as a specimen was available from only a single child. However, the prepubertal transplanted boy was sampled both before and during rhGH treatment (see Table 1). The number of subjects in each pool is small and in the particular cases mentioned, only one child was available for assessment, therefore we have not performed any statistical analyses of the data.

4.2.2 Sample preparation

The blood samples were collected into plain tubes containing no anti-coagulant, allowed to clot at room temperature for approximately one hour and centrifuged at 3,000 rpm at 4° C. The

Table 4.1 Categories of prepubertal and pubertal male and female children

Category	normal	CRF	SSGC	CRF/rhGH	Transplant	Transplant/rhGH	Dialysis	Dialysis/rhGH
Prepubertal males (no.)	**4**	**4**	**5**	**4**	**1**	**1**		
Average age (years)	7.6	6.5	9.2	5.3	9.5	11.0		
(range)	(5.7-9.6)	(3.0-10.0)	(7.3-12.5)	(4.0-7.0)				
Prepubertal females (no.)	**2**	**4**	**1**		**1**			
Average age (years)	9.9	7.2	10.0		4.3			
(range)	(9.4-10.3)	(3.3-11.0)						
Pubertal males (no.)	**2**	**4**	**2**	**4**	**2**	**2**	**2**	**2**
Average age(years)	15.7	15.8	14.7	14.9	11.4	13.5	16.4	15.1
(range)	(14.8-16.5)	(14.0-19.0)	(13.5-15.8)	(13.3-16.6)	(11.0-11.8)	(12.0-15.0)	(16.2-16.6)	(15.0-15.2)
Pubertal females (no.)	**3**	**3**	**1**		**2**			
Average age (years)	13.0	14.9	12.0		17.2			
(range)	(12.9-13.6)	(13.4-16.6)			(16.0-18.4)			

serum was aspirated and stored in tubes at -80° C. Equal volumes of each child's serum was used to provide pooled samples representative of each group (Table 1). These were well vortexed and stored at -80° C for further analysis.

4.2.3 Analytical gel chromatography

Individual patient samples were delipidated and incubated with ^{125}I-IGF-I at 4° C overnight and at 37° C for 1 minute and processed according to the method for fast protein liquid chromatography (Chapter 2.6). In addition, pooled serum samples were incubated with ^{125}I-IGF-I at 4° C overnight and then separated into the different molecular weight complexes by FPLC. Fractions (1-85) were collected, the radioactivity counted in a gamma-counter (Chapter 2.6) and then stored in tubes at -80° C for further analysis.The column was calibrated with known molecular weight markers before and after the samples were run (Chapter 2.6).

4.2.4 Western immunoblotting

Western immunoblotting was used to identify which IGFBPs were present in the large and small molecular weight IGF-IGFBP complexes in both individual and pooled FPLC fractions. Proteins contained in alternate individual FPLC fractions from 21 to 33 (of the 1-85 0.5 ml fractions), which contained the ^{125}I-IGF-I binding peaks (as determined by the molecular weight markers, see Chapter 4.3.1), were further separated by SDS PAGE using the Hoefer Mighty Small II minigel system (Hoefer Scientific Instruments, San Francisco, CA, USA). Following transfer to nitrocellulose filters (Hoefer Semiphor semi-dry transfer unit, Hoefer

Scientific Instruments, San Francisco, CA, USA), the proteins were immunoblotted with the anti-hIGFBP-3 antibody (R100), the anti-hIGFBP-1 antibody (A2), and the anti-hIGFBP-2 antibody (UBI) using the enhanced chemiluminescent method (see Chapter 2.4). Three second to 5 minute exposures of the incubated nitrocellulose to X-ray film (Dupont, Wilmington, MA, U.S.A.) were used to demonstrate the binding protein bands. The molecular weight of IGFBPs-1, -2, -3 and -4 have previously been identified (Chapter 2.5.1). The individual FPLC fractions making up the area under the peaks for molecular weights 150 and 30-69 kDa were pooled using equal volumes from each fraction. Therefore fraction (#) 22-24 were combined to form the large complex pool and the small complex pool was made up of # 25-30. The constituent proteins in the pooled fractions were separated by SDS PAGE (Chapter 2.4) and immunoblotted for IGFBP-1, -2, -3.

4.3 Results

4.3.1 Analytical gel chromatography

Thyroglobulin, with a known molecular weight of 669 kDa, the approximate molecular weight of the type II IGF receptor appeared at # 17-18 on the chromatogram. γ-globulin, with an established molecular weight of 150 kDa, the molecular weight of the large IGF-IGFBP ternary complex appeared at # 23. Bovine serum albumin (69 kDa) and carbonic anhydrase (29 kDa) were used to predict the elution positions of the binding proteins occurring in the small molecular weight binary complex, *i.e.* 30 - 69 kDa. They appeared at # 25-26 and # 28-29 respectively. ^{125}I-IGF-I was also run down the column where it eluted at # 32-33.

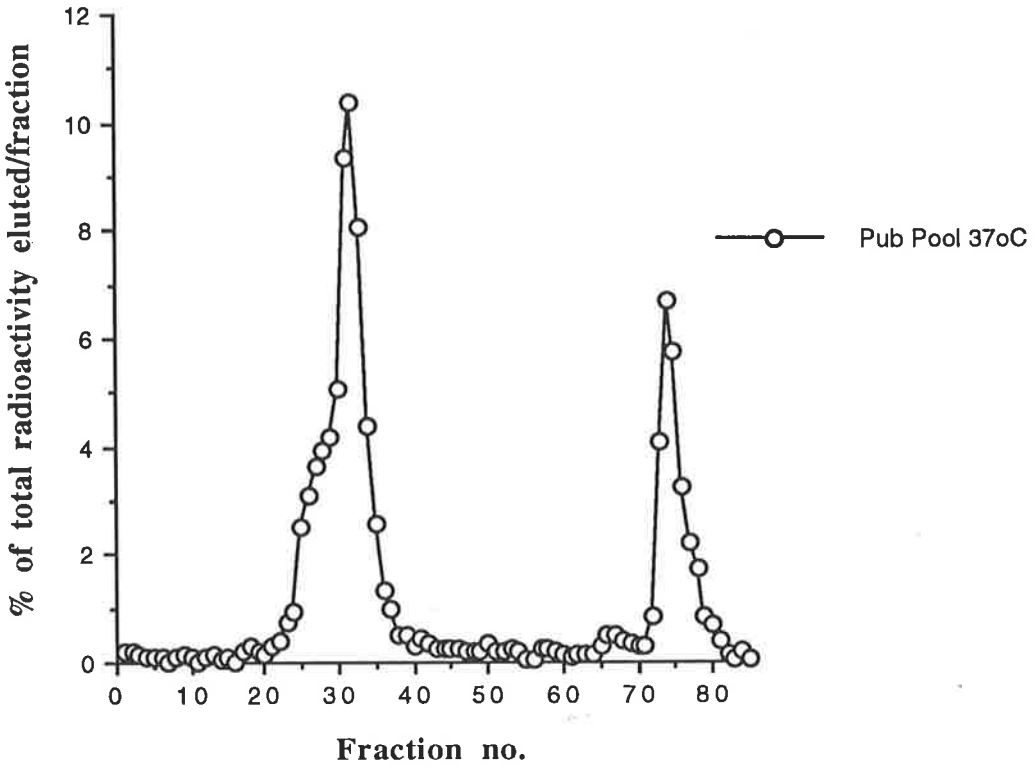
Fig. 4.1, is a representative chromatogram of serum incubated with ^{125}I-IGF-I for 1 minute at 37° C and shows the percentage of total radioactivity counted in each fraction. A small peak at # 16 - 20 is equivalent to the type II IGF receptor (669 kDa), followed by a large peak encompassing #21-42. A fifth peak at # 73 - 82 is fragmented ^{125}I-IGF-I or free radiolabelled iodine which is no longer bound to the growth factor. Fig. 4.2 is a chromatogram of the same serum as used in Fig. 4.1, but incubated overnight with ^{125}I-IGF-I at 4° C. In this case five distinct peaks of radioactivity were noted. A small peak at # 16 - 20, equivalent to the type II IGF receptor, was followed by three larger peaks at # 21 - 25, # 26 - 30 and # 31 - 40, representing 150, 30 - 69 kDa regions and the unbound ^{125} IGF-I respectively. These peaks contain the intact ^{125}I-IGF-I. When compared with Fig. 4.1, it was noticeable that there had been a shift in binding over time with the overnight incubation, from the unbound or "free" ^{125}I-IGF-I peak (# 30-40) in Fig. 1, to the 150 (#22 - 24) and 30-69 kDa binding peaks (# 25 - 30) in Fig. 4.2. This change in binding pattern with time more clearly depicted the different IGF-IGFBP complexes, therefore we have elected to show the chromatograms of the longer incubation time for all the children in the study, although both 1 minute (37° C) and overnight (4° C) incubations were performed on all the samples.

The counts per minute in each FPLC fraction contained in the binding peaks representing the large and small IGF-IGFBP complexes and unbound ^{125}I-IGF-I was calculated as a percentage of the total intact counts recorded from the beginning of the increase in radioactivity above the baseline, (arbitrarily determined to be # 16 in each case), to where the graph reached the

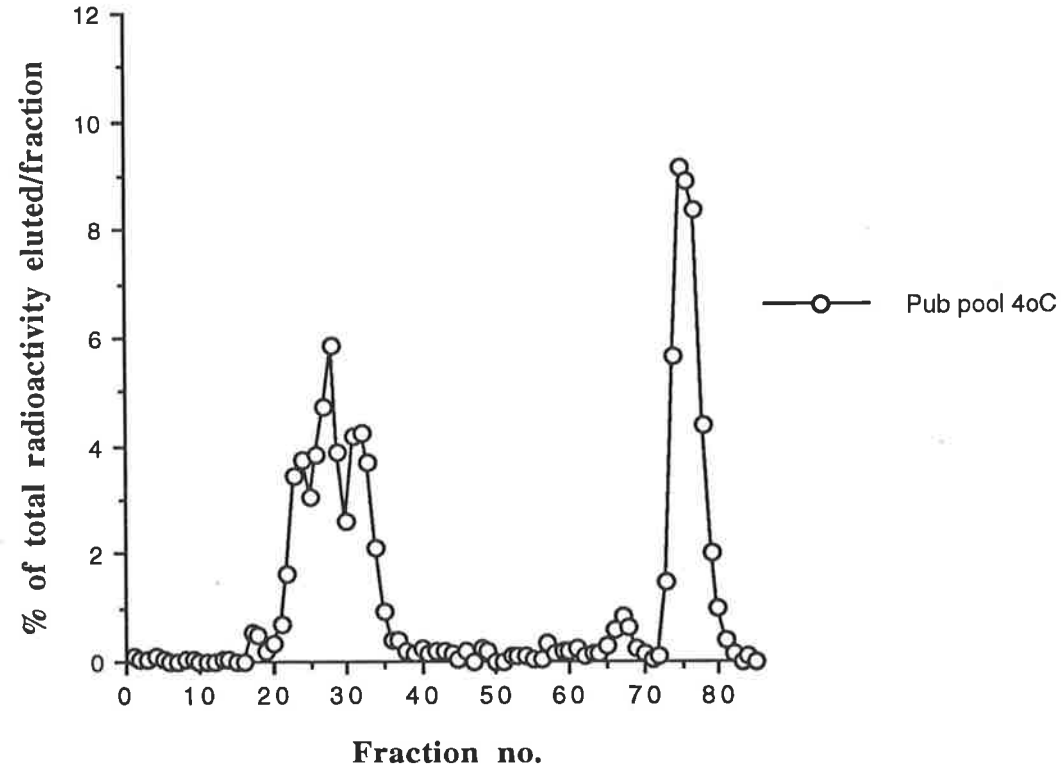
Fig. 4.1 *Elution of radioactivity from Superose-12 chromatography of pubertal pooled male serum incubated with radiolabelled IGF-I at 37° C for 1 minute.* The plots show the recovery of radioactivity in 0.5 ml fractions as a percentage of total eluted radioactivity. Samples were chromatographed as described in Chapter 2.7. Molecular weight markers used to calibrate the column and their elution fraction numbers are discussed in Chapter 4.3.1

Fig. 4.2 *Elution of radioactivity from Superose-12 chromatography of pubertal pooled male serum incubated with radiolabelled IGF-I at 4° C overnight.* The plots show the recovery of radioactivity in 0.5 ml fractions as a percentage of total eluted radioactivity. Samples were chromatographed as described in Chapter 2.7. Molecular weight markers used to calibrate the column and their elution fraction numbers are discussed in Chapter 4.3.1

Pubertal pooled male serum incubated with 125I IGF-I for 1 minute at 37oC



Pubertal pooled male serum incubated with 125I IGF-I at 4oC overnight



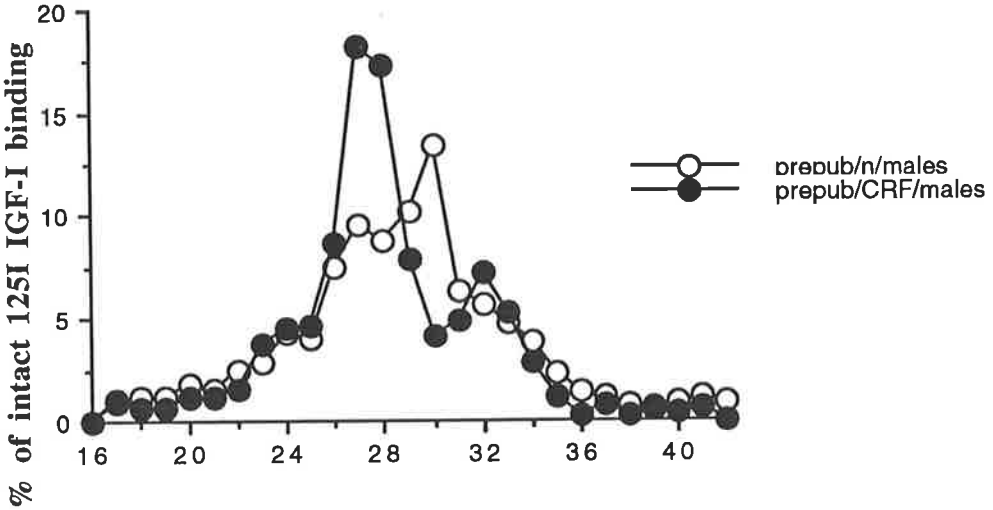
baseline after the free ^{125}I-IGF-I peak (arbitrarily determined to be # 42 in each case) on the chromatogram. The percentage for each fraction in the peak was then summed to obtain a total comprising the "area under the curve" (AUC) for each binding peak. This determined the percentage of the total counts of intact ^{125}I-IGF-I found in each of the respective peaks. The percentage of binding in the type-II IGF receptor region (669 kDa) was calculated by adding # 16-21, the large molecular weight complex was the sum of # 22-25, while binding in the 30-69 kDa region was found in # 26-30. The percentage of unbound ^{125}I-IGF-I was drawn from the remainder of the intact binding region in each case *viz* # 31-42. We were therefore able to compare numerically, as well as graphically, the binding profiles of the different groups of children (*eg* rhGH therapy, dialysis and transplantation). As the aim of this study was to compare ^{125}I-IGF-I binding patterns between different patient groups, the area of interest is confined to # 16-42 of the chromatogram which represents the region of intact ^{125}I-IGF-I.

Prepubertal normal, CRF and SSGC male pools

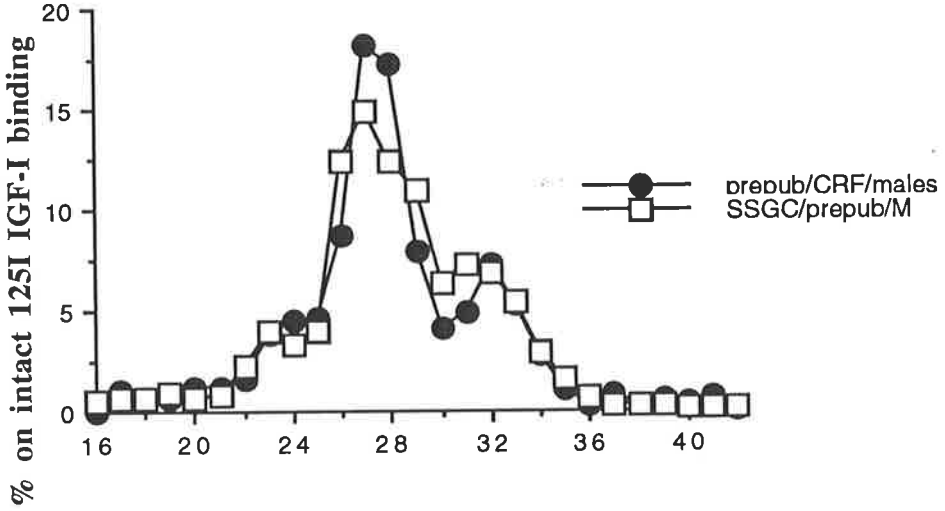
There was little difference between the prepubertal normal (13.6%), CRF (14.5%) and SSGC (13.5%) male pools in comparing the 150 kDa binding regions (Fig. 4.3 a, b and c). Comparison of the small molecular weight binding peaks of the chromatograms revealed more binding in the CRF (56.2%) and SSGC (56.9%) than in the normal (49.6%) pool, while the amount of unbound ^{125}I-IGF-I in the CRF (24.5%) was lower than that of the normal pool (30.1%) but similar to the SSGC pool (25.5%). There was slightly more ^{125}I-IGF-I binding in the region of the Type-II IGF-I receptor in the normal (6.7%) pool compared with the CRF (4.8%) and SSGC (4.3%), which were similar.

Fig. 4.3 *Comparison of percentage of intact ^{125}I-IGF-I binding from Superose-12 chromatography of normal, CRF and SSGC prepubertal pooled male serum incubated at 4° C overnight.* The plots show the percentage of intact radioactivity in 0.5 ml fractions as a percentage of total intact radioactivity in #16-42 of the chromatogram. Samples were chromatographed as described in Chapter 2.7. Molecular weight markers used to calibrate the column and their elution fraction numbers are discussed in Chapter 4.3.1. a) Comparison between chromatograms from normal and CRF prepubertal pooled male serum; b) Comparison between chromatograms from CRF and SSGC prepubertal pooled male serum; c) Comparison between chromatograms from normal and SSGC prepubertal pooled male serum.

