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GROWTH HORMONE AND TISSUE GROWTH FACTORS IN THE PATHOGENESIS OF DIABETIC RETINOPATHY

by

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A thesis for the degree of Doctor of Medicine (M.D.)

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'Tu ch'hai la bocca dolce ...

Tu che il zucchero porti in mezzo al core ...'

You, whose mouth is sweet ...

And who instil sugar into the depths of the heart ...

Serenade, Mozart's 'Don Giovanni'

Abstract

The aim of this work was to examine the role of Growth Hormone (GH) and tissue growth factors in the pathogenesis of diabetic retinopathy, and to investigate the mechanism of excessive GH secretion in diabetes. Levels of GH and insulin-like growth factor-I (IGF-I) (the principal mediator of the growth promoting activity of GH) were measured in diabetic patients with retinopathy and control subjects. The concentration of basic fibroblast growth factor (bFGF), a potent angiogenic growth factor, was also examined in vitreous and retinal extracts.

24h studies confirmed increased GH and normal IGF-I levels during poor diabetic control suggesting impaired IGF-I production. Serum IGF-I was inversely correlated with HbA_1 concentration. Reduced feedback inhibition by IGF-I when control is poor could contribute to excessive GH release. In addition, pretreatment with GH failed to suppress the GH response to GH-releasing hormone (GHRH) in some patients indicating altered GH feedback control. Relative resistance to somatostatin was inferred from the results of treatment with octreotide, a potent somatostatin analogue. GH hypersecretion in diabetes is likely to be a product of several different mechanisms.

No correlation was found between the development of background retinopathy and changes in serum IGF-I concentration, in patients commencing continuous subcutaneous insulin infusion. A significant increase in mean serum IGF-I occurred at the onset of proliferative retinopathy in patients treated conventionally (281 $\pm$ 54 versus 196 $\pm$ 58 micrograms/l; $p < 0.05$).

Cultured retinal endothelial cells were demonstrated to release IGF-I but not bFGF into the cell medium. A different release mechanism is likely for bFGF which was abundant in whole retinal extracts. In the diseased diabetic retina, cell damage could release bFGF which would then induce cell competence in surviving endothelial cells and fibroblasts. By allowing the cells to complete the cell cycle, the increase in IGF-I levels could play a crucial role in promoting cell proliferation and neovascularisation.

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Statement by the Author

All studies contained in this thesis were designed and carried out by the author with the advice and guidance of the author's supervisor, Professor Eva M. Kohner. All were approved by the Ethical Committee of the Royal Postgraduate Medical School and Hammersmith Hospital. The assays were performed in the Hormone Assay Laboratory, Hammersmith Hospital. All assays for growth hormone and insulin-like growth factor -I were carried out by the author assisted by Mr Roger Brooks and Mr Nick Tuson under the supervision of Dr Jacqueline Burrin (Principal Biochemist).

There have been 4 major areas of collaboration : -

In the study of insulin-like growth factor-I levels in patients treated by continuous insulin infusion, the author was responsible for the medical management of the patients, the hormonal assays, and the collection and analysis of the data. Retinopathy grading was performed by the author and Dr Marcus Sleightholm. Vitreous fluorophotometry data was analysed by Dr Walter Plehwe.

In the study comparing growth hormone and insulin-like growth factor-I levels with the stimulatory activity of serum on endothelial cell growth, cell growth and proliferation were assessed by by Dr Richard Petty and colleagues at the Section of Vascular Biology, MRC Clinical Research Centre, Northwick Park Hospital. The study was performed at the author's suggestion. The author obtained the serum samples, measured the levels of growth hormone and insulin-like growth factor-I, and analysed the results.

In the section involving the measurement of growth factors in vitreous and bovine retinal extracts, the author's contribution was the suggestion to measure insulin-like growth factor-I in these samples, perform the assays, and analyse the results. Retinal extracts were prepared by Dr David Kinshuck. The assay for basic fibroblast growth factor was performed by Mr Roger Brooks with some assistance from the author.

Finally in the study of the growth hormone response to galanin, the author instigated, designed and carried out the study with the help of Dr Stephen Gilbey and Professor Steve Bloom. Plasma galanin levels were measured by Dr Donald O'Halloran.