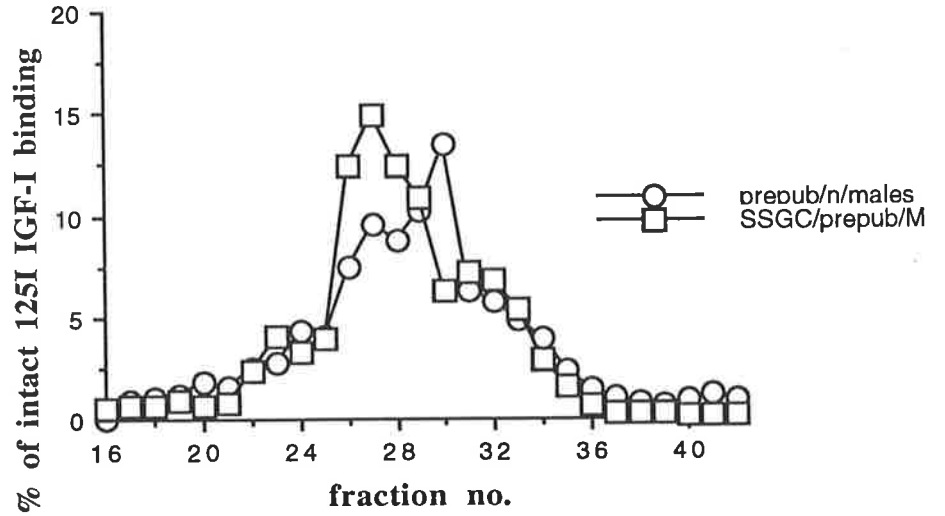
a **Comparison between prepubertal normal and CRF males**



b **Comparison between prepubertal CRF and SSGC males**



c **Comparison between prepubertal normal and SSGC males**



Prepubertal CRF male with/ without rhGH treatment and normal pools

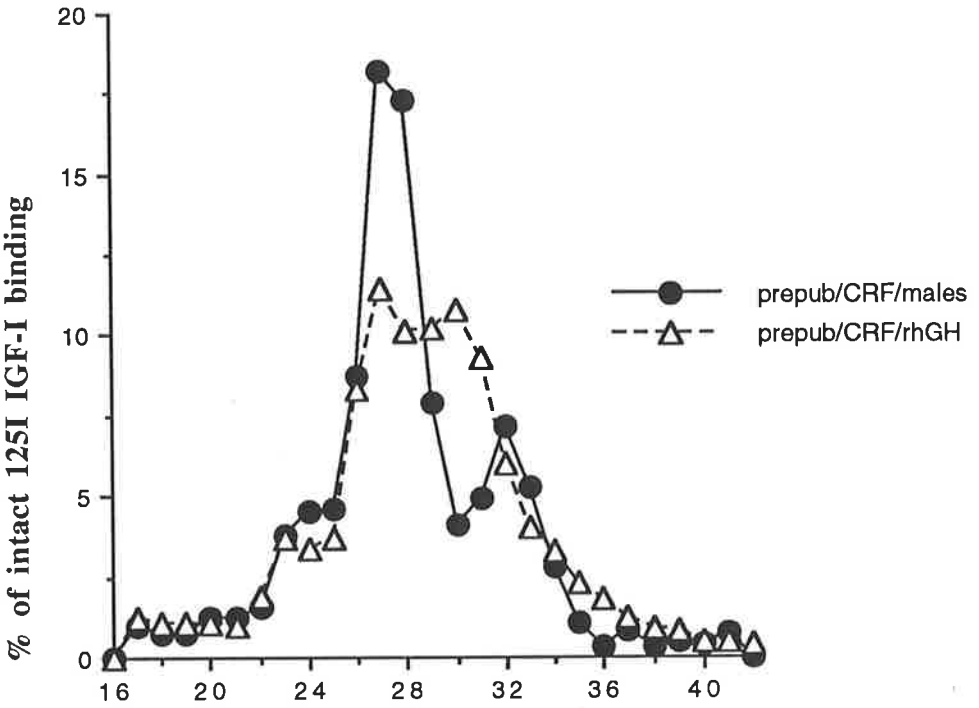
The binding profile of the rhGH-treated prepubertal CRF pool revealed a reduction in the percentage of binding in the small complex (50.8%) compared with the untreated CRF group (56.2%), and an increase in the amount of unbound ^{125}I-IGF-I (31%) compared to the group not on rhGH (24.5%) (Fig. 4.4 a). The chromatogram from the rhGH-CRF pool more closely approximated that of the prepubertal normal pool (Fig. 4.4 b). rhGH therapy did not result in much change in the amount of binding in the 150 kDa complex (12.7%) compared with the untreated CRF (14.5%) and normal (13.6%) groups, and the percentage binding in the 669 kDa region was also similar (5.5% in the rhGH-CRF pool).

Prepubertal transplanted male with/without rhGH treatment and normal pool

An increase in binding in the large complex was the significant difference between the prepubertal transplanted boy (21.8%) and the normal pool (13.6%) (Fig. 4.5 a). The amount of binding found in the small complex in this child before treatment (43.5%) was less than the normal pool (49.6%), but the percentage of unbound ^{125}I-IGF-I was very similar (31.3% in the transplanted boy and 30.1% in the normal pool) (Fig. 4.5 a). The addition of rhGH therapy in the transplanted boy did not alter the amount of binding in the 150 kDa complex (21.6% on rhGH compared with 21.8% before rhGH). However, it did result in a further reduction in binding in the 30-69 kDa complex (36.3%) compared with before treatment (43.5%) (Fig. 4.5 b), and an increase in the percentage of unbound ^{125}I-IGF-I (36.8%). Compared with the normal pool (49.6%), rhGH treatment resulted in a further fall in the percentage of small

Fig. 4.4 *Comparison of percentage of intact ^{125}I-IGF-I binding from Superose-12 chromatography of normal and CRF prepubertal pooled male serum, with and without rhGH treatment, incubated at 4° C overnight.* The plots show the percentage of intact radioactivity in 0.5 ml fractions as a percentage of total intact radioactivity in #16-42 of the chromatogram. Samples were chromatographed as described in Chapter 2.7. Molecular weight markers used to calibrate the column and their elution fraction numbers are discussed in Chapter 4.3.1. a) Comparison between chromatograms from CRF and rhGH-treated CRF prepubertal pooled male serum; b) Comparison between chromatograms from normal and rhGH-treated CRF prepubertal pooled male serum.

a **Comparison between prepubertal CRF males with/without rhGH**



b **Comparison between prepubertal normal and CRF/rhGH males**

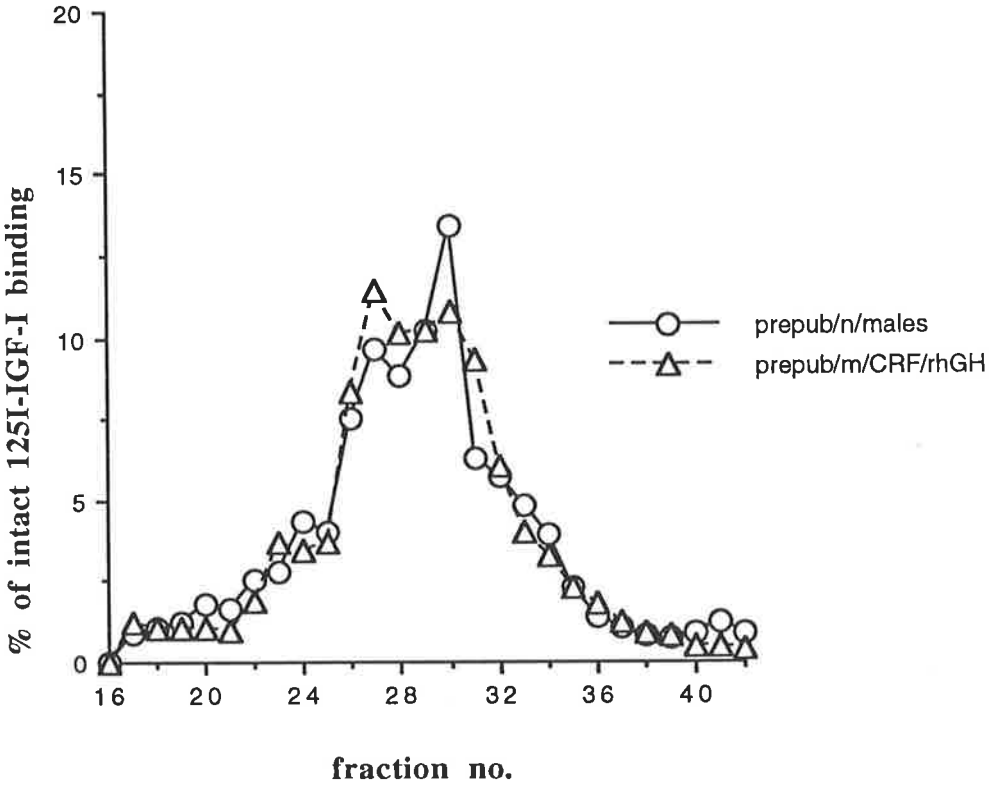
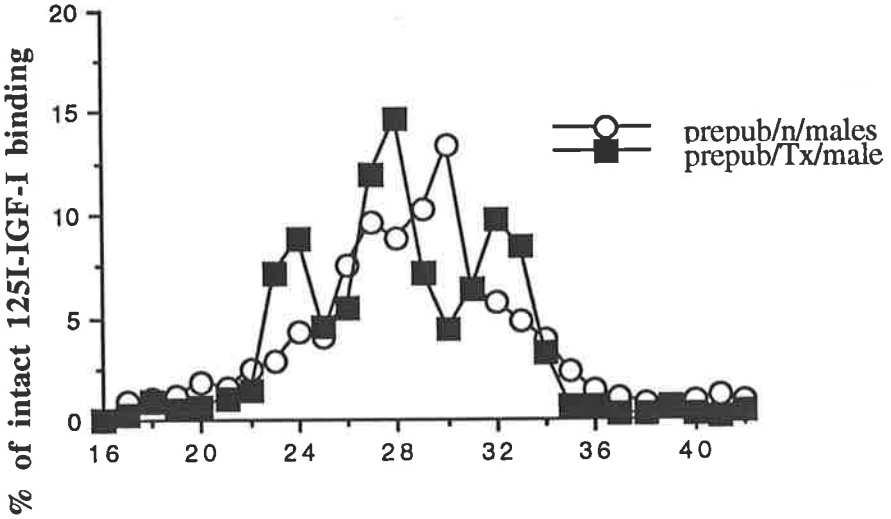
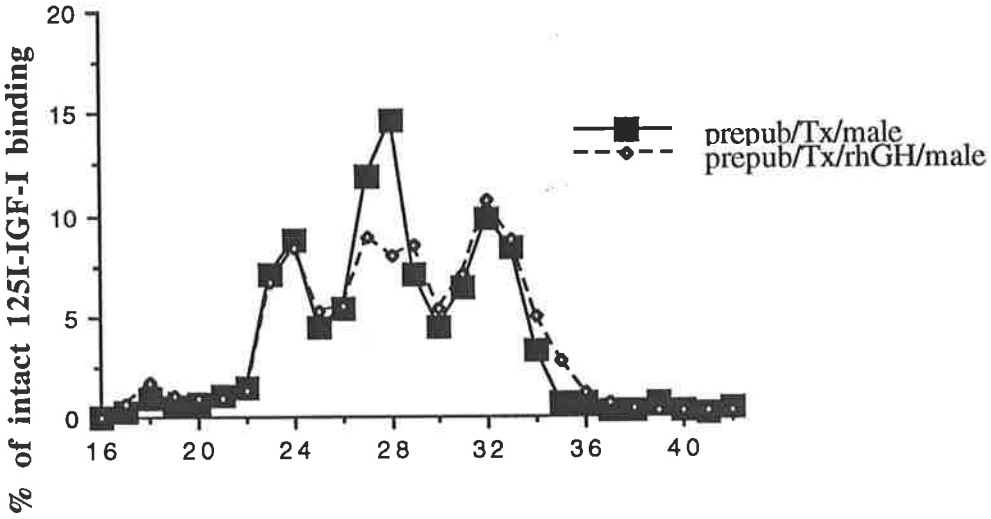


Fig. 4.5 *Comparison of percentage of intact ^{125}I-IGF-I binding from Superose-12 chromatography of normal pooled and transplanted prepubertal male serum, with and without rhGH treatment, incubated at 4° C overnight.* The plots show the percentage of intact radioactivity in 0.5 ml fractions as a percentage of total intact radioactivity in #16-42 of the chromatogram. Samples were chromatographed as described in Chapter 2.7. Molecular weight markers used to calibrate the column and their elution fraction numbers are discussed in Chapter 4.3.1. a) Comparison between chromatograms from normal pooled and transplanted prepubertal male serum; b) Comparison between chromatograms from transplanted and rhGH-treated transplant prepubertal male serum; c) Comparison between chromatograms from normal pooled and rhGH-treated transplant prepubertal male serum;

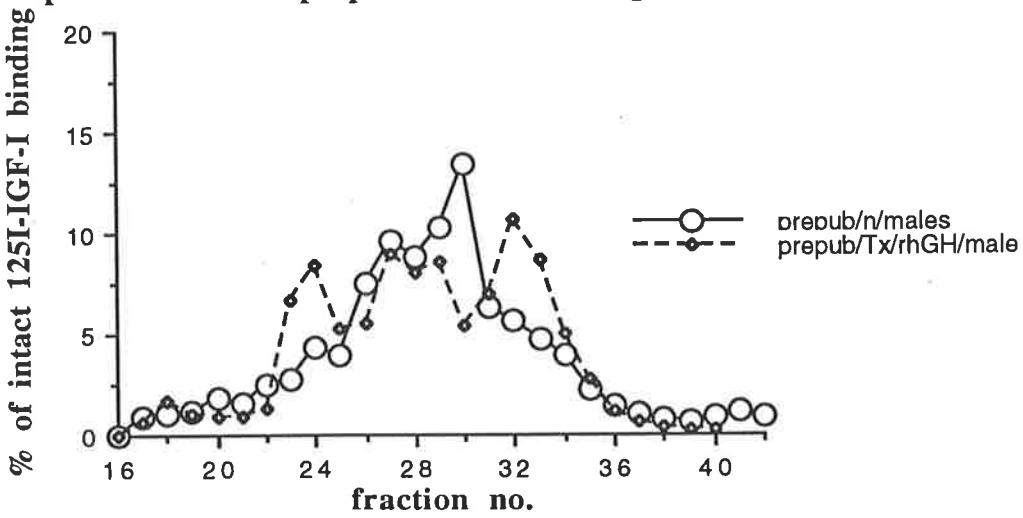
a **Comparison between prepubertal normal and transplant male**



b **Comparison between prepubertal transplant male without/with rhGH**



c **Comparison between prepubertal and transplant/rhGH male**



complex binding (36.3%) in this boy and increased the amount of "free" ^{125}I-IGF-I (36.8% compared with 30.1%) (Fig. 4.5 c). Binding in the region of the Type-II receptor increased with rhGH therapy in the transplant boy (5.3%) compared with before rhGH (3.5%), and more closely approximated that seen in the normal pool (6.7%).

Pubertal normal, CRF and SSGC male pools

In contrast to the prepubertal males, where there was very little difference in binding in the 150 kDa complex between all three groups, the pubertal normal males had substantially more binding in this region (22.8%) than either the CRF (14.0%) or SSGC (16.5%) pools (Fig. 4.6 a, b, and c). The percentage binding in the small IGF-IGFBP complex in the CRF males (52.0%) was considerably higher than the normal (40.2%) and SSGC (35.3%) groups, while the proportion of unbound or "free" tracer in the CRF males (31.3%) was similar to the normal (32.5%) but less than the SSGC (45.0 %) pools. The 669 kDa region in the normal pool (4.5%) revealed more binding than either the CRF (2.6%) or SSGC (3.0%) groups.

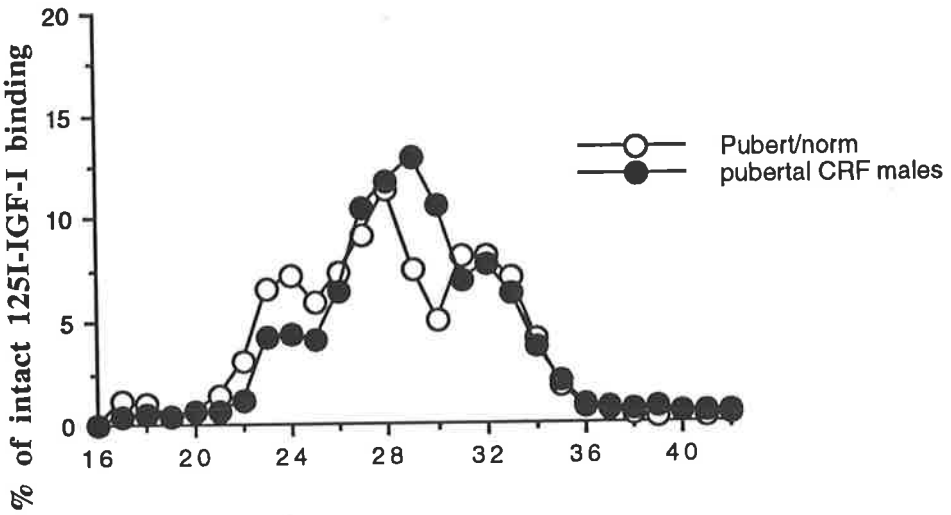
Pubertal CRF males with/without rhGH treatment and normal pools

rhGH treatment in the pubertal CRF boys resulted in a "normalising" of the binding profile compared with the CRF pool without therapy (Fig. 4.7 a and b). There was an increase in binding in the large complex in the rhGH-CRF pool (20.1%) compared to the untreated CRF pool (14.0%) (Fig. 4.7 a), so that it more closely resembled the normal pattern (22.8%) (Fig 4.7 b). In addition, there was a shift in binding from the small complex (45.3%) compared with

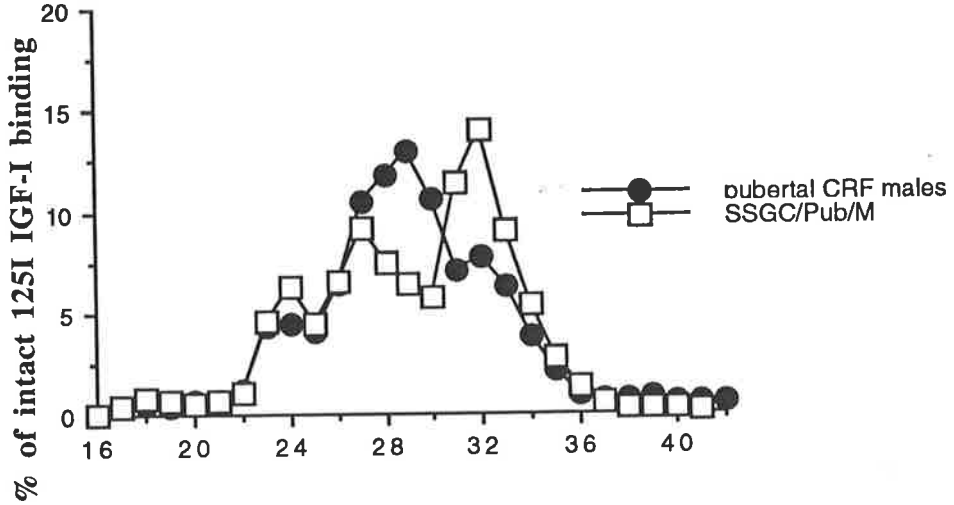
Fig. 4.6 *Comparison of percentage of intact ^{125}I-IGF-I binding from Superose-12 chromatography of normal, CRF and SSGC pubertal pooled male serum incubated at 4° C overnight.* The plots show the percentage of intact radioactivity in 0.5 ml fractions as a percentage of total intact radioactivity in #16-42 of the chromatogram. Samples were chromatographed as described in Chapter 2.7. Molecular weight markers used to calibrate the column and their elution fraction numbers are discussed in Chapter 4.3.1.

a) Comparison between chromatograms from normal and CRF pubertal pooled male serum; b) Comparison between chromatograms from CRF and SSGC pubertal pooled male serum; c) Comparison between chromatograms from normal and SSGC pubertal pooled male serum.