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Hyer SL, Sharp PS, Brooks RA, Burrin JM, Kohner EM. (1989) Continuous subcutaneous octreotide infusion markedly suppresses IGF-I levels whilst only partially suppressing GH secretion in diabetics with retinopathy. Acta Endocrinologica 120:187-194.

Hyer SL, Sharp PS, Brooks RA, Burrin JM, Kohner EM. (1989) A 2 year follow-up study of serum IGF-I in diabetics with retinopathy. Metabolism (accepted for publication).

Hyer SL, Sharp PS, Sleightholm M, Burrin JM, Kohner EM. (1989) Progression of diabetic retinopathy and changes in serum insulin-like growth factor I (IGF-I) during continuous subcutaneous insulin infusion (CSII). Hormone and Metabolic Research (accepted for publication).

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Presentations of the work contained in this thesis

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British Endocrine Society
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Oxford, July 87

Serum IGF-I concentration in diabetic retinopathy
British Diabetic Association
Liverpool, Sept 87

Growth hormone suppression in diabetic retinopathy
European Association Study of Diabetes
Leipzig, Sept 87

Changes in serum IGF-I and Diabetic Retinopathy during CSII
Aidspit Study Group
Igls, Austria, Jan 88

Changes in serum IGF-I in patients with diabetic retinopathy followed prospectively over 2 years
Somatomedin and Growth Club
London, May 88

Serum growth hormone and IGF-I levels in patients with diabetic retinopathy after treatment with continuous infusion of octreotide
International Diabetes Federation
Sydney, Nov 88

Endothelial proliferative activity in serum of diabetics with retinopathy is not related to serum growth hormone (GH) or insulin-like growth factor I (IGF-I) concentration.
Medical Research Society
London, Jan 89

Introduction

Impressed by (1) the improvement in the state of the retina and the reduction in capillary fragility in diabetic patients who had undergone ablative pituitary surgery and (2) the finding of elevated growth hormone (GH) levels in the serum of diabetics in their usual state of glycaemic control, Lundbaek and co-workers (Lundbaek et al., 1970, Lundbaek, 1976) proposed that GH is a causal factor in the development of diabetic microvascular complications. Since that time, a substantial amount of evidence has been produced demonstrating abnormalities of GH regulation in diabetes, but the evidence for the hypothesis that GH is directly involved in the development of diabetic microangiopathy, and in particular, diabetic retinopathy, remains inconclusive.

With the appreciation that the growth promoting actions of GH are mediated by the somatomedins, in particular insulin-like growth factor -1 (IGF-I), and with the availability of specific assays for their measurement, it has become possible to look at circulating levels of this growth factor in diabetics with retinopathy. The results of studies to date have been conflicting and no prospective measurements have been reported. In this work, studies of IGF-I levels will be described in diabetics with varying severity of retinopathy and in patients followed prospectively over two years.

The results of these studies will determine if levels of IGF-I are elevated in diabetics with retinopathy, and in particular, if an increase in concentration precedes the development of proliferative retinopathy, i.e. new vessel formation. The relation between IGF-I levels and retinopathy changes will be further explored in patients treated by continuous subcutaneous infusion in whom transient deterioriation of retinopathy commonly occurs.

More direct evidence for a role of GH in the development of diabetic retinopathy will be sought by the selective suppression of GH in patients with retinal complications. The effects of cholinergic antagonists and the long acting somatostatin analogue, octreotide, on GH secretion and on the appearance of the retina will be examined.

A major problem with the GH hypothesis is the absence of retinopathy in non-diabetic acromegalic patients. It is now appreciated that retinal extacts contain highly potent angiogenic growth factors, allowing a more refined version of Lundbaek's original GH hypothesis to be postulated; GH can now be envisaged as interacting either directly or indirectly via IGF-I, with retinal derived growth factors liberated by the diseased diabetic retina, to promote the process of new vessel formation. Local concentrations of these growth factors will therefore be investigated.

The levels of basic fibroblast growth factor, the more potent of the retinal derived growth factors, and IGF-I, will be investigated in vitreous samples of patients undergoing vitrectomy and in the media conditioned by cultured retinal endothelial cells.

Turning to possible causes of GH hypersecretion in diabetes, various aspects of the regulatory pathways involved in the control of GH release, will be assessed. GH feedback inhibition in diabetic patients will be investigated. In addition, the pituitary response to galanin, a recently described neuropeptide which stimulates GH secretion, will be studied in diabetics with retinopathy.

The aim of this work is thus twofold; (1) to examine the hypothesis that GH and tissue growth factors are important in the development of diabetic retinopathy, particularly in new vessel formation, and (2) to investigate the mechanism of excessive GH secretion in diabetes. In chapters 1-3, the literature is reviewed, beginning with epidemiological and clinical evidence implicating these growth factors in the pathogenesis of diabetic retinopathy.

CHAPTER 1: Growth hormone as a factor in the pathogenesis of proliferative diabetic retinopathy

1.1 Introduction

The most frequent microvascular complication of insulin dependent diabetes is retinopathy (Krolewski et al., 1987). After a lag period of about four years, the risk of background retinopathy, that is microaneurysms, exudates and haemorrhages, increases rapidly with the duration of diabetes, so that after 14 years of diabetes almost all patients whose diabetes was diagnosed before the age of 30 years, have some background changes (Krolewski et al., 1986). By contrast, the risk of developing proliferative retinopathy is minimal in the first 10 years of insulin-dependent diabetes, but rises abruptly after 10-15 years and thereafter remains constant for the next 25 years, at a rate of 3/100 previously unaffected patients/year (Figure 1.1) (Krolewski et al., 1987).

Changes in concentration of peptide hormones, in particular growth hormone (GH) and its mediator of growth promoting activity - insulin-like growth factor I (IGF-I) (section 2.2), could potentially contribute to this interesting epidemiological pattern as well as explain a number of related clinical observations.

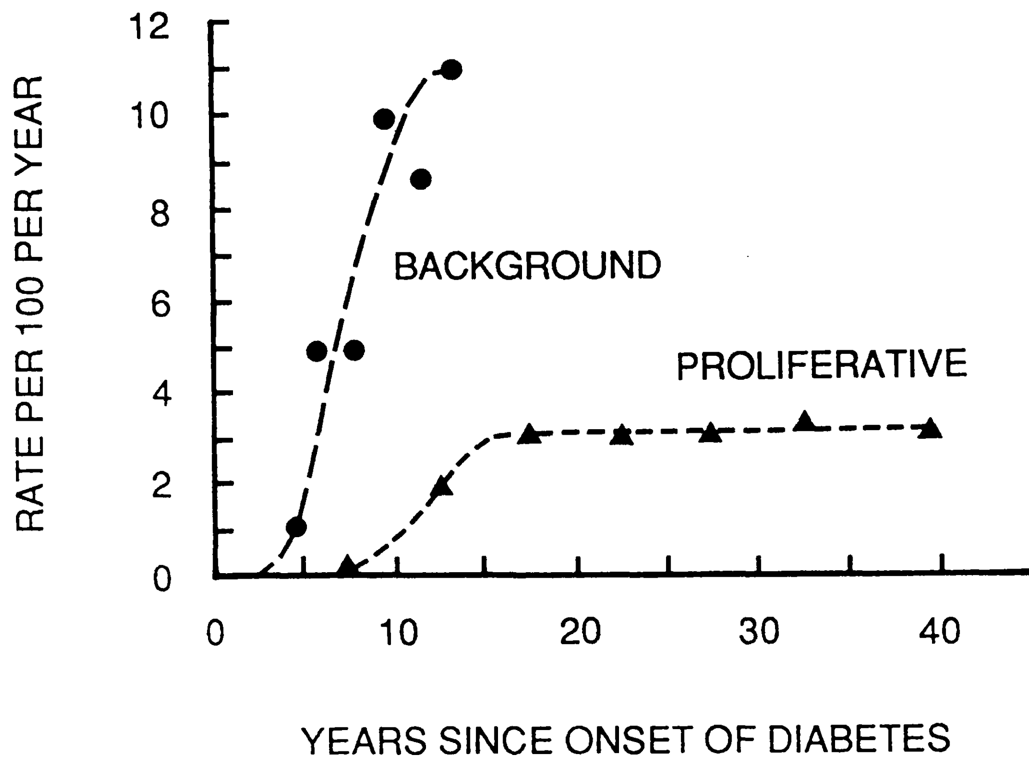


Figure 1.1 Incidence rate of background and proliferative retinopathy according to duration of Type I diabetes (Reproduced from Krolewski et al, 1987).