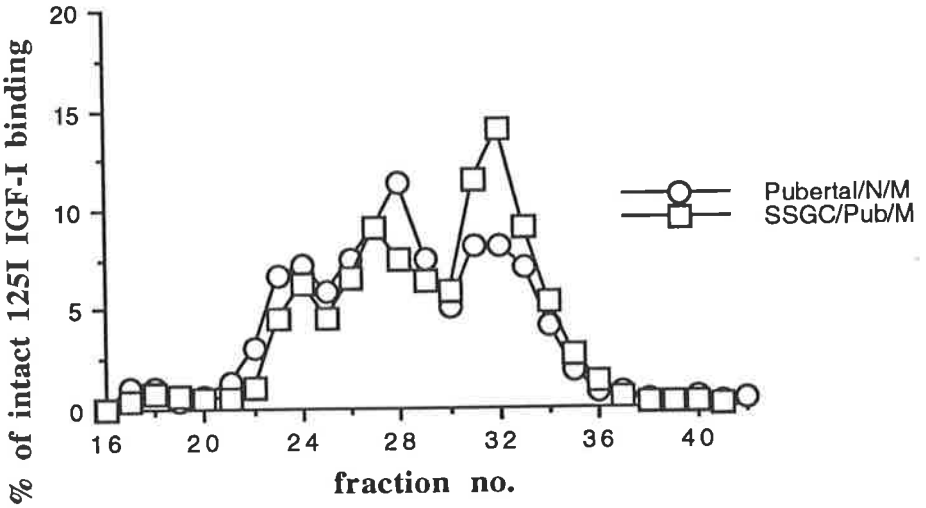
a **Comparison between pubertal normal and CRF males**



b **Comparison between pubertal CRF and SSGC males**



c **Comparison between pubertal normal and SSGC males**



the non-treated group (52%), so that it approximated the normal pool (40.2%), which resulted in a similar amount of unbound ^{125}I-IGF-I (31.6%), to the untreated pool (31.3%) and the normal pool (32.3%). There was a similar amount of binding evident in the 669 kDa region with the rhGH-CRF (3.0%) and the CRF (2.6%), compared with the normal (4.5%) pools.

Pubertal males on dialysis with/without rhGH treatment

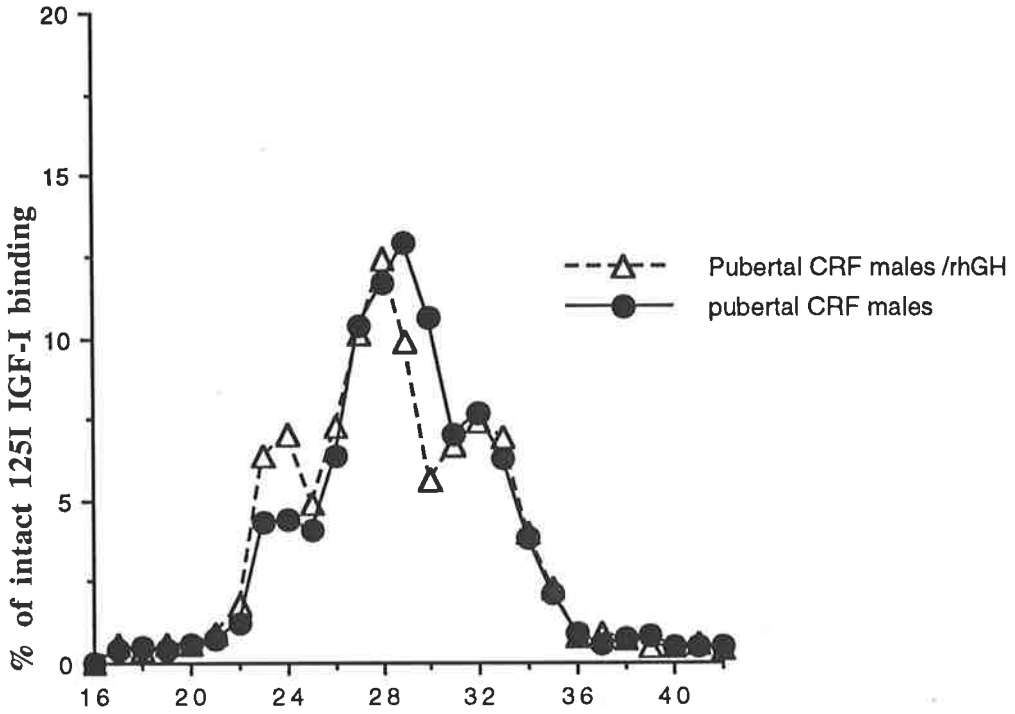
Binding of the tracer in the 150 kDa complex region was lower in the pubertal dialysis pool (17.6%) than in the normal group (22.8%), but a greater percentage of binding was evident in the small complex region (52.8%) compared to the normal pool (40.2%) (Fig. 4.8 a). rhGH treatment of the pubertal boys on dialysis (Fig. 4.8 b) resulted in an increase in binding in the large complex (19.9%), more closely approximating the normal profile (22.8%), and a decrease in the small complex binding (46.6%) compared to the untreated dialysis boys (52.8%). rhGH therapy had little effect on the amount of unbound ^{125}I-IGF-I (28.8%) compared to the untreated dialysis pool (28.3%) (Fig. 4.8 b) but was less than that seen in the normal pool (32.5%) (Fig. 4.8 c). Binding in the 669 kDa region with rhGH (4.8%) was not much different from the normal pool (4.5%), but increased compared to the untreated pool (1.3%).

Pubertal transplanted males with/without rhGH treatment

An increase in binding in the large complex was noted in the pubertal transplanted pool (25.2%) compared to the normal boys (22.8%), together with a reduction in binding in the

Fig. 4.7 *Comparison of percentage of intact ^{125}I-IGF-I binding from Superose-12 chromatography of CRF, rhGH-treated CRF and normal pubertal pooled male serum incubated at 4° C overnight.* The plots show the percentage of intact radioactivity in 0.5 ml fractions as a percentage of total intact radioactivity in #16-42 of the chromatogram. Samples were chromatographed as described in Chapter 2.7. Molecular weight markers used to calibrate the column and their elution fraction numbers are discussed in Chapter 4.3.1. a) Comparison between chromatograms from CRF and rhGH-treated CRF pubertal pooled male serum; b) Comparison between chromatograms from normal and rhGH-treated CRF pubertal pooled male serum.

a **Comparison between pubertal CRF males with/without rhGH**



b **Comparison between pubertal normal and CRF/rhGH males**

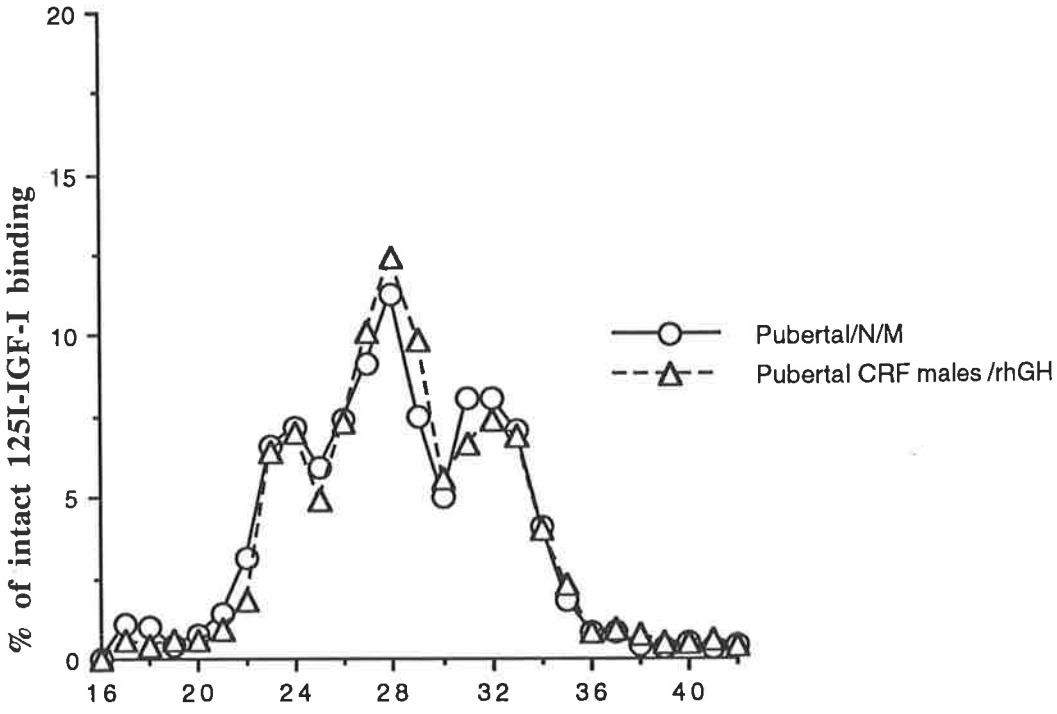
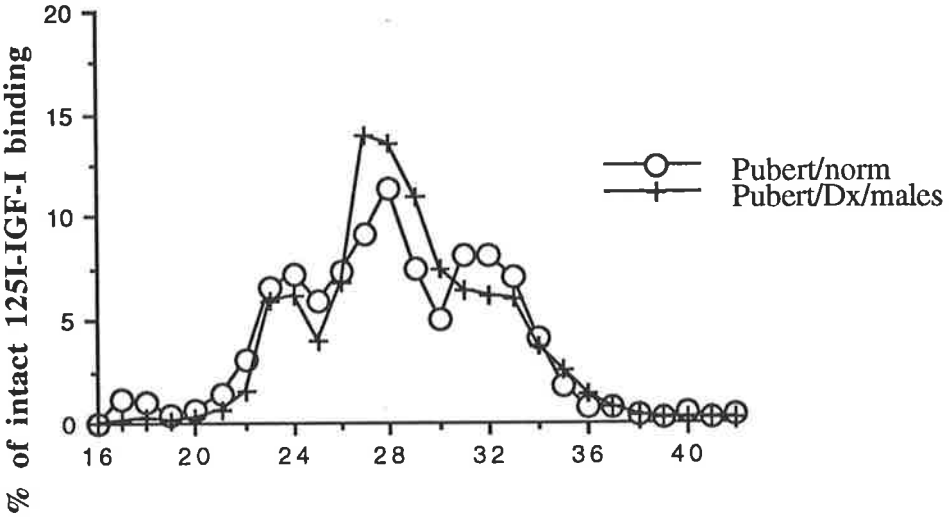
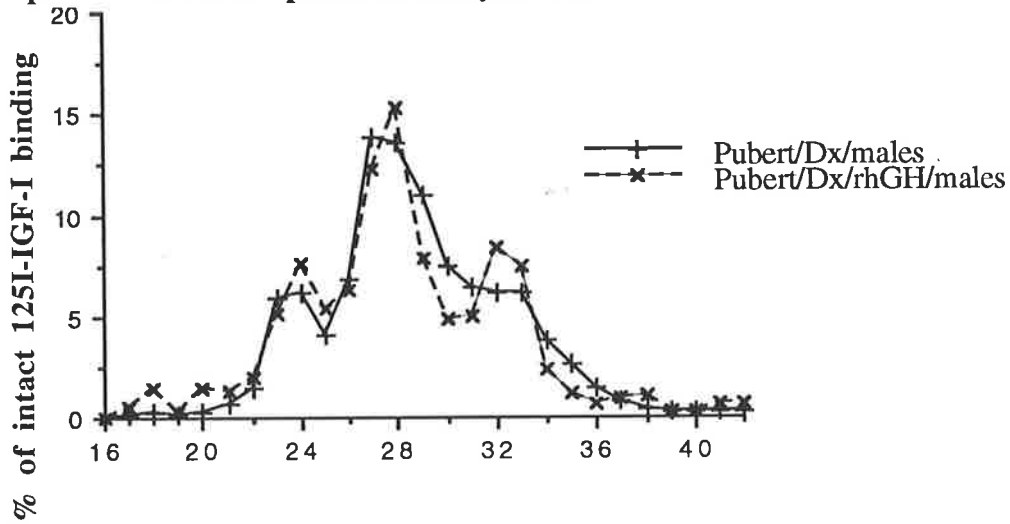


Fig. 4.8 *Comparison of percentage of intact ^{125}I-IGF-I binding from Superose-12 chromatography of normal, dialysis and rhGH-treated dialysis pubertal pooled male serum incubated at 4° C overnight.* The plots show the percentage of intact radioactivity in 0.5 ml fractions as a percentage of total intact radioactivity in #16-42 of the chromatogram. Samples were chromatographed as described in Chapter 2.7. Molecular weight markers used to calibrate the column and their elution fraction numbers are discussed in Chapter 4.3.1. a) Comparison between chromatograms from normal and dialysis pubertal pooled male serum; b) Comparison between chromatograms from dialysis and rhGH-treated dialysis pubertal pooled male serum; c) Comparison between chromatograms from normal and rhGH-treated dialysis pubertal pooled male serum.

comparison between pubertal normal and dialysis males



Comparison between pubertal dialysis males without/with rhGH



Comparison between pubertal normal and dialysis/rhGH males

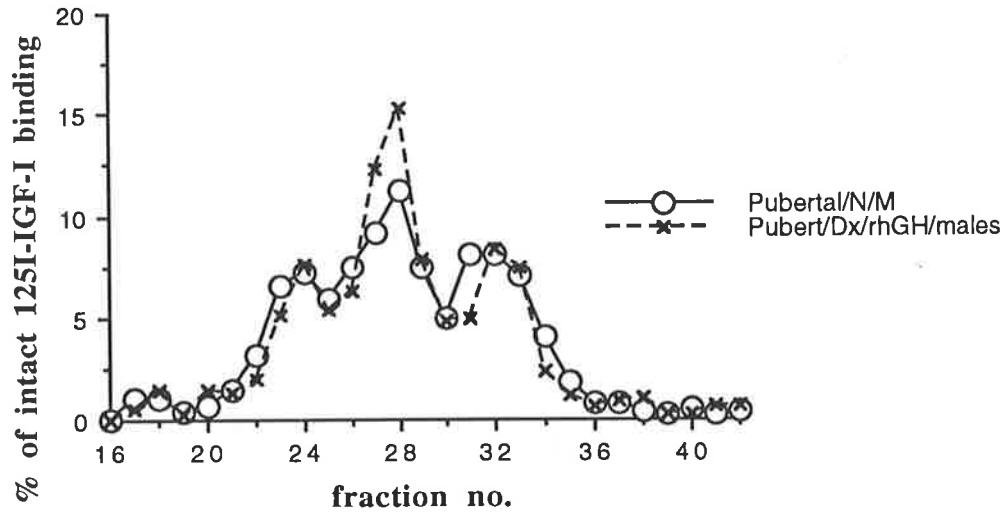
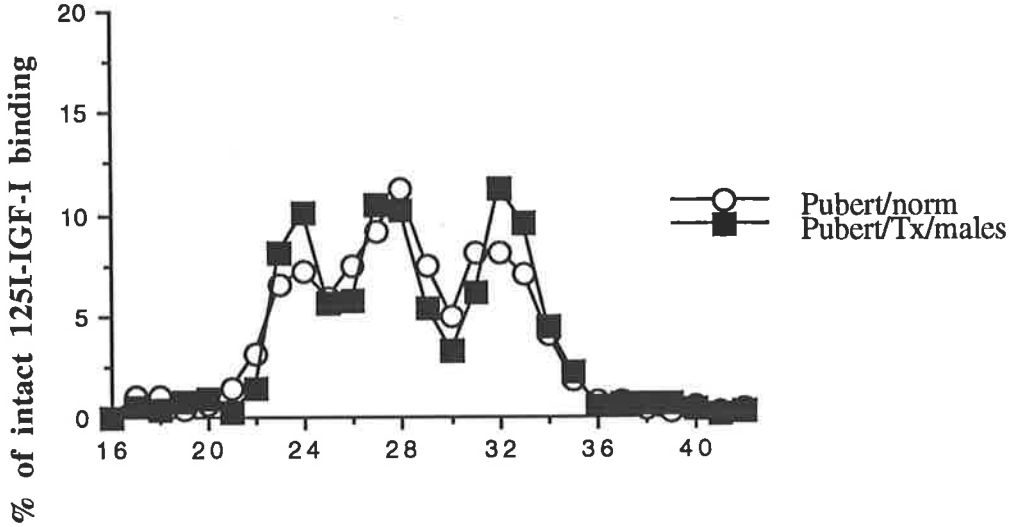
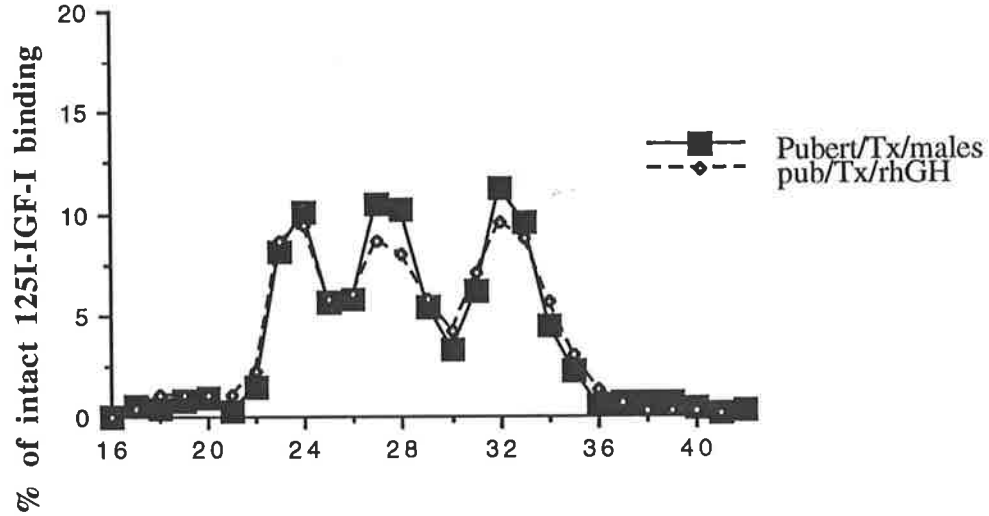


Fig. 4.9 *Comparison of percentage of intact ^{125}I-IGF-I binding from Superose-12 chromatography of normal, transplanted and rhGH-treated transplanted pubertal pooled male serum incubated at 4° C overnight.* The plots show the percentage of intact radioactivity in 0.5 ml fractions as a percentage of total intact radioactivity in #16-42 of the chromatogram. Samples were chromatographed as described in Chapter 2.7. Molecular weight markers used to calibrate the column and their elution fraction numbers are discussed in Chapter 4.3.1. a) Comparison between chromatograms from normal and transplanted pubertal pooled male serum; b) Comparison between chromatograms from transplanted and rhGH-treated transplanted pubertal pooled male serum; c) Comparison between chromatograms from normal and rhGH-treated transplanted pubertal pooled male serum.