These include the relation of proliferative retinopathy and puberty (Frank et al., 1982), the relative frequency of proliferative retinopathy in insulin-dependent diabetes (IDDM) compared to non-insulin dependent diabetes (NIDDM) (Klein et al., 1984a; Klein et al., 1984b), the transient worsening of background retinopathy when glycaemic control is tightened (Lauritzen et al., 1983; Kroc Collaborative Study Group, 1984; Dahl-Jorgensen et al., 1985) and the effects of pituitary ablation and GH deficiency on diabetic retinopathy (Kohner et al., 1976; Sharp et al., 1987; Merimee, 1978).

1.2 GH/IGF-I and the epidemiology of diabetic retinopathy

(1) The relation of proliferative retinopathy and puberty

Although it is possible to demonstrate retinopathy by fluorescein angiography in diabetic children (Kohner, 1982), proliferative retinopathy is rare before the age of puberty regardless of duration of diabetes (Palmberg et al., 1981; Frank et al., 1982). After puberty, diabetics particularly aged 15-25 years are susceptible to a rapidly progressive 'florid' proliferative retinopathy which untreated leads to blindness within approximately 2 years (Kohner et al., 1976). A slower progressive proliferative retinopathy may affect patients of any age after many years of diabetes.

The reason for the much more aggressive form of retinopathy in young adults soon after puberty is not known but hormonal mechanisms are likely to be important. Puberty in both normal adolescents and diabetics is associated with a marked increase in GH (Finkelstein et al., 1972; Miller et al., 1982), and IGF-I (Blethen et al., 1981; Salardi et al., 1986), although the increase is less in diabetics (Taylor et al., 1988).

Serum IGF-I may be particularly elevated in the rapidly accelerated form of retinopathy, disproportionately raised in relation to GH levels (Merimee et al., 1983). It is possible that sex hormone changes at puberty may further potentiate the growth promoting effects of GH and/or IGF-I.

(2) The relative frequency of proliferative retinopathy in IDDM vs NIDDM

Patients with non-insulin dependent diabetes (NIDDM) have a lower prevalence of proliferative retinopathy compared to patients with insulin-dependent diabetes (IDDM) of similar duration (Klein et al., 1984a; Klein et al., 1984b). Decreased GH secretion in NIDDM would be predicted by the GH hypothesis to explain this lower prevalence. Since NIDDM patients are older at diagnosis of diabetes compared to IDDM patients and hence older for a similar duration of diabetes, and since GH levels diminish with age (particularly after age 40 years) (Finkelstein et al., 1972), they are indeed likely to have reduced GH secretion.

Furthermore, the association of NIDDM and obesity will also contribute to reduced GH compared to IDDM (Williams et al., 1984; Kopelman et al., 1988). In a preliminary report from the UK Prospective Diabetes Study (Kohner et al., 1987b), all retinopathy in NIDDM at entry was inversely related to body weight. Other mediators, such as hyperinsulinaemia, may also be important.

(3) The pattern of incidence rates of background and proliferative retinopathy and relation with glycaemic control

The rarity of proliferative retinopathy in the first 10 years of IDDM (Krolewski et al., 1986; Klein et al., 1984a) presumably reflects a minimum latent period in which sufficient changes take place in the retinal vessels or their supporting structures, so that neovascularisation can subsequently be induced. By this stage, most patients would have some evidence of background retinopathy. Interestingly, this interval was not related to glycaemic control in the first 5 years of diabetes (Krolewski et al, 1986).

Histological changes taking place at this time include narrowing and occlusion of capillaries and loss of mural pericytes (Cogan et al., 1961; Ashton, 1974). GH could contribute to the process of capillary damage and vascular occlusion in several ways. GH interacts with both the coagulation process and platelet activity, increasing levels of factor VIII related antigen, plasminogen activator and platelet release of prostaglandin E_2 (Colwell et al., 1976; Sarji et al., 1977; Sundkvist et al., 1984).