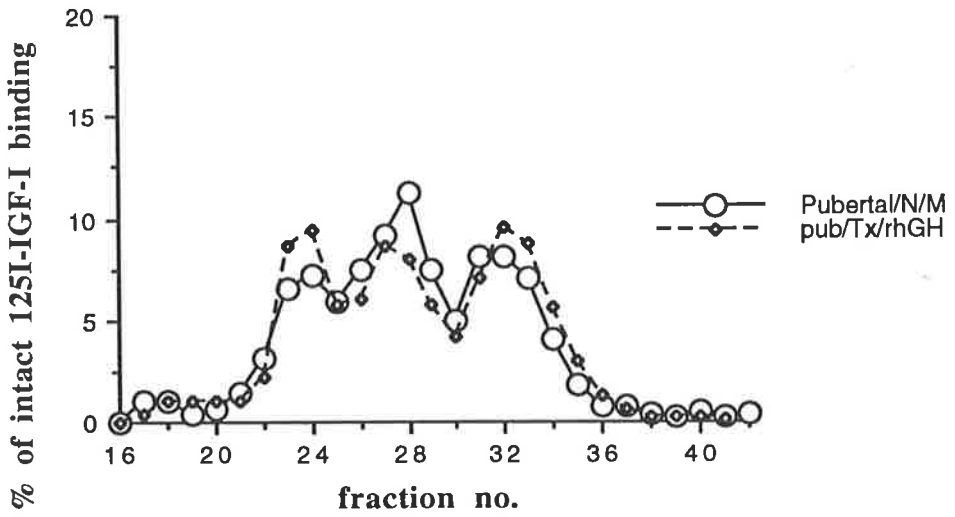
a **Comparison between pubertal normal and transplanted males**



b **Comparison between pubertal transplant males with/without rhGH**



c **Comparison between normal and transplanted/rhGH males**



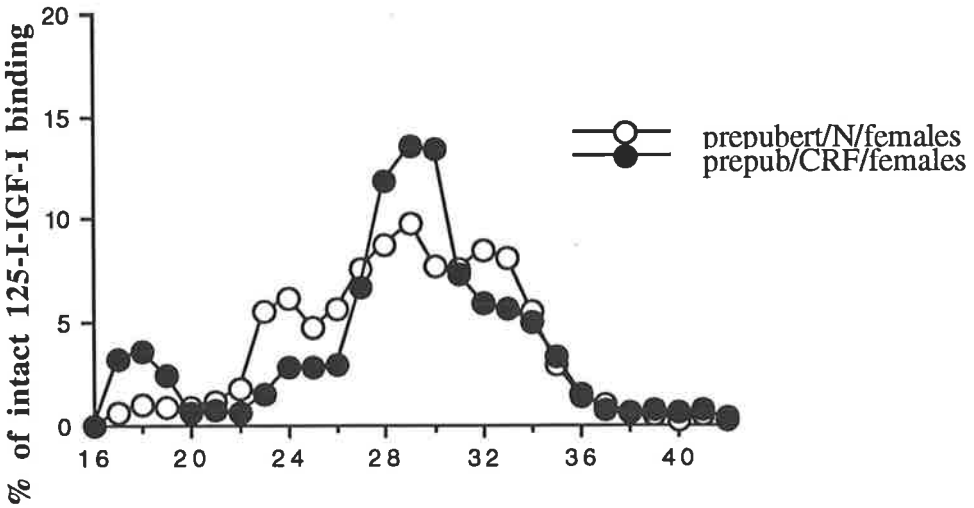
small complex (35.1%) compared with the normal pool (40.2%). The amount of "free" ^{125}I-IGF-I in the transplant (36.8%) was greater than the normal (32.5%) pools (Fig. 4.9 a). rhGH treatment had no effect on the binding in the 150 kDa complex (25.2%) compared with the untreated group (25.2%), but resulted in a slight decrease in binding in the 30-69 kDa (33.9%) and the unbound region (35.1%) of the chromatogram compared to the untreated group (Fig. 4.9 b). An increased amount of binding was noted in the 669 kDa region in the rhGH treated transplanted pool (5.8%) compared to the normal (4.5%) and untreated transplant pool (2.9%).

Prepubertal normal, CRF and SSGC females

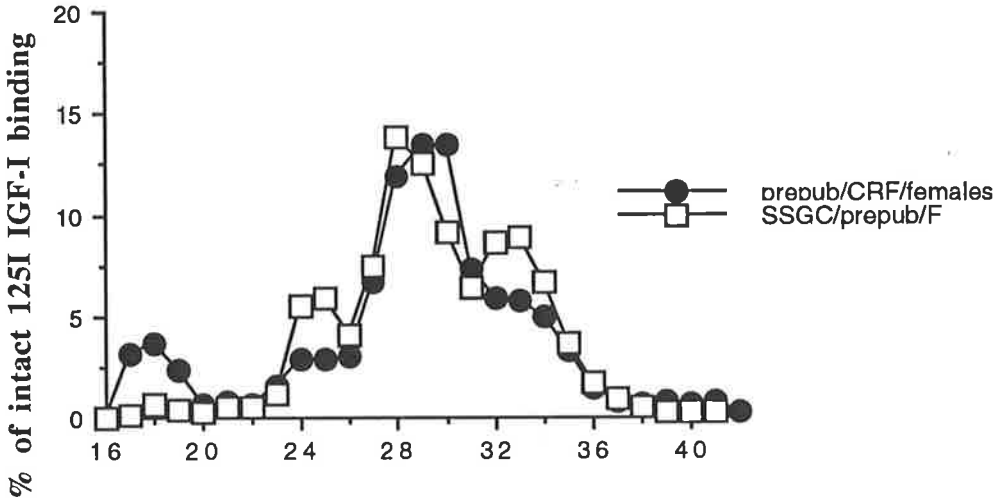
^{125}I-IGF-I binding in the large complex region in the prepubertal CRF female pool (8.1%) was substantially lower than the prepubertal normal girls (18.3%) (Fig. 4.10 a), and the SSGC girl (13.1%) (Fig. 4.10 c). The CRF pool revealed more tracer binding in the 30-69 kDa region (48.5%) compared to the normal group (39.6%), but was similar to the binding noted in the SSGC female (47.1%). While the percentage of unbound tracer in the normal pool (37.5%) and SSGC girl (38.1%) were approximately the same (Fig. 4.10 c), there was less "free" ^{125}I-IGF-I in the CRF group (32.6%) (Fig. 4.10 a and b). A marked increase in binding in the 669 kDa region was noted in the prepubertal CRF female pool (10.7%) compared to the normal (4.6%) and SSGC (1.9%) girls.

Fig. 4.10 *Comparison of percentage of intact ^{125}I-IGF-I binding from Superose-12 chromatography of normal, CRF and SSGC prepubertal pooled female serum incubated at 4° C overnight.* The plots show the percentage of intact radioactivity in 0.5 ml fractions as a percentage of total intact radioactivity in #16-42 of the chromatogram. Samples were chromatographed as described in Chapter 2.7. Molecular weight markers used to calibrate the column and their elution fraction numbers are discussed in Chapter 4.3.1. a) Comparison between chromatograms from normal and CRF prepubertal pooled female serum; b) Comparison between chromatograms from CRF and SSGC prepubertal pooled female serum; c) Comparison between chromatograms from normal and SSGC prepubertal pooled female serum.

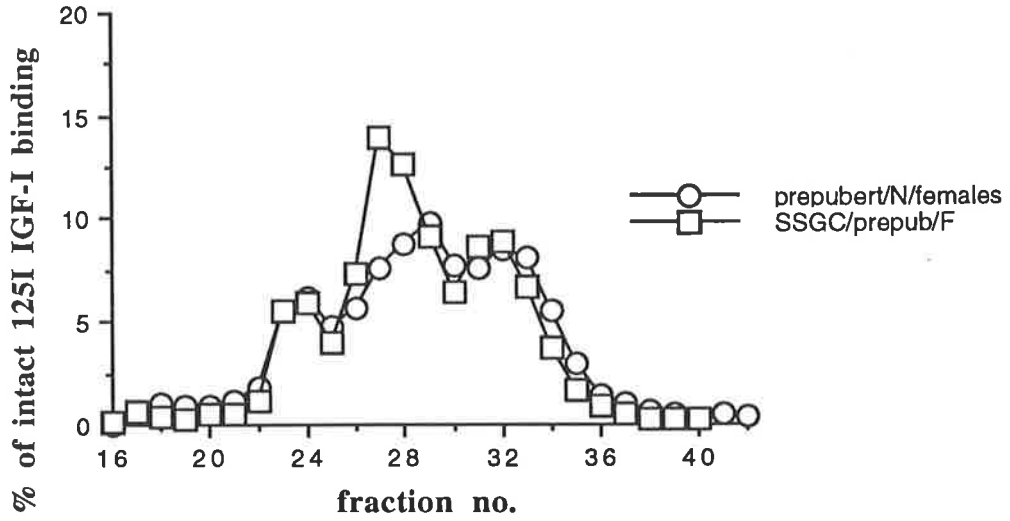
a **Comparison between prepubertal normal and CRF females**



b **Comparison between prepubertal CRF and SSGC females**



c **Comparison between prepubertal normal and SSGC females**



Prepubertal transplanted female

There was a substantially increased amount of binding seen in the 150 kDa region in the transplanted girl (24.8%) compared to the normal prepubertal pool (18.3%), but the percentage binding in the small IGF-IGFBP complex (39.5%) was approximately the same as the normal group (39.6%). The amount of unbound ^{125}I-IGF-I was similar in the transplanted girl (32.5%) and in the normal pool (29.9%) (Fig. 4.11 a) while binding in the Type II IGF-I receptor region was similar in both groups (3.2%-transplant; 4.6%-normal).

Pubertal transplanted females

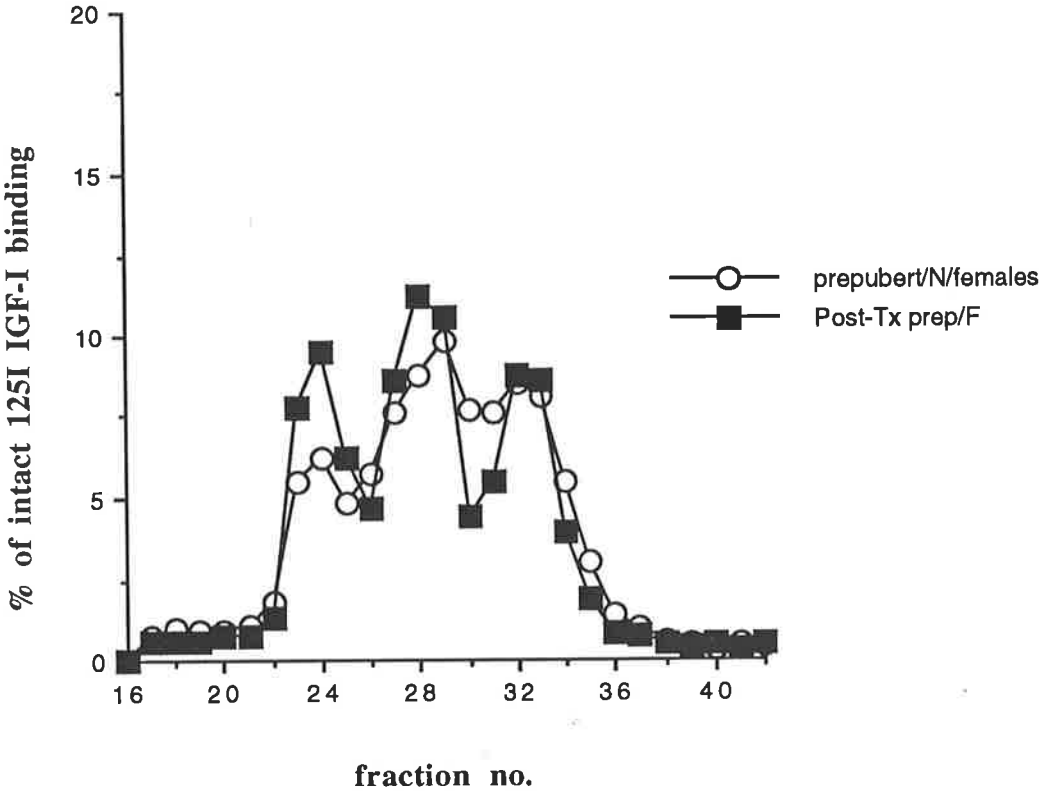
The chromatogram of the pubertal transplant female pool demonstrated increased binding in the 150 kDa binding peak (19.3%) compared with the normal pubertal girls (16.3%) (Fig. 4.11b). There was also considerably more binding in the small IGF-binding region of the transplanted pool (43.8%) when compared with the normal group (23.0%). However, there was much less "free" ^{125}I-IGF-I in the transplant group (34.2 %) compared with the normal girls (54.4 %). The normal pubertal female pool revealed more binding in the 669 kDa region (6.3%) than the transplant pool (2.7%).

Pubertal normal, CRF and SSGC females

Binding in the large complex region was more closely approximated in the pubertal CRF (14.4%) and normal pools (16.3%) (Fig. 4.12 a). There was, however, substantially more

Fig. 4.11 *Comparison of percentage of intact ^{125}I-IGF-I binding from Superose-12 chromatography of normal pooled and transplanted, prepubertal and pubertal female serum incubated at 4° C overnight.* The plots show the percentage of intact radioactivity in 0.5 ml fractions as a percentage of total intact radioactivity in #16-42 of the chromatogram. Samples were chromatographed as described in Chapter 2.7. Molecular weight markers used to calibrate the column and their elution fraction numbers are discussed in Chapter 4.3.1. a) Comparison between chromatograms from normal pooled and transplanted prepubertal female serum; b) Comparison between chromatograms from normal and transplanted pubertal pooled female serum.

a **Comparison between prepubertal normal and transplanted females**



b **Comparison between pubertal normal and transplanted females**

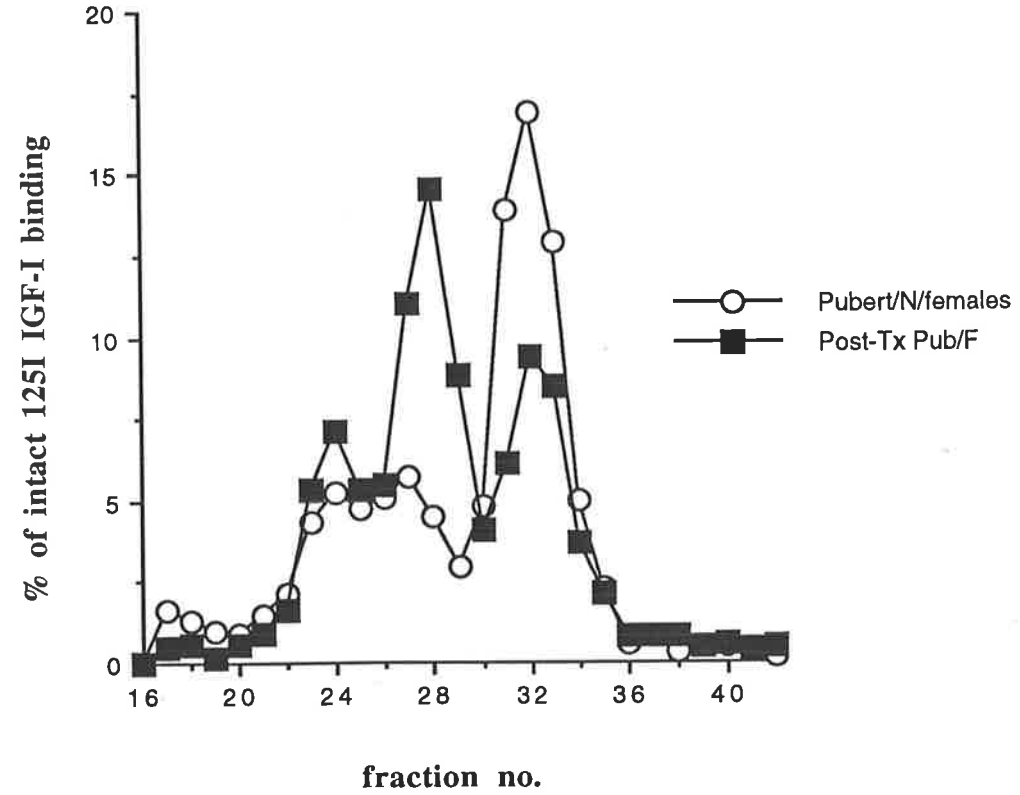
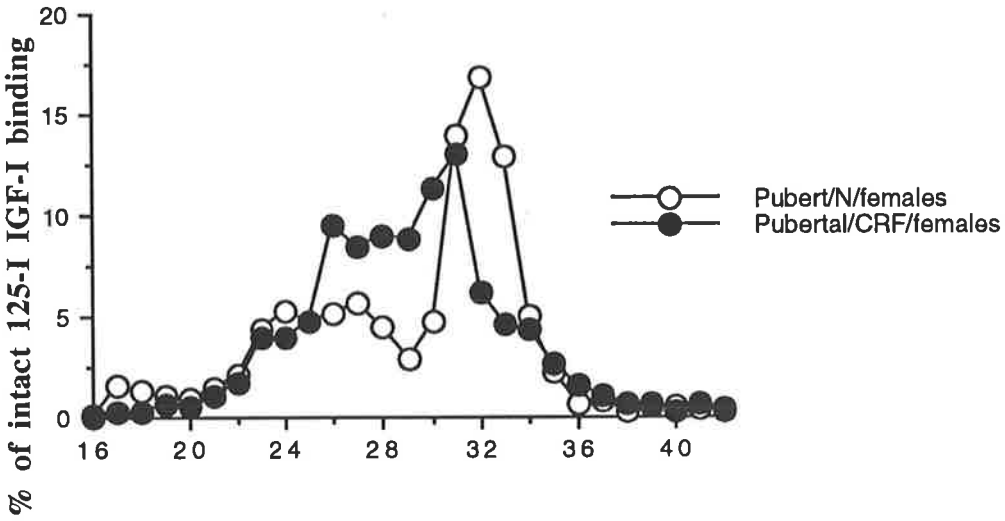


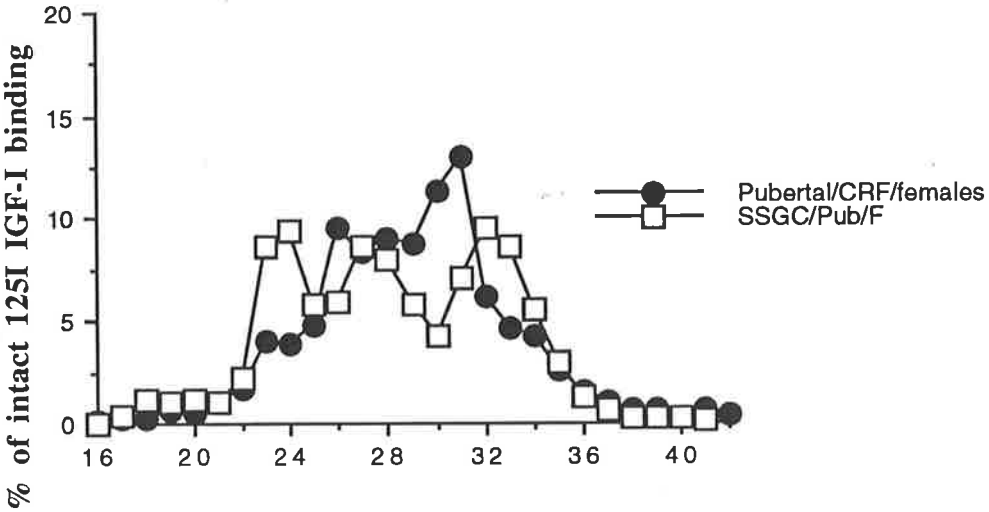
Fig. 4.12 *Comparison of percentage of intact ^{125}I-IGF-I binding from Superose-12 chromatography of normal, CRF pooled and SSGC pubertal female serum incubated at 4° C overnight.* The plots show the percentage of intact radioactivity in 0.5 ml fractions as a percentage of total intact radioactivity in #16-42 of the chromatogram. Samples were chromatographed as described in Chapter 2.7. Molecular weight markers used to calibrate the column and their elution fraction numbers are discussed in Chapter 4.3.1.

a) Comparison between chromatograms from normal and CRF pooled pubertal female serum; b) Comparison between chromatograms from CRF and SSGC pooled pubertal female serum; c) Comparison between chromatograms from normal and SSGC pooled pubertal female serum.