These changes would favour microthrombus formation and capillary closure. GH also alters the glycosaminoglycan composition of the blood vessel wall (Brosnon et al., 1971), and increases capillary basement membrane thickness (Lundbaek et al., 1970), although the relevance of these latter changes to new vessel formation is not known. There is no evidence in vitro of a stimulatory effect of GH on cultured retinal endothelial cells studied under serum-free conditions (King et al., 1985). This does not exclude a role for GH interacting with serum components or locally produced factors. This will be further discussed in Chapter 7.

Increased GH levels may be present in diabetics with proliferative retinopathy despite very mild carbohydrate intolerance (Barnes et al., 1985) consistent with the lack of an association of proliferative retinopathy with the level of glycaemic control in the first few years of diabetes.

After 10 years, the risk of proliferative retinopathy is no longer related to duration of diabetes but does strongly correlate with the quality of diabetic control immediately preceding the appearance of new vessels (Krolewski et al., 1986). Again, GH can be invoked as one of the possible mediators of the detrimental effects of hyperglycaemia. Poor glycaemic control has long been shown to be associated with excessive GH secretion (Molnar et al., 1972; Hayford et al., 1980).

1.3 Transient worsening of retinopathy with tight glycaemic control

Initial experience with intensified insulin treatment by means of continuous subcutaneous insulin infusion (CSII) suggested that rapid improvement in retinopathy could occur in some patients (White et al., 1981). However subsequent reports (Lawson et al., 1982; Tamborlane et al., 1982) and three controlled trials in early background retinopathy (Lauritzen et al., 1983; Kroc Collaborative Study Group, 1984; Dahl-Jorgensen et al., 1985) have established that transient deterioriation of retinopathy commonly occurs in the first few months of CSII treatment and may be more severe in patients with initially more advanced retinopathy (Lawson et al., 1982).

This initial worsening of retinopathy usually consists of the development of cotton wool spots with haemorrhages and intraretinal microvascular abnormalities but new vessels may also appear (Lawson et al., 1982; Lauritzen et al., 1983). An analagous situation of progressive retinopathy and the development of proliferative changes with improved diabetic control was reported in the treatment of three children with Mauriac's syndrome (stunted growth, hepatomegaly and delayed puberty associated with poorly controlled diabetes) (Daneman et al., 1981). More recently, the rapid development of florid proliferative retinopathy was reported after pancreatic transplantation in a previously poorly controlled young diabetic patient (Ramsay et al., 1988).

The mechanism causing these changes is uncertain. In the Oslo study, patients developing retinal cotton wool spots showed the greatest reductions in glycosylated haemoglobin and blood glucose values (Dahl-Jorgensen et al., 1985). The increase in insulin levels associated with the institution of tight control could promote endothelial cell proliferation as capillary endothelial cells in vitro are stimulated by insulin far more than endothelial cells from large vessels (King et al., 1983). Alternatively, the rise in insulin might divert essential metabolites away from the retina to other insulin-dependent tissues (Forrester, 1987).

Other hormonal changes accompanying intensified insulin treatment include a rise in serum IGF-I concentration within 8-16 weeks of CSII treatment despite a fall in resting and exercise stimulated GH levels over the same period (Tamborlane et al., 1981). In another study (Amiel et al., 1984), serum IGF-I levels rose within one week of commencement of CSII associated with a fall in 24h GH levels. There is experimental evidence that insulin can increase IGF-I production acting on the GH receptor or at a post-receptor site (Baxter et al., 1980b; Maes et al., 1986). It is conceivable that proliferative changes developing during intensified insulin therapy, could result from the increased IGF-I levels induced by insulin.

IGF-I levels may rise with improved diabetic control before the increased GH levels have fallen (Cohen et al., 1988), so that endothelial cells would be temporarily exposed to increased insulin, GH and IGF-I concentrations. Hyperglycaemia itself may inhibit endothelial cell proliferation (Lorenzi et al., 1985). By restoring glucose levels to normal, these cells would be released from this inhibitory control and thus more able to proliferate in response to growth factor stimulation.