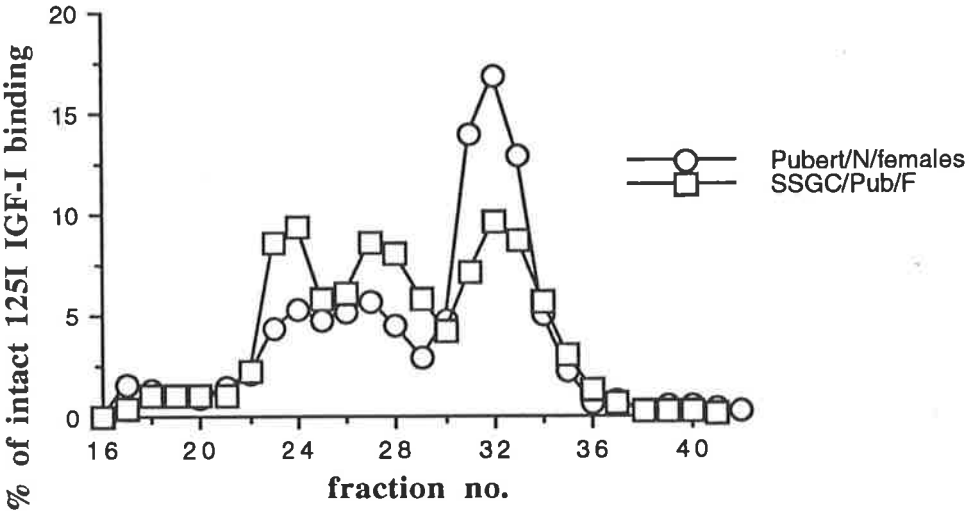
a **Comparison between pubertal normal and CRF females**



b **Comparison between pubertal CRF and SSGC females**



c **Comparison between pubertal normal and SSGC females**



binding of ^{125}I-IGF-I in the large complex in the pubertal SSGC girl (26.0%) (Fig. 4.12 b and c). The CRF pool (47.0%) revealed a greater percentage of binding in the small complex than in the normal pool (23.0%) and the SSGC girl (32.6%) (Fig. 4.12 a and b). The amount of unbound ^{125}I-IGF-I in the CRF pool (35.9%) was less than the normal pool (54.4%) and the SSGC female (36.8%) (Fig. 4.12 a and b). There was less binding in the 669 kDa region in the CRF pool (2.7%) than either the normal pool (6.3%) or the SSGC girl (4.6%).

4.3.2 Identification of the IGFBPs in the large and small IGF-binding complexes

IGFBP-3 WIB

Western immunoblotting of alternate individual FPLC fractions with the anti-hIGFBP-3 antibody revealed bands at 44-46, 46-48 and 30 kDa (Fig. 4.13). The IGFBP-3 doublet (44-46 and 46-48 kDa) was found in #23, #25 and # 27, but it did not appear in #29 which corresponded to the 30 kDa molecular weight marker on the chromatogram (Chapter 4.3.1). The 30 kDa IGFBP-3 form was, however, present in # 23- # 29. These results reveal that all three IGFBP-3 bands are present in both the large (150 kDa) and small (30-69 kDa) IGF-IGFBP complexes. Pooled FPLC fractions, from the 150 and 30-69 kDa binding peaks (AUC) of the chromatogram, also revealed by WIB that the IGFBP-3 doublet and the 30 kDa IGFBP-3 form were present in both binding complexes in prepubertal normal, SSGC and CRF, as well as rhGH-treated CRF boys (Fig. 4.14 a). This was also the case in the pubertal normal, SSGC, CRF and rhGH-CRF males (Fig. 4.14 b) and prepubertal females (Fig. 4.14 c).

Fig. 4.13 *Western immunoblot of serum and alternate individual FPLC #21-33 of CRF prepubertal pooled male serum with a specific anti-h IGFBP-3 antibody* (see Chapter 4.2.4). Lane 2, serum; Lane 3, #21; Lane 4, #23; Lane 5, #25; Lane 6, #27; Lane 7, #29; Lane 8, #31; Lane 9, #33. Molecular weight markers; 150 kDa ran in #23; 69 kDa ran in #25; 30 kDa ran in #29 and 7.5 kDa ran in #33. ^{14}C molecular weight markers are shown in the left column.

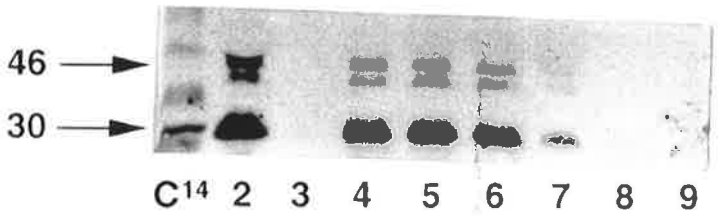

46
30
C14 2 3 4 5 6 7 8 9

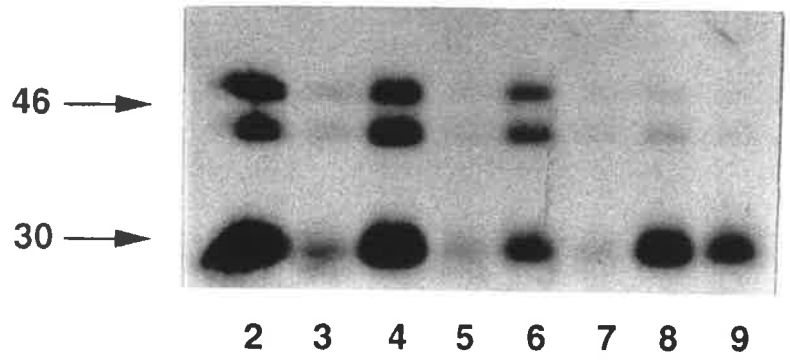
Fig. 4.14

a) *Western immunoblot of pooled FPLC # contained in the 150 and 30-69 kDa binding peak of normal, SSGC, CRF and rhGH-treated CRF pooled prepubertal male serum with a specific anti-h IGFBP-3 antibody* (see Chapter 4.2.4). Lane 2, normal (150kDa); Lane 3, normal (30-69 kDa); Lane 4, SSGC (150 kDa); Lane 5, SSGC (30-69 kDa); Lane 6, CRF (150 kDa); Lane 7, CRF (30-69 kDa); Lane 8, rhGH-treated CRF (150 kDa); Lane 9, rhGH-treated CRF (30-69 kDa).

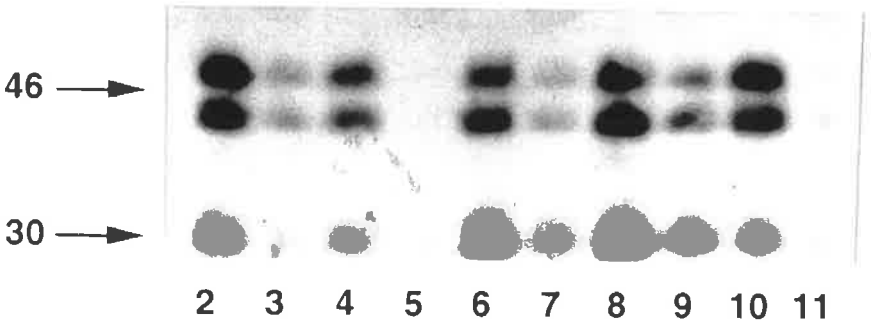
b) *Western immunoblot of pooled FPLC # contained in the 150 and 30-69 kDa binding peak of normal, SSGC, SSGC rhGH-treated, CRF and rhGH-treated CRF pooled pubertal male serum with a specific anti-h IGFBP-3 antibody* (see Chapter 4.2.4). Lane 2, normal (150kDa); Lane 3, normal (30-69 kDa); Lane 4, SSGC (150 kDa); Lane 5, SSGC (30-69 kDa); Lane 6, CRF (150 kDa); Lane 7, CRF (30-69 kDa); Lane 8, rhGH-treated CRF (150 kDa); Lane 9, rhGH-treated CRF (30-69 kDa); Lane 10, rhGh-treated SSGC (150 kDa); Lane 11, rhGH-treated SSGC (30-69 kDa).

c) *Western immunoblot of pooled FPLC # contained in the 150 and 30-69 kDa binding peak of normal, SSGC, CRF and transplanted pooled prepubertal and pubertal female serum with a specific anti-h IGFBP-3 antibody* (see Chapter 4.2.4). Lane 2, prepubertal CRF (150kDa); Lane 3, prepubertal CRF (30-69 kDa); Lane 4, prepubertal SSGC (150 kDa); Lane 5, prepubertal SSGC (30-69 kDa); Lane 6, prepubertal transplant (150 kDa); Lane 7, prepubertal transplant (30-69 kDa); Lane 8, pubertal normal (150 kDa); Lane 9, pubertal normal (30-69 kDa); Lane 10, pubertal CRF (150 kDa); Lane 11, pubertal CRF (30-69 kDa).

a



b



c

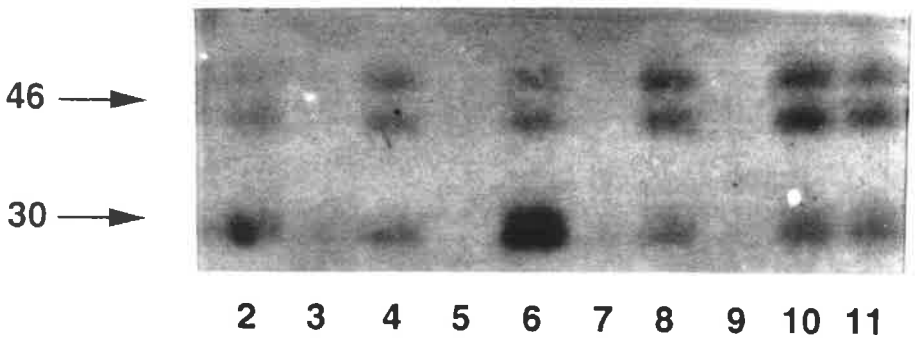


Fig 4.15

a) *Western immunoblot of serum and alternate individual FPLC #21-33 of CRF prepubertal pooled male serum with a specific anti-h IGFBP-1 antibody* (see Chapter 4.2.4). Lane 2, serum; Lane 3, #21; Lane 4, #23; Lane 5, #25; Lane 6, #27; Lane 7, #29; Lane 8, #31; Lane 9, #33. Molecular weight markers; 150 kDa ran in #23; 69 kDa ran in #25; 30 kDa ran in #29 and 7.5 kDa ran in #33. ^{14}C molecular weight markers are shown in the left column.

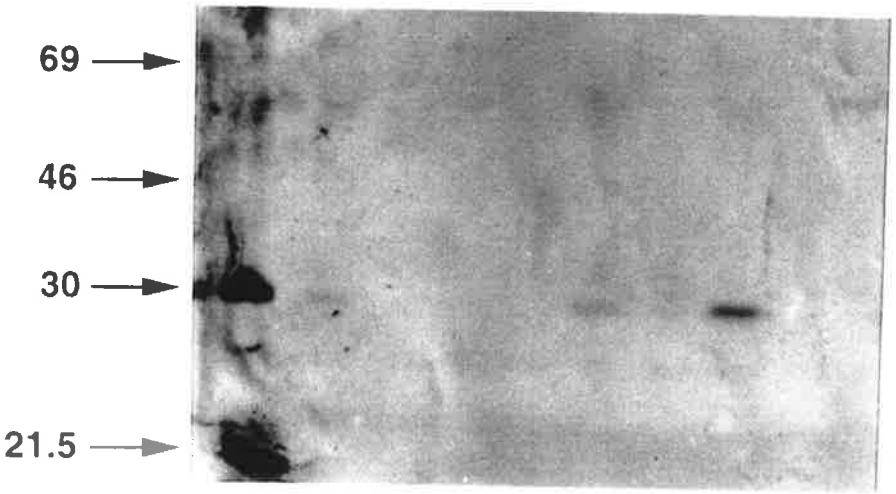
b) *Western immunoblot of pooled FPLC # contained in the 150 and 30-69 kDa binding peak of normal, SSGC, CRF and rhGH-treated CRF pooled prepubertal male serum with a specific anti-h IGFBP-1 antibody* (see Chapter 4.2.4). Lane 2, normal (150kDa); Lane 3, normal (30-69 kDa); Lane 4, SSGC (150 kDa); Lane 5, SSGC (30-69 kDa); Lane 6, CRF (150 kDa); Lane 7, CRF (30-69 kDa); Lane 8, rhGH-treated CRF (150 kDa); Lane 9, rhGH-treated CRF (30-69 kDa); Lane 10, rhGH-treated SSGC (150 kDa); Lane 11, rhGH-treated SSGC (30-69 kDa).

c) *Western immunoblot of serum and alternate individual FPLC #21-33 of CRF prepubertal pooled male serum with a specific anti-h IGFBP-2 antibody* (see Chapter 4.2.4). Lane 2, serum; Lane 3, #21; Lane 4, #23; Lane 5, #25; Lane 6, #27; Lane 7, #29; Lane 8, #31; Lane 9, #33. Molecular weight markers; 150 kDa ran in #23; 69 kDa ran in #25; 30 kDa ran in #29 and 7.5 kDa ran in #33. ^{14}C molecular weight markers are shown in the left column.

a


46
30
2 3 4 5 6 7 8 9 C14

b


69
46
30
21.5
C14 2 3 4 5 6 7 8 9 10 11

c


46
30
C14 2 3 4 5 6 7 8 9

IGFBP-1 WIB

Protein bands identified by the anti-hIGFBP-1 antibody in the individual fractions were seen at 29-30 kDa on the WIB (Fig. 4.15 a). These occurred in # 27 and #29 from the chromatogram, indicating that IGFBP-1 does not participate in the formation of the large 150 kDa IGF-IGFBP complex but is present in the small binding complex. The 29 kDa IGFBP-1 band was identified by WIB only in the small binding region of the chromatogram in the pooled FPLC fractions (Fig. 4.15 b)

The anti-hIGFBP-2 antiserum identified protein bands in individual FPLC fractions at 34 kDa on the WIB which were present in #27 and #29, suggesting that IGFBP-2 is not found in the large IGF-IGFBP binding complex but contributes to the binding in the small molecular weight complex (Fig. 4.16).

4.4 Discussion

The FPLC data from this study demonstrate binding of ^{125}I-IGF-I in serum following *in vitro* incubation. Comparison between the incubation at 37°C for 1 minute with the incubation at 4° C overnight showed that binding of ^{125}I-IGF-I to the unoccupied sites in the small binding peak occurs more rapidly than to the 150 kDa complex and that this binding is greater in CRF patients than in normal children. rhGH treatment in CRF boys resulted in a "normalising" of the binding in the small complex and the amount of unbound ^{125}I-IGF-I. Increased binding in the large complex, associated with transplantation, was unaffected by rhGH, although a

decrease in the amount of binding in the small binding region was evident. We have also shown that the 30 kDa IGFBP-3 form participates in the formation of the ternary complex, while also occurring in the small binding region together with IGFBPs-1 and -2.

It is apparent from animal studies that there is a rapid flux of IGFs through the circulation in amounts far greater than that assayable as "free" peptide (Lewitt, Saunders and Baxter, 1993) and this movement between IGFBP-bound and unbound forms of growth factor, and between the circulation and the tissues, needs to be considered when comparing the differences between the binding profiles in the present study. Indeed, this was apparent when comparing the FPLC chromatograms of a 1 minute with the overnight incubation of the same serum, where there was a shift in radioactivity, with time, from the 7.5 kDa peak to the smaller 30-69 kDa and the 150 kDa ternary complex. The longer incubation allows for more complete equilibration of free and bound IGFs. It is also important to remember that ^{125}I-IGF-I incubated with serum *in vitro* s only able to bind to the unsaturated binding sites, *i.e.* those IGFBPs not already associated with endogenous IGF-I in the serum. In addition, the methods involved in separating out the various binding complexes may have an effect on the binding which does not maintain the *in vivo* conditions (Frystyk, Skjaerbaek, Dinesen, *et al*, 1994). However, as all the patient samples in our study were treated identically, we believe that comparison between the groups is valid.

The most significant and consistent difference between the chromatograms of the normal and CRF pooled serum was the increase in the amount of binding seen in the 30-69 kDa binding region. This was the case in all CRF sera, *i.e.* prepubertal and pubertal male and female patients. This finding was described in 1982 by Goldberg *et al*, who also showed an increase in small complex binding of ^{125}I-IGF-I by neutral gel chromatography (Goldberg, Trivedi,

Delmez, *et al*, 1982). Increased high affinity IGF-binding sites in adult CRF serum have been found to interfere with IGF RIAs, and Powell *et al* suggested that the IGFBPs be evaluated for their role in the growth failure in children with CRF (Powell, Rosenfeld, Baker, *et al*, 1986). This increased unsaturated IGF-binding, associated with poor growth has been confirmed by several other studies (Blum, Ranke, Kietzmann, *et al*, 1991; Lee, Hintz, Sperry, *et al*, 1989; Powell, Rosenfeld, Sperry, *et al*, 1987), including a report by the European Study Group for Nutritional Treatment of Chronic Renal Failure in Childhood (Tonshoff, Blum, Wingen, *et al*, 1995). A recent report has outlined certain criteria for establishing the role for IGFBPs-1, -2 and -3 as growth inhibitors in CRF, and it appears that IGFBP-1 meets most of these criteria while IGFBP-2 meets some of them (Powell, Liu, Baker, *et al*, 1996).

rhGH treatment in the prepubertal and pubertal CRF males in this study resulted in a more "normal" profile due mainly to a reduction in the binding in the small complex. This apparently occurred by slightly different mechanisms in the pubertal, compared with prepubertal groups. The shift in ^{125}I-IGF-I binding in the pubertal boys occurred from the 30-69 kDa to the 150 kDa region, with little alteration in the "free" peak, while in the prepubertal group, there was a shift from the small complex to the unbound region of the chromatogram with little effect on the large complex.

The percentage of ^{125}I-IGF-I binding in the large complex in the pubertal CRF boys before rhGH was similar to the prepubertal CRF pool. This may reflect a delayed rise in endogenous growth hormone levels in these children at puberty (Scharer, Schaefer, Trott, *et al*, 1989), which, in addition to the partial GH resistance seen in uremia (Mehls, Scheafer and Tonshoff, 1991) results in the persistence of prepubertal levels of IGFBP-3. This binding protein is necessary for the formation of the 150 kDa ternary complex together with IGF-I or -II and

ALS. IGFBP-3 is known to be regulated by GH, and IGFBP-3 levels may in fact reflect total GH secretion (Blum and Ranke, 1990). It is possible that the delayed puberty of CRF results in a reduction in the formation of the GH-dependant ternary complex, which is normalised by rhGH therapy. rhGH treatment in the pubertal dialysis patients in our study also resulted in a similar shift from the 30-69 kDa binding region to the large complex with no effect on the amount of unbound ^{125}I-IGF-I.

Baxter and co-workers, after fractionating normal serum by Superose-12 chromatography, using a similar method to our study, showed that 10% of the IGFBP-3 forms in the small complex had been modified, so as to inhibit their ability to bind ^{125} I-ALS, suggesting that this was due to a form of limited proteolysis. In CRF serum there was an increase to 50% of immunoreactive IGFBP-3 occurring in the small complex, with an additional proportion of even smaller molecular weight size, presumably representing fragments of IGFBP-3. ALS binding studies showed that IGFBP-3 in the ± 35kDa complex in CRF binds ALS very poorly. This increase in noncomplexing forms of IGFBP-3 in CRF serum is presumably due to reduced renal clearance (Baxter, Dai, Holman, *et al*, 1994).

Elevated serum concentrations of IGFBP-1, -2 and -4 (Chapter 3.3.7) and IGFBP-3 fragments (Chapter 2.5.1) are presumably responsible for the increased binding seen in the 30-69 kDa region of the chromatograms in our children with CRF. We have shown that these IGFBPs are present in the FPLC fractions corresponding to the small complex (Fig. 4.13; 4.15 a and b; and 4.16). The 30 kDa IGFBP-3 form, which appears in both large and small IGF-IGFBP complexes, may contribute to the overall increase in IGFBPs in the small binding peak. In addition, smaller 19 and 14 kDa IGFBP-3 fragments have been identified by cross-linking and immunoprecipitation in the 35 kDa peak (Powell, Liu, Baker, *et al*, 1993). These forms of

IGFBP-3 may be part of the normal physiological metabolism of the native IGFBP-3, possibly by deglycosylation, but which accumulate in CRF. There have been no reports of increased proteolytic activity in CRF, as compared with pregnancy serum (Hossenlopp, Segovia, Lassarre, *et al*, 1990). An abundant 29 kDa form of IGFBP-3 in CRF serum has also been reported by Kale and co-workers which decreases further to 19 kDa with endoglycosidase F treatment (Kale, Liu, Hintz, *et al*, 1996). We have shown that the 29-30 kDa IGFBP-3 form is immunoreactive, being identified by WIB (Chapter 2.5.1), but has a reduced affinity for radiolabelled IGFs in that it does not appear on WLB (Chapter 2.4.2). This may be due to partial proteolysis of the IGFBP-3, similar to that which occurs in pregnancy, which results in a reduced ability to bind iodiated IGF-I (Suikkari and Baxter, 1991). It is also possible, however, that the decreased binding activity of the 30kDa form of IGFBP-3 is due to the harsh conditions of the electrophoresis procedure, which does not reflect the *in vivo* situation (Baxter, Suikkari and Martin, 1993). FPLC conditions may not be as severe as WLB and therefore may not result in a similar reduction in ^{125}I-IGF-I affinity, with binding to the lower molecular weight IGFBP-3 thus contributing to the binding capacity we have observed in this region. There is some evidence *in vitro*, that a reduced affinity of lower molecular weight forms of IGFBP-3 for IGFs may occur following preincubation of IGFBP-3 with fibroblasts, possibly allowing for more ready access of IGFs to the IGF receptors on the target tissues (Conover, Bale, Clarkson, *et al*, 1992). This suggests a facilitatory, rather than an inhibitory role for these small IGFBP-3 forms, however there are no data to support a similar role for the soluble IGFBP-3 fragments such as are found in CRF (Powell, Liu, Baker, *et al*, 1996).

The significant increase in binding in the large complex seen on the chromatograms in the prepubertal and pubertal transplanted males and females was initially surprising. Previous reports have shown decreased GH secretion in renal transplant patients, associated with

prednisolone therapy (Hokken-Koelega, Stijnen, Keizerschrama, *et al*, 1993; Ingulli, Singh, Moazami, *et al*, 1993; Rees and Chantler, 1990) and therefore a reduction, rather than an increase in the GH-dependent ternary complex of IGF-IGFBP-3-ALS would be expected. In addition, it has been reported that IGFBP-3 gene transcription may be reduced by glucocorticoid administration (Villafuerte, Koop, Pao, *et al*, 1995), resulting in a reduction in circulating levels of IGFBP-3, which would be expected to further depress ternary complex formation. However, short-term dexamethasone administration in normal male volunteers has resulted in a marked increase in IGF-I concentration and significant increase in IGFBP-3 levels, while reducing IGF bioactivity (Miell, Taylor, Jones, *et al*, 1993). A study of prednisolone-treated transplanted children with growth retardation by Hokken-Koelega *et al*, found normal serum IGFs, but elevated levels of IGFBP-3 (Hokken-Koelega, Stijnen, Keizerschrama, *et al*, 1993), and a further study of children treated with alternate-day or daily prednisolone also showed increased IGFBP-3 levels and decreased IGF bioavailability (Hokken-Koelega, de Muinck Keizer-Schrama and Drop, 1994). A negative correlation between serum IGFBP-3 levels and GFR was found in the latter study and the increased IGFBP-3 concentration was considered to be due to a reduction in renal graft function. There does not appear to be a clear explanation at this stage for the increased ^{125}I-IGF-I binding in large complex in our transplanted children and further investigation of the effects of immunosuppresive therapy on the GH-IGF-I-IGFBP-3 axis is necessary.

Binding in the small complex region in serum of the transplanted patients was not elevated compared with normal, suggesting normal concentrations of the lower molecular weight IGFBPs in these children, possibly due to "normal" clearance by the renal graft. The pubertal female pool was the exception, with significantly increased binding in the 30-69 kDa region, and although their GFRs were not measured, a reduction in renal function must be considered.

Prednisolone is thought to inhibit the efficacy of rhGH treatment for increasing height velocity in renal transplant patients, possibly as a result of increased IGFBP-3 levels, which have been described in transplanted children on low-dose daily steroid therapy (Ingulli, Singh, Moazami, *et al*, 1993) and which may inhibit IGF-I action. rhGH therapy did not alter the binding in the large complex in the prepubertal and pubertal males with transplants, although a decrease in binding in the 30-69 kDa region was seen in both groups.

The SSGC were part of a cohort of children with idiopathic short stature who participated in a longitudinal trial (Kabi International Growth Study) to assess the response to rhGH. The aetiology of the idiopathic short stature was heterogeneous and children with familial short stature and maturational delay were included (Cowell, 1996). The increase in ^{125}I-IGF-I binding in the small IGF-IGFBP complex in this group of children suggests an increase in the smaller molecular weight IGFBPs. A recent study of constitutionally short children and adolescents also showed increased small IGFBPs in association with decreased serum IGF-I levels compared to normals, which possibly results in decreased IGF-I availability, and may contribute to the short stature (Lindgren, Segovia, Lassarre, *et al*, 1996).

4.5 Conclusions

We have shown that an increase in small molecular weight IGFBPs, including an altered form of IGFBP-3, in prepubertal, and pubertal male and female children, with CRF, on dialysis and after transplantation results in an elevated IGF-binding capacity. This could result in a reduced IGF bioavailability in the tissues and be associated with growth retardation. rhGH is able to

alter this state by inducing a significant increase in endogenous serum IGF-I (Chapter 3.3.2) and a fall in IGFBP-1 (Chapter 3.3.6) concentrations. This possibly leads to an increased ratio of IGFs to total IGFBPs, resulting in a decrease in the small molecular weight binding capacity and an increase in IGF bioavilability to receptor sites in the tissues. The elevated small molecular weight binding capacity noted in the SSGC children may also play a part in their poor growth.

CHAPTER 5

GROWTH HORMONE EFFECTS ON IGFS AND IGFBPS IN CHILDREN WITH CHRONIC RENAL FAILURE AND TRANSPLANTATION

5.1 Introduction

Elevated concentrations of low molecular weight IGF binding proteins have been implicated in the reduced IGF bioactivity in the serum of children with CRF (Blum, Ranke, Kietzmann, *et al*, 1991; Goldberg, Trivedi, Delmez, *et al*, 1982; Lee, Hintz, Sperry, *et al*, 1989) and may contribute to the growth retardation in these patients. This poor growth has been successfully treated with rhGH as shown in Chapter 3.3.1. Growth failure also occurs frequently in transplanted children, often influenced by age, pre-treatment height velocity (Wuhl, Haffner, Tonshoff, *et al*, 1993) and corticosteroid therapy, which, in most cases, is part of a routine immunosuppressive treatment regime (Broyer, Guest and Gagnadoux, 1992). rhGH treatment has also been shown to improve growth velocity in these children (Jabs, Vandop and Harmon, 1993; Johansson, Sietnieks, Janssens, *et al*, 1990). In this study, we examined sequential IGF and IGFBP profiles in 3 children, each of whom received a renal transplant during a two-year rhGH treatment period, to investigate the possible roles of these peptides in the improved growth response and to determine the effect of transplantation on these parameters.

5.2 Patients and methods

Three children, included in a 2-year study-group of 11 children, who were treated with a combination of a protein- and phosphate-restricted diet and rhGH, were investigated retrospectively. Two boys (Patients 3 and 4; Chapter 3.2), included in the group of ten males in the study and a girl, whose data were not included in the report (van Renen, Hogg, Sweeney,

et al, 1992) due to her requirement for dialysis just after commencing the trial, comprised the present study group. These children were the only subjects in the study group to receive transplants. The pretreatment data of subject 1 (Patient 3; Chapter 3.2), subject 2 (Patient 4; Chapter 3.2) and subject 3 (female) are shown in Table 5.1.

Subject 3 required renal replacement therapy (haemodialysis) after only 2 weeks of rhGH treatment. Subject 2 commenced haemodialysis after 12 months of treatment and Subject 1 was transplanted without previous dialysis. Each child received a transplant within 4 weeks of the 18 month time-point of the study and was commenced on daily prednisolone treatment (oral prednisolone, 25 mg twice/day, commencing on day 3 with progressive reduction of the dose from the second week) as part of the standard immunosuppressive regime. rhGH treatment was discontinued in each patient for a period of 4-7 days after transplantation. Treatment was restarted with the full informed consent of the parents and children, in consultation with the caring physicians, when serum creatinine levels had stabilized and there was no evidence of rejection.

Serum samples from the children had been collected during routine venepuncture, at intervals over a period of approximately 5 years before the start of treatment with rhGH. Blood was collected before the first rhGH injection and 3-monthly thereafter. A pool of normal prepubertal serum and serum from a normal pubertal boy were used as controls (Chapter 3.2). Pooled serum from three normal 13-year-old females was used as the control for the female subject. Pubertal control serum was included on the Western ligand blots (Fig. 5.3) to assess possible precocious changes induced by the treatment and adult female control serum was also included on the WLB of Subject 3. Informed, signed consent was obtained from the children

Table 5.1 Subject data before rhGH treatment.

	Sex	Chronological age (years)	Bone Age *a* (years)	GFR *b* (ml/min/1.73m2)	Height SDS *c*	Puberty (Tanner Stage)
Subject 1	Male	7.7	5.2	16.0	-3.4	1
Subject 2	Male	10.0	8.3	11.0	-2.1	1
Subject 3	Female	15.3	13.5	8.0	-3.7	2

a Bone Age determined using the method of Greulich and Pyle.
b GFR measured by diethylene triamine penta-acetic acid (DTPA) clearance.
c SDS, Standard deviation score

Table 5.2 Changes in height velocity with rhGH (cm/year)

	Sex	Before rhGH	12 months/rhGH	18 months/rhGH (before transplant)
Subject 1	Male	5.6	10.1	7.6
Subject 2	Male	3.3	8.1	5.6
Subject 3	Female	4.3	5.8	5.8

and/or their parents and approval for the collection was obtained from the Ethics Committee of the Women's and Children's Hospital (Chapter 3.2)

5.3 Serum analysis

5.3.1 Western ligand blotting

IGFBPs were characterised by WLB analysis according to the method described in Chapter 2.4. Serum from each of the three children, representative of approximately 5 years before rhGH treatment, together with serum collected during treatment, before transplantation and following transplantation from each child, was loaded sequentially onto the gels. We were thus able to visualize the IGFBP profile of each child "through time" and observe the effects of rhGH therapy and transplantation.

5.3.2 Western immunoblotting

Immunoblotting, using specific anti-IGFBP antisera was performed, following the method discussed in Chapter 2.5, in order to identify the individual protein bands seen on the ligand blots. The nitrocellulose filter from the ligand blot from Subject 1 was cut in half, so that the first 6 lanes could be immunoblotted with the antibody to IGFBP-3 and the subsequent 7 lanes with the IGFBP-1 antibody. The filter from Subject 2 was also halved and the first 6 lanes were immunoblotted with the IGFBP-1 antibody, while the following 7 lanes were incubated with

the IGFBP-3 antibody. This was to demonstrate the effect of rhGH treatment and transplantation on both IGFBPs, as the ligand blots were not able to be repeated due to lack of serum.

5.3.3 Fast protein liquid chromatography

Serum IGFBP complexes of different molecular weights and free IGF were separated using size-exclusion FPLC under neutral pH conditions as described in Chapter 2.6. We have used the shorter incubation time, *ie* 1 minute at 37° C, in these samples as the work in this Chapter preceeded the experiments performed in Chapter 4.

5.3.4 High performance liquid chromatography

IGFs were separated from the IGFBPs, before quantitation of IGF-I and -II, using size-exclusion liquid chromatography under acidic conditions (pH 2.8) as described in Chapter 2.7.1

5.3.5 Quantitation of serum IGFs and IGFBPs.

Serum IGF-I concentrations were measured by RIA and IGF-II by RRA according to the method outlined in Chapter 2.7.2 and serum levels of IGFBP-1, IGFBP-3 and the ALS were measured by specific RIA (see Chapter 2.7.4).

Because this study comprised only three children, we have made no attempt to analyse the data statistically, and individual values (average ± SD) have been presented (Table 5.2). The IGF-I and -II values reported for each child at each stage of the study is an average (± SD) of the values obtained during the three stages, i.e. 5 years before rhGH treatment, from the start of rhGH treatment to immediately before the transplant, and after the transplant to the end of the 2-year study period (no IGF result was available for patient 2 after transplantation). Similarly the values reported for IGFBP-3, ALS and IGFBP-1 are an average (± SD) of the values obtained (or a value alone where a single sample was available for analysis) before rhGH treatment, during treatment but before transplantation and after transplantation.

5.4 Results

5.4.1 Height velocity

All three children showed a marked increase in height velocity after the first 12 months of rhGH therapy (Table 5.2). This rise in height velocity was not maintained in all patients during the second year of treatment before transplantation, although the increase was still greater than before rhGH treatment. It was not possible to assess the velocity after transplantation accurately as only 6 months data were available.

5.4.2 Characterization of IGFBPs by WLB and WIB

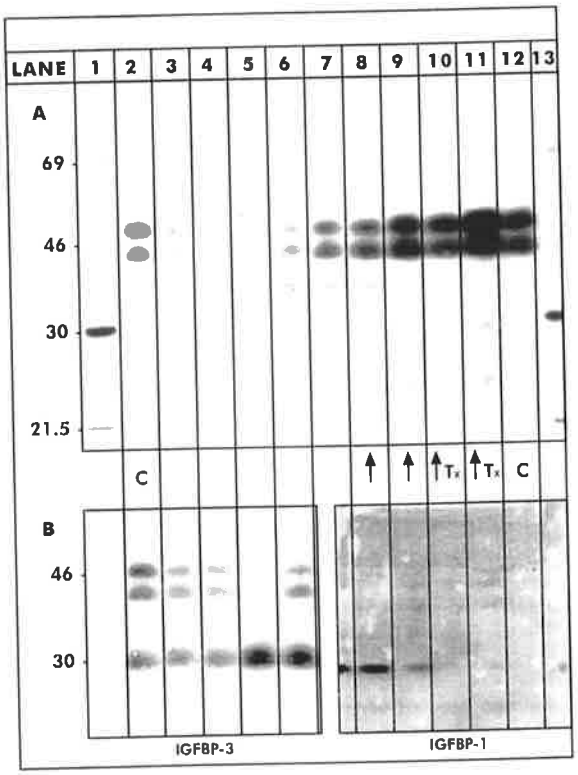
Five distinct protein bands which bound ^{125}I-IGF-I were identified on ligand blotting (Fig 5.1 (a), (b) and (c)) as also described in Chapters 2 and 3. A band at 46-48 and one at 44-46 kDa, comprising a doublet, were the dominant bands seen in each case. The density of this doublet increased with age, was not greater than the age-matched normal control serum and did not appear to be consistently altered by rhGH treatment or transplantation. Immunoblotting with the antibody to IGFBP-3 resulted in positive identification of the 44-48 kDa bands as IGFBP-3 (Fig 5.1, (a) - B and Fig 5.1, (b) - B). A further band, recognised by the hIGFBP-3 antibody, was observed on the immunoblots at 30 kDa. This band, which was also present in the normal control serum, was not clearly visualised by WLB. It appeared on immunoblot to be slightly increased in intensity before rhGH treatment in Subject 1 (Fig 5.1 (a) - B; lanes 5 and 6). In

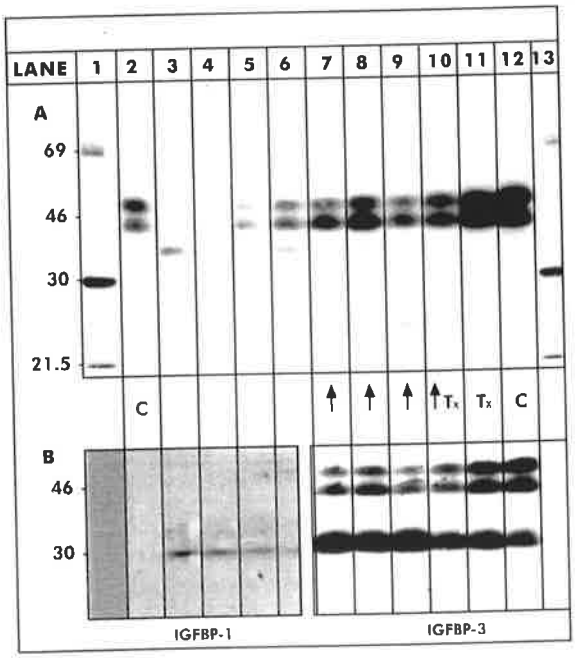
Fig 5.1 Western ligand blots (**A**) and immunoblots (**B**) of sequential serum samples from Subject 1, 2 and 3. The ligand used in (**A**) was ^{125}I-IGF-I, and the antibody used in each immunoblot is indicated below the blot (**B**). C, control sera; ↑, rhGH treatment; Tx, transplanted. Lanes 1 and 13, ^{14}C molecular weight markers. There was no immunblot for Subject 3.

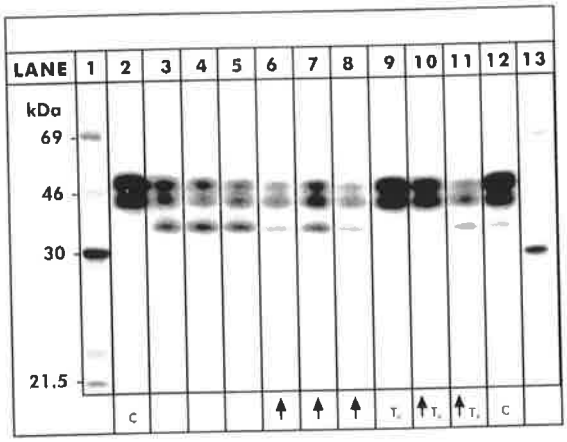
a) Subject 1 Lane 2, prepubertal control serum;Lanes 3 - 7, patient serum at ages 3.0, 4.0, 5.0, 6.6 and 7.6 years respectively, with GFR of 18 - 16 ml/min/1.73m^2; Lanes 8 and 9, patient sera at ages 8.2 and 8.8 years, with GFR stable at 11 ml/min/1.73m^2; Lanes10 and 11, patient sera at ages 9.6 and 10.6 years (post-transplant); Lane 12, pubertal control serum.

b) Subject 2 Lane 2, prepubertal control serum; Lanes 3 - 6, patient serum at ages 5.3, 6.3, 7.5 and 10.0 years respectively with GFR of 27, 23, 19 and 11 ml/min/1.73m^2; Lanes 7 - 8, patient sera at ages 10.3 and 10.8 years with GFR stable at 11 ml/min/1.73m^2 ; and Lane 9, patient serum at 11.0 years (on dialysis); Lanes 10 and 11, patient sera at age 11.8 and 12.0 years (post-transplant); Lane 12, pubertal control serum.

c) Subject 3 Lane 2, pubertal control serum;. Lanes 3 - 5, patient sera at ages 10.5, 11.6 and 12.3 years respectively with GFR of 15, 14 and 13 ml/min/1.73m^2; Lanes 6 - 8, patient sera at ages 15.5, 16.0 and 16.3 years respectively (on dialysis); Lanes 9 - 11, patient sera at ages 16.8, 17.0 and 17.3 years respectively (post-transplantation); Lane 12, adult control serum


LANE 1 2 3 4 5 6 7 8 9 10 11 12 13
A
69
46
30
21.5
C
Tx
Tx
C
B
46
30
IGFBP-3
IGFBP-1


LANE 1 2 3 4 5 6 7 8 9 10 11 12 13
A
69
46
30
21.5
C
Tx
Tx
C
B
46
30
IGFBP-1
IGFBP-3


LANE 1 2 3 4 5 6 7 8 9 10 11 12 13
kDa
69
46
30
21.5
C

Subject 2, this band appeared to decline in intensity following transplantation (Fig 5.1 (b) - B; lanes 10 and 11). No other bands could be identified as IGFBP-3.