Whether hormonal mechanisms can also account for the transient background changes such as the appearance of cotton wool spots during intensified insulin treatment, is not known. Such changes are perhaps more likely to arise from retinal ischaemia consequent upon loss of hyperglycaemia-enhanced retinal blood flow (Kohner et al., 1987c). The possible association of retinopathy changes during CSII and changes in serum IGF-I will be further examined in chapter 6.

1.4 GH deficiency and pituitary ablation effects on diabetic retinopathy

Interest in the possibility that hormones of the anterior pituitary were somehow involved in the development of diabetic retinopathy arose from the case report of a 30 year old woman with poorly controlled diabetes whose retinopathy progressively improved and finally disappeared following post-partum pituitary infarction (Poulsen, 1953).

Since at that time no alternative form of treatment was available for proliferative retinopathy, this observation together with the availability of corticosteroid replacement, prompted trials of hypophysectomy and 90-Yttrium pituitary implantation for the treatment of diabetic retinopathy (Luft et al, 1955; Joplin et al., 1965; Bradley et al., 1965; Oakley et al., 1969; Kohner et al., 1970, Panisset et al.,1971). Over 1000 patients worldwide have undergone pituitary ablation by either hypophysectomy or yttrium implant for the treatment of diabetic retinopathy. Two controlled series (Lundbaek et al., 1969; Kohner et al., 1972) showed rapid amelioration of microaneurysms, haemorrhages and cotton wool spots, but more importantly new vessels, especially those arising from the disc, improved markedly.

This was confirmed in a long term follow-up of patients undergoing pituitary yttrium implants (Sharp et al., 1987). A dramatic and progressive fall in mean disc new vessel grade was found after 5 years and by 10 years, there was no disc neovascularisation in any eye. This study was limited by lack of a control group. Nevertheless, it is clear from this and previous studies that pituitary ablation was greatly beneficial in halting the progression or reversing proliferative retinopathy. Pituitary ablation was particularly effective in the treatment of rapidly accelerating florid proliferative retinopathy (Kohner et al., 1976). It appeared to have no effect on hard exudates or fibrous proliferation (Kohner et al., 1982).

There are two reasons to indicate that it was removal of GH that accounted for the benefit of this procedure in diabetic retinopathy. Firstly, post ablation, all patients received replacement therapy with glucocorticoids, thyroxine and sex steroids without adverse effect on the retinopathy, but were not replaced with GH. Secondly, it was observed that the efficacy of pituitary ablation in the treatment of retinopathy correlated with the degree of completeness of the ablation (Joplin et al., 1967; Adams et al., 1974) and in particular, to the degree of GH deficiency achieved (Wright et al., 1969).

A further piece of evidence supporting the concept that GH is related to the development of microangiopathy comes from a 10-12 year follow-up study of ateliotic GH-deficient dwarfs with diabetes (Merimee, 1978). These dwarfs (aged 39-76 years) with no immunoassayable GH after provocation with arginine or insulin-induced hypoglycaemia, had no detectable retinopathy by direct and indirect fundoscopy, initially and at follow-up. By comparison, 41% of diabetic controls matched for age and sex showed evidence of retinopathy. Although it is probable that the dwarfs had milder diabetes than the diabetic controls (none had elevated fasting glucose levels, integrated blood glucose concentration was lower), this alone could not explain the complete absence of retinopathy at follow-up.

1.5 Clinical studies of GH secretion in diabetics with retinopathy

Soon after a reliable radioimmunoassay for circulating GH became available, it became clear that 24 hour GH concentration was increased in both male and female newly diagnosed insulin-dependent diabetics (Hansen, 1972; Hayford et al., 1980) and that values returned towards normal (although not necessarily completely normal) as good glycaemic control was achieved (Hansen and Johansen, 1970; Hansen, 1973; Arias et al., 1984). Diabetic patients were found to show increased or inappropriate responses to physiological stimuli including exercise (Hansen, 73; Passa et al., 1974), and sleep (Hansen et al., 1981).