A second distinct band was seen on WLBs at 34 kDa (Fig 5.1 (a), (b), and (c) - A) which had previously been identified as IGFBP-2 (Chapter 2.5.1). This band appeared to be much more intense in the three chronic renal failure children compared with the normal controls, both prepubertal and pubertal, in which it was either of low intensity or not visualized at all. It was, however, also observed in the adult female control serum. This band decreased in intensity following transplantation in all three CRF children, although it did reappear to some extent in the female patient (Fig 5.1 (c)). A further faint band was noted at 29 kDa on WLB, which did not appear to be affected by rhGH treatment. It was identified on immunoblotting by specific antibody as IGFBP-1 (Fig 5.1 (a) - B and (b) - B), was not observed in normal controls and disappeared following transplantation in subjects 1 and 2. A fourth distinct protein band was seen at 24 kDa in the CRF subjects, which was not seen in the normal controls. The density of the band did not appear to change with rhGH treatment or following renal transplantation. This band had previously been identified as IGFBP-4 by WIB (Chapter 2.5.1).

5.4.3 IGF, IGFBP, and ALS Assays

rhGH therapy resulted in a four-fold increase in the mean serum IGF-I level, which remained elevated after transplantation (Table 5.3). The mean serum IGF-II value rose 1.6 times on rhGH treatment, but after transplantation fell to below pre-treatment levels in Subject 1 and remained elevated in Subject 3. The mean IGFBP-3 value, which was within the reference

Table 5.3 Results of IGF and IGFBP RIA [a]

	Subject	IGF-I (ng/ml)	IGF-II (ng/ml)	IGFBP-3 (ug/ml)	ALS (ug/ml)	IGFBP-1 (ng/ml)
	1	27.8 (10.5)	644 (88)	4.1 (0.8)	23.2 (5.0)	648 (290)
Chronic renal failure	2	63.5 (23.2)	562 (154)	3.0 (0.9)	17.8 (6.3)	375 (195)
(not on rhGH treatment)	3	64.1 (5.2)	513 (79)	2.4 (0.2)	14.2 (2.7)	469 (171)
	1	174 (97)	990 (24)	8.5 (3.4)	49.3 (5.7)	581 (380)
Chronic renal failure	2	297 (55)	960 (81)	7.5 (1.5)	41.8 (8.4)	148 (37)
(during rhGH treatment)	3	186 (64)	969 (325)	5.1 (0.8)	33.8 (4.9)	556 (115)
	1	180	423	5.8 (2.6)	49.2 (2.9)	79.7 (51.8)
Post transplantation	2	N/A	N/A	4.4	49.8	75.0
(during rhGH treatment)	3	183 (37)	734 (405)	4.2 (0.9)	36.7 (3.8)	137 (40)

N/A, Not available

[a] Standard deviation score in parentheses

range for age-matched normal children (2-5 μg/ml) before rhGH therapy (Baxter and Martin, 1986), more than doubled on treatment, but following transplantation approximately 18 months later, decreased to the upper limit of the age-matched normal range (2-5 μg/ml). Before rhGH treatment, the ALS level (Table 5.3) was also within the normal range (9-35 μg/ml) (Baxter, 1990) and displayed a similar proportional increase to IGFBP-3 during rhGH therapy. Unlike IGFBP-3, however, ALS remained elevated after transplantation. IGFBP-1 levels, which were elevated compared with normal children (see Table 3.1) fell minimally on rhGH therapy in subjects 1 and 2 but increased in subject 3 before transplantation, but decreased substantially in all the children afterwards (Table 5.3).

5.4.4 ^{125}I-IGF-I binding profiles by FPLC

Three major regions of radioactivity were observed on the chromatograms, indicating binding of the ^{125}I-IGF-I at approximately 150 kDa and at 30-50 kDa, with a third peak at 7.5 kDa representing the unbound ^{125}I-IGF-I. With the 60 second incubation time used in this study, binding of ^{125}I-IGF-I in the 150 kDa binding protein peak before rhGH therapy was minor and appeared to be similar in each child. Subjects 1 and 3 had approximately the same percentage of radioactivity associated with the 30-50 kDa region of the chromatogram, while Subject 2 had less binding in this region and therefore relatively more radioactivity appeared in the "free" ^{125}I-IGF-I region. The elution profiles of all three children altered dramatically on

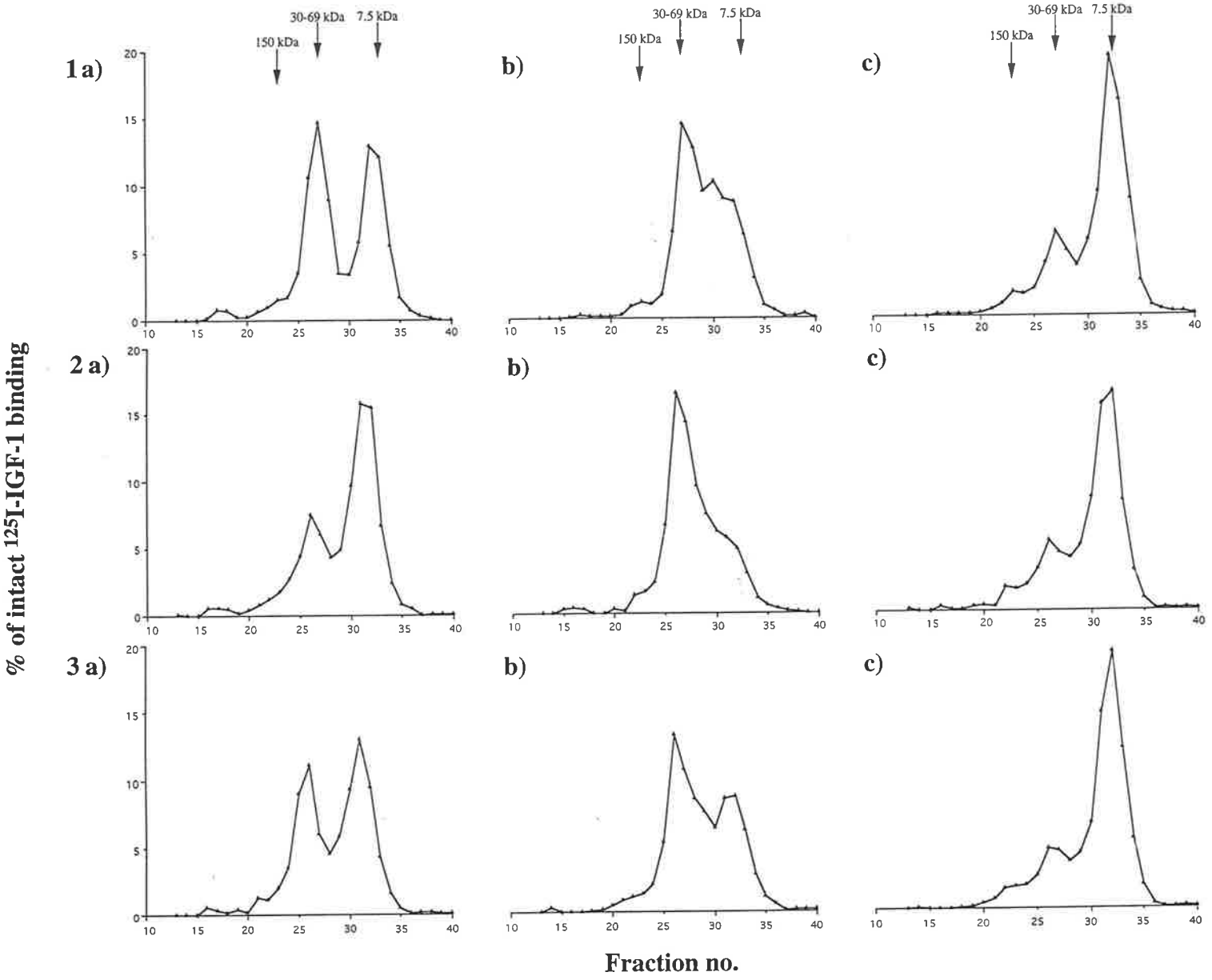
rhGH treatment and with progressive deterioration of renal function. The 150 kDa binding region did not differ much from the pre-treatment profile. However, the notable change was

the decrease in the "free" ^{125}I-IGF-I peak, with a relative increase in both the 30-50 kDa peak and in the amount of binding in the region between the 30-50 kDa and the unbound ^{125}I-IGF-I peaks. Following transplantation, there was better definition of the 150 kDa peak, with a significant decrease in the 30-50 kDa binding peak. The 7.5 kDa ^{125}I-IGF-I peak increased in each case to levels equal to, or greater than, those found before rhGH therapy. The pattern of an increase in the 30-50 kDa peak, with a decrease in the 7.5 kDa ^{125}I-IGF-I peak during rhGH therapy and declining renal function, followed by a reversal of this situation after transplantation, was remarkably consistent in all three patients.

5.5 Discussion

The success of rhGH treatment in increasing growth velocity in CRF (Lippe, Fine, Koch, *et al*, 1988; Rees, Rigden, Ward, *et al*, 1990; Tonshoff, Mehls, Schauer, *et al*, 1989; van Renen, Hogg, Sweeney, *et al*, 1992) and renal transplantation (Jabs, Vandop and Harmon, 1993; Johansson, Sietnieks, Janssens, *et al*, 1990) has previously been reported. In this study-group of three patients, velocity almost doubled before transplantation, but decreased slightly afterwards, although it was still greater than before rhGH therapy. The mechanism of this growth effect is thought to be stimulation by rhGH of IGF production by the liver and other tissues (Rees, Rigden, Ward, *et al*, 1990; Tonshoff, Mehls, Schauer, *et al*, 1989), reportedly overcoming the peripheral "growth hormone resistance" of uremia. The effect of rhGH on the

Fig 5.2. Size distribution of radioactivity in serum from Subjects 1, 2, and 3, before rhGH treatment, during rhGH treatment and after transplantation, is shown in panels 1a, b and c; 2 a, b and c and 3 a, b and c respectively. Serum was incubated for 60 seconds with ^{125}I IGF-I and then fractionated on a Superose 12 size-exclusion column at pH 7.4. 0.5 ml fractions were collected at a flow rate of 1ml/min.The molecular size of the relevant radioactive regions during the three stages of treatment is indicated at the top of panel a). These were estimated from the molecular mass standards used to calibrate the column.


1 a)
b)
c)
2 a)
b)
c)
3 a)
b)
c)
150 kDa
30-69 kDa
7.5 kDa
% of intact 125I-IGF-1 binding
Fraction no.

IGFs and IGFBPs in children with CRF has been reported (Hokken-Koelega, Stijnen, De Muinck Keizer-Schrama, *et al*, 1991; Rees, Rigden, Ward, *et al*, 1990; Tonshoff, Tonshoff, Mehls, *et al*, 1992). However, as far as we are aware, there are no reports of rhGH effects on these parameters and on the ALS followed during the progression of CRF and subsequent transplantation. Although the use of rhGH in children after renal transplantation is controversial, with acute rejection and graft loss being reported (Ingulli and Tejani, 1995), our experience with three children, in whom rhGH treatment was recommenced within 4-7 days of the transplant, is that no deleterious effects were evident as assessed by clinical examination and biochemical parameters throughout the study period.

Most of the growth promoting effects of growth hormone are thought to be mediated by the IGFs, particularly IGF-I since it is a more potent mitogen than IGF-II on an equimolar basis (Powell, Rosenfeld, Sperry, *et al*, 1987). Factors that regulate the production of the IGFs and IGFBP-3 are: age, nutritional state and growth hormone (Baxter, 1991). The IGF assay results in these three children showed a four-fold increase in IGF-I and a 1.6 times elevation of IGF-II as a result of rhGH treatment. This degree of elevation is almost identical to that noted in the results of the 5 prepubertal boys (which includes subjects 1 and 2) discussed in Chapter 3.3.2 and similar to the findings of Hokken-Koelega *et al* in their group of 23 prepubertal male and female children (Hokken-Koelega, Stijnen, De Muinck Keizer-Schrama, *et al*, 1991). It is of interest to note that the IGF-I value before rhGH treatment in Subject 3 (Tanner stage 2) did not show any physiological increase (as evidenced by the SD), as would normally be expected during Tanner stage I-II in normal girls (Argente, Barrios, Pozo, *et al*, 1993).

Elevated concentrations of small molecular weight proteins, possibly due to reduced renal clearance, have been proposed to increase the IGF-binding capacity of CRF and dialysis serum (Blum, Ranke, Kietzmann, *et al*, 1991; Lee, Hintz, Sperry, *et al*, 1989; Powell, Rosenfeld, Sperry, *et al*, 1987; Tonshoff, Blum and Mehls, 1996), resulting in a reduction in the availability of IGFs to target tissues and consequent growth retardation. The serum concentration of IGFBP-3, which occurs in highest molar concentration in adult life, has been reported to be elevated in CRF (Blum, Ranke, Kietzmann, *et al*, 1991). IGFBP-3 data from our patients before rhGH treatment, by WLB or quantitative serum RIA, demonstrated no difference compared with normal subjects and are in keeping with those of Hodson *et al* (Hodson, Brown, Roy, *et al*, 1992). Powell and co-workers also found no difference in concentration and size distribution of their 41 and 38 kDa IGFBP-3 by WLB between CRF and normal sera from adolescents and children, however, they did find elevated IGFBP-3 levels by RIA (Powell, Liu, Baker, *et al*, 1993) as have several other authors (Blum, Ranke, Kietzmann, *et al*, 1991; Hokken-Koelega, Stijnen, De Muinck Keizer-Schrama, *et al*, 1991; Tönshoff, Tönshoff, Mehls, *et al*, 1992). An inverse correlation between elevated IGFBP-3 values and GFR has been reported (Tönshoff, Tönshoff, Mehls, *et al*, 1992), and the authors suggest that reduced renal clearance leads to elevated amounts of IGFBP-3 in the serum of patients with diminished kidney function. We were not able to comfirm this, as in Chapter 3..3.4, as our three patients, each with markedly reduced renal function at the start of the study (Table 5.1), were found to have IGFBP-3 RIA levels within the normal range. These conflicting results may be explained by the use of different antibodies in the assays which may not recognise IGFBP-3 fragments in the serum.

The 30 kDa form of IGFBP-3, not detected by the ^{125}I IGF-I radioligand on the WLB, but clearly identified by immunoblot, may represent an immunologically active IGFBP, with a significantly reduced affinity for IGF peptides. This form of IGFBP-3 was also present in normal control sera and did not disappear from the immunoblot in the CRF patients after transplantation, although it did appear to decrease in intensity. As discussed in Chapter 4, this, in fact, may be an altered form or fragment of IGFBP-3, possibly due to deglycosolation of native IGFBP-3, which appears to be increased in the small molecular weight fractions of CRF sera from where it is able to enter the extravascular space where it has been identified in peritoneal dialysis fluid (Kale, Liu, Hintz, *et al*, 1996). The function of this form of IGFBP-3 is not yet clear. It may facilitate the actions of the IGFs or, in excess quantities, prevent IGF-receptor interaction.

The function of the ALS has been extensively studied (Baxter, 1991). Much of the circulating ALS appears to be uncomplexed and, when quantitated by RIA, the mean level is more than double the concentration of IGFBP-3 or total IGFs. The association constant for ALS binding to IGF-IGFBP-3 complexes is low, and the relatively high concentration of ALS in the serum may be important in keeping most of the IGFs in the 150kDa complex. Serum levels of ALS in our patients before rhGH therapy were within the normal reference range, but levels increased in parallel to those of IGFBP-3 with rhGH treatment. These levels were, however, maintained after transplantation, while IGFBP-3 fell. This suggests limited clearance, before transplantation, of IGFBP-3 at the high serum concentrations which resulted from rhGH treatment. The maintenance of elevated ALS levels post-transplantation was probably due to the relatively larger molecular size of this peptide (85kDa) compared to IGFBP-3, thus precluding it's clearance by the transplanted kidney.

Serum IGFBP-1, when measured over 24 hours in children, reveals a marked diurnal rhythm independent of GH and appears to be acutely regulated by hormonal and nutritional status (Baxter, 1991). Fasting IGFBP-1 levels in our patients were elevated compared with normal subjects and decreased slightly with rhGH treatment. These results are similar to those of Hokken-Koelega *et al*, who found pre-rhGH IGFBP-1 concentrations as much as 5-15 times above normal, which fell during rhGH therapy (Hokken-Koelega, Stijnen, De Muinck Keizer-Schrama, *et al*, 1991). Lee and co-workers, also demonstrated significantly elevated levels of IGFBP-1 in CRF compared with normal controls (Lee, Hintz, Sperry, *et al*, 1989). We have previously shown that, while fasting serum glucose levels remain within normal limits during rhGH treatment, this occurs at the expense of a rise in serum insulin levels (van Renen, Hogg, Sweeney, *et al*, 1992) with an inverse relationship between IGFBP-1 and insulin levels (see Chapter 3.3.6). Following transplantation, serum concentrations of insulin tend to be higher than in CRF and increase by 85% during the first year on rhGH therapy (Tönshoff, Heinrich and Mehls, 1991). In our subjects, following transplantation, there was a significant decrease in IGFBP-1 RIA levels to one fifth of the baseline value. This effect was confirmed by the complete disappearance, after transplantation, of IGFBP-1from the WIB. These data suggest that elevated serum insulin levels, combined with improved renal clearance, may be responsible for the dramatic fall in serum IGFBP-1 RIA levels following transplantation.

IGFBP-2 is a specific IGFBP with a greater affinity for IGF-II than for IGF-I (Baxter, 1991). Although the regulation and clinical significance of IGFBP-2 are still poorly understood, it has been shown to be elevated in infancy, end-stage renal disease and in patients with IGF-II-producing tumours, and to decline with increasing age (Schwander and Mary, 1993). Increased IGFBP-2 synthesis by the liver in CRF has recently been reported which may be reversed with

transplantation. IGFBP-2 decreased in intensity on WLB following transplantation, suggesting increased clearance by the kidneys, although this apparent decrease in IGFBP-2 levels would ideally need to be quantitated by specific RIA (Powell, Liu, Baker, *et al*, 1996).

The shift of ^{125}I IGF-I from the free 7.5kDa peak, in the FPLC profiles, to the 30-50 kDa binding protein peak during rhGH treatment, suggested that previously unbound ^{125}I-IGF-I was now located in the small molecular weight binding region, due to an increase in binding proteins of this molecular size. The increased IGFBP-3 levels, induced by rhGH treatment, and possibly not associated with ALS in the large complex with the short incubation time used in this study, could have contributed to the increased binding in this region. The reverse shift, seen in all three children following transplantation, from the 30-50 binding peak to the free 7.5kDa IGF-I peak, suggested less binding of ^{125}I-IGF-I due to a fall in the concentration of smaller molecular weight IGFBPs after the transplant. The chromatograms in these three children (Fig. 5.2) were derived after the short ^{125}I-IGF-I incubation *i.e.* 1 minute at 37° C (Chapter 2.6 and 4.3.1) and therefore the binding profiles on rhGH and after transplantation appear vastly different to those of the pooled serum in Chapter 4.3.1 which had been incubated overnight allowing for a further shift in binding towards the 150 kDa complex. Although FPLC elution profiles are not quantitative, they illustrate the increased binding in the smaller molecular weight region of the serum of these CRF children with deteriorating renal function and during rhGH treatment. As discussed in Chapter 4.4, ^{125}I-IGF-I incubated with serum is only able to associate with those IGFBPs not already bound to endogenous IGF-I, however, the identical preparation of the patient samples, we believe, makes comparisons between the different treatment modalities valid.

The improved height velocity in the three study patients, demonstrated during the first year on rhGH therapy, was less in the second year before transplantation despite the maintainance of elevated IGF-I levels. Height velocity appeared to slow further after transplantation, although accurate velocities cannot be calculated on only 6 month data. This pattern of slowed growth velocity after the first year of rhGH treatment, although still significantly higher than pre-treatment values, has been described in children with CRF and transplantation, as well as in idiopathic growth hormone deficiency and Turners syndrome (Van Es, Albertsson-Wikland, Berg, *et al*, 1991). It has been hypothesised that the elevated serum IGFBP concentrations in CRF, particularly small molecular weight proteins, bind the IGFs resulting in a diminished IGF bioavailability and therefore a reduction in IGF bioactivity resulting in growth retardation (Blum, 1991; Lee, Hintz, Sperry, *et al*, 1989; Tonshoff, Blum, Wingen, *et al*, 1995). The altered growth response in transplanted children may be the result of growth hormone resistance caused by concomitant immunosuppressive therapy, particularly corticosteroids. Hokken-Koelega *et al* (Hokken-Koelega, Stijnen, Keizerschrama, *et al*, 1993) found that transplanted children, receiving either alternate day, or daily prednisolone therapy had decreased GH secretion, but normal IGF-I and -II levels. Ingulli and co-investigators (Ingulli, Singh, Moazami, *et al*, 1993) recently described a strong correlation between steroid administration and failure to achieve accelerated growth. This was the result of a blunted peak GH level in prednisolone treated patients after rhGH treatment compared with patients not receiving prednisolone, although they did respond to higher doses of rhGH. However, these authors did find that these same patients demonstrated an elevated peak IGF-I level on rhGH, causing some speculation on the interaction between prednisolone, GH and IGF-I (Ingulli, Singh, Moazami, *et al*, 1993). Several studies, (Hokken-Koelega, Stijnen, Keizerschrama, *et*

al, 1993; Mehls, Tonshoff, Kovacs, *et al*, 1993) have shown that glucocorticoid treatment had no effect on IGF-I or IGFBP-1 levels, but that IGFBP-3 levels were elevated. The results from this study do not support these IGFBP-3 results, although this is quite possibly due to the small number of children which we were able to assess and the specificity of the IGFBP-3 RIA.

5.6 Conclusions

In conclusion, our results show that rhGH treatment in CRF leads to an improved growth velocity in association with an increased production of growth hormone dependent IGFs (both IGF-I and IGF-II), IGFBP-3 and the ALS. Following transplantation, however, the growth response diminished, despite maintenance of the increased IGF-I RIA levels and a decrease in IGFBP-3 RIA values. This may be attributed to the concomitant use of corticosteroid immunosuppressive therapy. We have demonstrated that levels of IGFBP-1 and -2 in CRF and, possibly, a low molecular weight form of IGFBP-3, decrease following transplantation, suggesting that elevated levels of small molecular weight IGFBPs in CRF may be due to decreased clearance by the diseased kidneys.

5.7 Acknowledgements

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CHAPTER 6

FINAL CONCLUSIONS

FINAL CONCLUSIONS:

The well described growth retardation observed in children with CRF has, in part, been attributed to abnormalities of the GH-IGF axis. rhGH has been most successful in improving height velocity in these children. In the studies presented in this thesis, we have been able to analyse serum IGF concentrations and IGFBP profiles, before and during rhGH in short children with CRF and transplantation. Although the number of children available for investigation was too small to result in statistically significant observations, we believe the results to be of sufficient interest to contribute to, and possibly promote, further research into this complex pathophysiological problem.

Increased IGFBP-1, -2 and -4, levels were identified in our patients before rhGH treatment, together with low-normal IGF-I, elevated IGF-II, normal IGFBP-3 and ALS concentrations using the techniques available to us. In addition, neutral size-exclusion gel chromatography revealed an increase in ^{125}I-IGF-I binding capacity in the small molecular weight binding peak in all the CRF and transplanted children, regardless of age and sex. These findings suggest that a decrease in IGF bioavailability in these patients may contribute to their growth retardation.

The addition of rhGH therapy to the management of our children led to an approximate doubling of mean height velocity, which was associated with a significant increase in serum IGF-I concentrations and in addition, a substantial fall in IGFBP-1 levels and a moderate rise in serum IGF-II, IGFBP-3 and ALS levels. This was associated with a decrease in binding in the small molecular weight IGF-IGFBP complex region. These results suggest that the improved growth in response to rhGH in CRF may be due to the significant increase in IGF-I and fall in

IGFBP-1 concentrations, resulting in an improved IGF/ total IGFBP ratio, and a consequent increase in IGF bioavailabity leading to improved growth.

APPENDIX A

4 x sodium dodecyl sulphate (SDS) loading buffer

3.02 % (w/v) Tris base, 8.0 % (w/v) SDS, 20 % (v/v) glycerol and 0.004 % (w/v) bromphenol blue. pH to 6.8.

Western buffer

4 M NaCL, 1 M Tris base (0.15M NaCl, 0.01M Tris stock)

TBS - Tween 20 (ECL wash buffer)

8 % (w/v) NaCl, 2.42 % (w/v) Tris base, 0.1 % (w/v) Tween 20. pH to 7.6.

FPLC buffer

50 mM sodium dihydrogen orthophosphate, 150 mM sodium chloride, 0.02 % w/v sodium azide, 10U/ml Heparin. pH 7.2. Filtered through 0.22µm HA filter and degas.

IGF-RIA Buffer

30 mM NaH_2PO_4, 10mM EDTA, 0.02% (w/v) sodium azide, 0.05% (v/v) Tween 20 at pH 7.5.

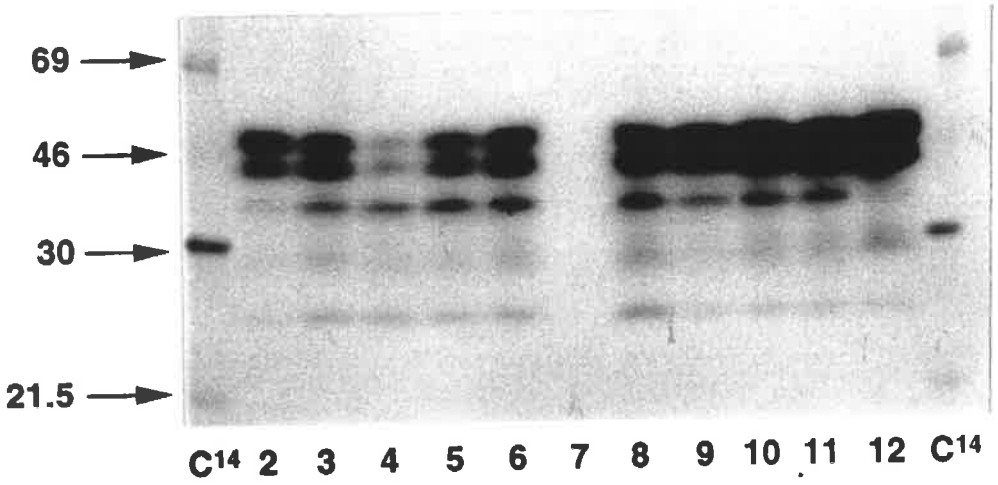
IGF-RRA Buffer

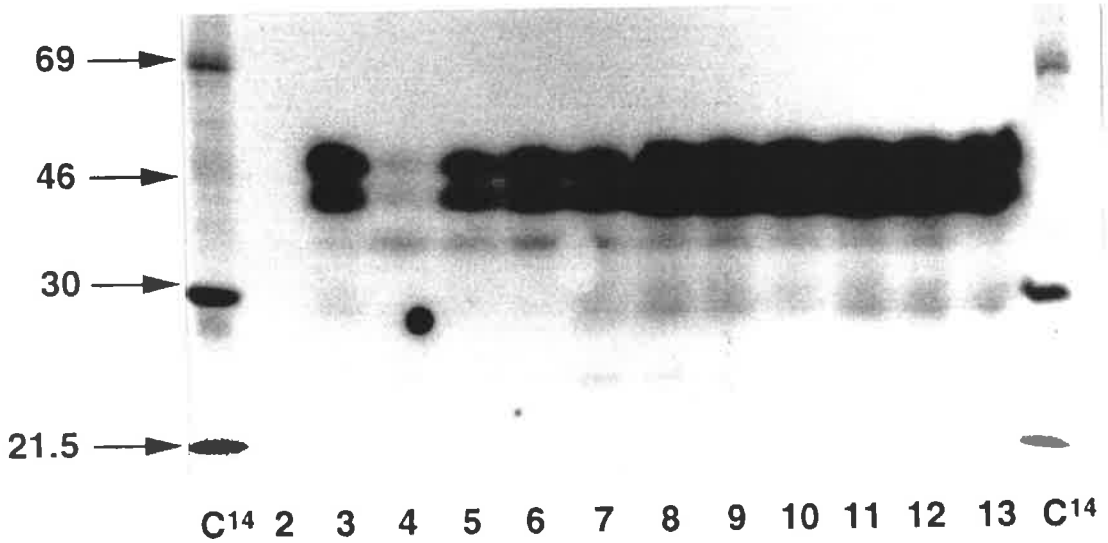
0.1M Tris base, 5.0 g/L BSA (RIA grade), 0.01M $CaCl_2$. pH 7.4.

APPENDIX B

Figure 1 *Western ligand blot of sequential serum samples from Patient 1.* (see Chapter 3.2). The ligand used was ^{125}I-IGF-I. ^{14}C molecular weight markers are shown in the left and right columns. Lane 2, prepubertal normal male; Lane 3, Patient 1 aged 3.5 years; Lanes 4-6, aged 5.0-5.6 years, on rhGH, stable GFR; Lane 7, blank, Lanes 8-9, aged 5.8-6.8 years on rhGH, stable GFR; Lanes 10-11, aged 7.8-8.0 years, stable GFR; Lane 12, pubertal normal male.

Figure 2 *Western ligand blot of sequential serum samples from Patient 5.* (see Chapter 3.2). The ligand used was ^{125}I-IGF-II. ^{14}C molecular weight markers are shown in the left and right columns. Lane 2, blank; Lane 3, prepubertal normal male; Lanes 4-7, Patient 5 aged 7.8-11.8 years, GFR of 54-31 ml/min/1.73m^2; Lanes 8-11, aged 12.0-13.5 years, on rhGH, GFR of 31-19 ml/min/1.73m^2; Lane 12, aged 14.2 years, haemodialysis; Lane 13, pubertal normal male.


69
46
30
21.5
C14 2 3 4 5 6 7 8 9 10 11 12 C14


69
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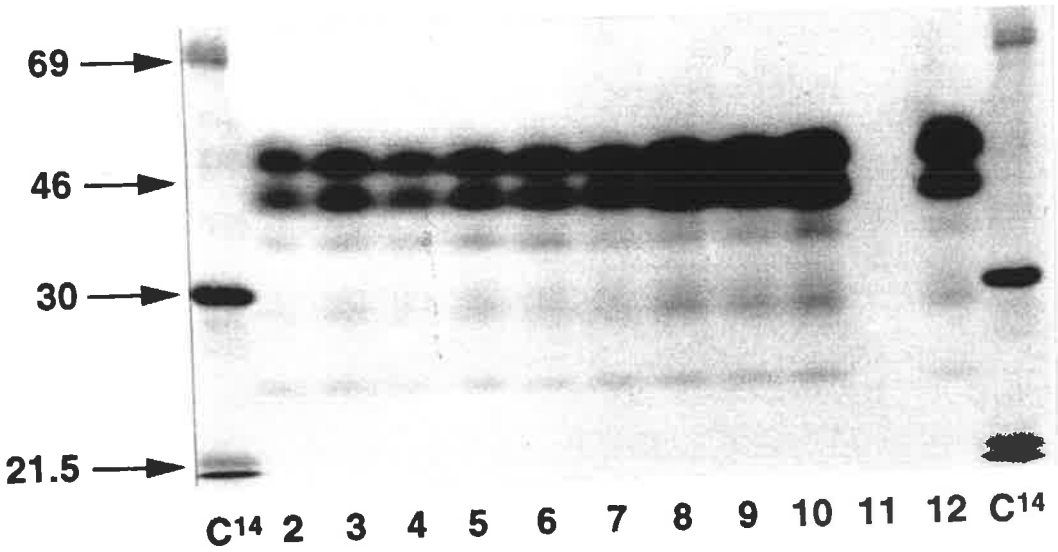
30
21.5
C14 2 3 4 5 6 7 8 9 10 11 12 13 C14

Figure 3 *Western ligand blot of sequential serum samples from Patient 6.* (see Chapter 3.2). The ligand used was ^{125}I-IGF-II. ^{14}C molecular weight markers are shown in the left and right columns. Lane 2, prepubertal normal male; Lanes 3-6, Patient 6 aged 8.2-12.3 years, GFR of 72-62 ml/min/1.73m^2; Lanes 7-10, aged 12.8-14.3 years, on rhGH, GFR of 61-52 ml/min/1.73m^2; Lane 11, blank; Lane 12, pubertal normal male.

Figure 4 *Western ligand blot of sequential serum samples from Patient 8.* (see Chapter 3.2). The ligand used was ^{125}I-IGF-I. ^{14}C molecular weight markers are shown in the left and right columns. Lane 2, prepubertal normal male; Lanes 3-7, Patient 8 aged 8.2-13.8 years, GFR of 38-36 ml/min/1.73m^2; Lanes 8-10, aged 14.2-15.8 years, on rhGH, GFR of 38-28 ml/min/1.73m^2; Lane 11, aged 16.8 years GFR of 28 ml/min/1.73m^2; Lane 12, pubertal normal male.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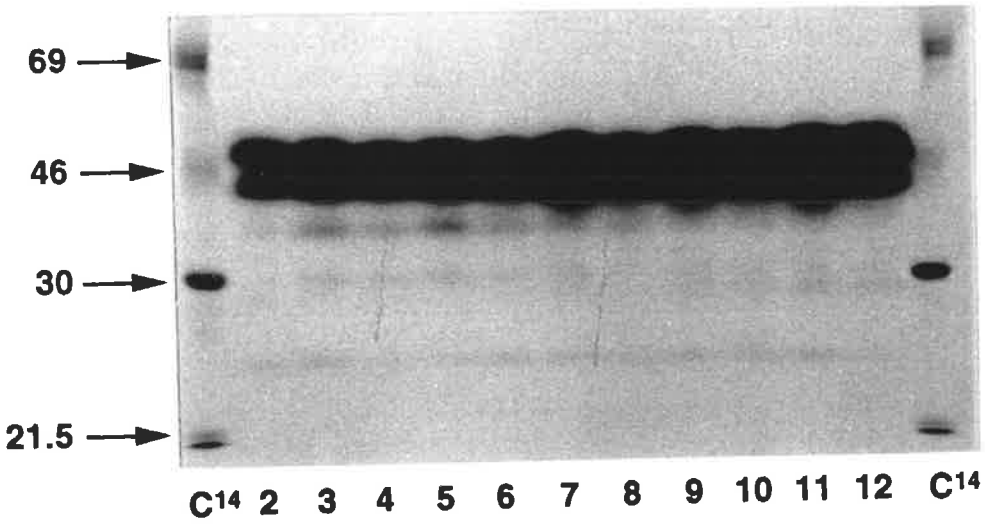
69
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C14 2 3 4 5 6 7 8 9 10 11 12 C14

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ADDENDA

[*Note: All references cited in this section can be found in the* ***References*** *section of the thesis*]

CHAPTER 1

Insert in section 1.4, page 14, line 5 after "(Baxter, 1991)." :

There is a high linear correlation between the molar sum of serum IGF-I and IGF-II, and serum IGFBP-3 (Blum, Ranke, Kietzmann *et al*, 1991).

CHAPTER 2

Insert in section 2.2.1, page 26, line 12 after "at -80° C." :

Because some of the serum samples had been subjected to prolonged storage, studies on the effects of storage and freeze-thawing were undertaken before the assays were performed. No significant degradation of the proteins of interest was observed (data not shown).

Insert in section 2.8.2, page 37, line 5 after "each assay." :

The intra-assay co-efficient of variation for this assay is 3.2%, while the inter-assay co-efficient of variation is 12.4% (Owens, Kind, Carbone *et al*,1994).

Insert in section 2.8.3, page 37, line 19 after "gamma counter." :

The intra- and inter-assay co-efficients of variation for this particular assay are 4.3% and 16.6% respectively (Owens, Kind, Carbone *et al*, 1994).

Insert in section 2.9, page 38, line 5 after "Australia)." :

The intra and inter-assay co-efficients of variation for the IGFBP-1 RIA are 4.7% and 12% respectively (Baxter, Martin and Wood, 1987), while the co-efficients of variation for the IGFBP-3 assay are 5.5% and 14.5% respectively (Baxter and Martin, 1986). The intra and inter-assay co-efficients of variation for the ALS assay are 3.3% and 5.4% respectively (Baxter, 1990).

Insert between sections 2.9 and 2.10, page 38. :

At the time that these experiments were performed, a radioimmunoassay for hIGFBP-2 was not available. Only a small amount of the IGFBP-2 antibody was available, which was used to detect IGFBP-2 using Western immunoblotting techniques.

CHAPTER 3

Insert in section 3.2, page 46, line 1 before "Patient results" :

Because serum levels of IGF-I and IGFBP-3 in particular, as well as some of the other IGFBPs *eg* IGFBP-2, are known to be age-dependent, these assay results should ideally be presented as Standard Deviation Scores (SDS). The SDS corrects individual values for the complicating effects of age and sex by using mean and standard deviation values derived from data from a normal population of the same age and sex. However, tables for the normal range, means and

standard deviations were not available for the IGF-I, -II and IGFBP-1 assays and although such tables were available for the IGFBP-3 and ALS assays, we elected, for the sake of consistency, not to use SDS scores to present these data.

Insert in section 3.4, page 67, line 10 after "both groups." :

Increased IGF-II levels in response to rhGH administration have also been reported by Powell and co-authors. They emphasize the contribution of this growth factor to the elevation in the ratio of total IGFs to total IGFBPs associated with the amelioration of growth retardation by rhGH in patients with CRF (Powell, Liu, Baker *et al*, 1996).

CHAPTER 5

Insert in section 5.5, page 126, line 14 after "(and Mehls, 1991)." :

Fasting serum insulin levels measured in these three children following transplantation were elevated compared with the pre-transplant values. Serum insulin levels in Subjects 1, 2 and 3 on rhGH treatment but before transplantation were 11.8, 16.7 and 23.5 U/ml respectively. However, following transplantation, these values rose to 14.8, 30.5 and 40.0 U/ml respectively.

CHAPTER 6

Insert on page 132, line 3 after"in these children." :

For the studies reported in this thesis, we seized the opportunity afforded by the availability of stored samples from our previous work on the improvement of growth in children with chronic

renal failure (Jureidini, Hogg, van Renen *et al* (1990); van Renen, Hogg, Sweeney *et al* (1992)). The data thus extend our previous clinical studies into a laboratory investigation of the mechanisms behind the growth retardation and its amelioration by growth hormone treatment in the same patients. The retrospective nature of the research in this thesis, and the small sample size available for investigation are recognised. The numbers of paediatric patients with chronic renal failure attending any one paediatric hospital are not large, especially of those qualifying for growth hormone treatment, and many studies reporting only radioimmunoassay data have therefore utilised multicentre trials to increase their sample sizes. The studies in this thesis, however, were not designed to focus on radioimmunoassay data, but rather on the nature of the changes in IGF binding protein profiles as determined by gel chromatography and Western blot techniques. These investigations could not have been undertaken on a larger scale.

Insert on page 132, line 7 after "significant observations," :

.....the radioimmunoassay results were found to be in agreement with much larger studies performed within the same time frame as this present work. This suggests that the findings in the subsequent chapters are therefore generalisable to the wider patient population, and.....