Responses to various pharmacological stimuli are also reported to be exaggerated or inappropriate including dopamine (Lorenzi et al., 1980), hypoglycaemia (Powell et al., 1966; Beaumont et al., 1971, Sonksen et al., 1972), glucose (Press et al., 1984a) arginine (Burday et al., 1968; Waldhausl, 1972), clonidine (Topper et al., 1985), TRH (Dasmahapatra et al., 1981; Blickle et al., 1982; Chiodera et al., 1984), LHRH (Giampietro et al, 1986) and insulin in the absence of hypoglycaemia (Sharp et al., 1984a).

Several studies have compared diabetics with and without retinopathy. Thus, higher fasting serum GH concentrations were found in male diabetics with retinopathy compared to age and weight matched males without retinopathy (Knopf et al., 1972) although the groups were not matched for glycaemic control.

Diabetics with retinopathy showed an increased response to moderate exercise compared to a control group of non-retinopathic diabetics (Passa et al., 1974; Sundkvist et al., 1984). A paradoxical rise in serum GH after TRH has been reported in some insulin-dependent diabetics and was more common in diabetics with retinopathy (Dasmahapatra et al., 1981).

The availability of synthetic GH releasing hormone (GHRH), a potent and specific stimulator of GH release in man, has resulted in a plethora of studies in diabetic subjects. Several investigators have demonstrated a normal GH response to GHRH in non-obese insulin-dependent diabetic patients without retinopathy (Press et al., 1984a; Richards et al., 1984; Kaneko et al., 1985; Giampietro et al., 1987; Kopelman et al., 1988) although it has been pointed out by Press et al. (1984a) that the 'normal' GH response is inappropriate for the level of glycaemia since non-diabetic subjects with a comparable blood glucose level show a suppressed GHRH-induced GH response. Indeed, diabetics studied at euglycaemia and during hyperglycaemia, show no significant change in the GHRH response (Sharp et al., 1984b). Normal weight non-insulin dependent diabetics also have normal GH responses to GHRH (Kopelman et al., 1988). Obesity probably accounts for previous reports of diminished GHRH responses in these patients (Richards et al., 1984).

The situation in diabetics with retinopathy is less clear with some studies finding an increased GHRH-stimulated GH response in retinopaths versus non-retinopaths (Kaneko et al., 1985), whilst others demonstrated an exaggerated GH response to GHRH in diabetics with or without retinopathy compared to normal volunteers (Pietschmann et al., 1987; Krassowski et al., 1988). These studies are difficult to interpret, in part because of the difficulties in standardising for glycaemic control. Thus differences in chronic glycaemia can alter the GHRH response despite the test being performed during normoglycaemia (Giampietro et al., 1987).

1.6 Objections to the GH hypothesis

Clearly, hypersecretion of GH, by itself, is not enough to cause retinopathy as non-diabetic acromegalic patients do not develop this complication (Ballintine et al., 1981). Furthermore, a patient with hypopituitarism was recently reported (Rabin et al., 1984) who became diabetic after undergoing total pancreatectomy at the age of 2 years and subsequently developed retinopathy together with nephropathy and neuropathy. This observation clearly challenges the concept that GH is essential for the development of these complications.

As the authors of this report point out, however, the patient only developed early background retinopathy despite severe nephropathy and neuropathy and a duration of diabetes exceeding 24 years, consistent with GH having a permissive effect on the development of proliferative retinopathy (Gerich, 1984).

Undoubtedly, pituitary ablation dramatically benefitted patients with proliferative retinopathy. It is now appreciated that pituitary ablation removes many other important hormones and growth factors besides GH which are not replaced. The anterior pituitary produces prolactin (which may have some GH-like effects), beta-endorphin, lipotrophin, insulin-like growth factors, epidermal growth factor, chondrocyte growth factor, glial growth factor, fibroblast growth factor, as well as plasminogen activators and autostimulatory growth factors (Dieguez et al., 1988a). Even when hormone supplements are given, pituitary hormones are not replaced physiologically and the normal homeostatic mechanisms are lost. Many of these growth factors probably act within the pituitary itself in an autocrine or paracrine fashion regulating somatotroph cell growth (Dieguez et al., 1988a).

GH and IGF-I represent the major circulating mediators of cell growth and remain the most likely candidates to explain the indirect effects of pituitary ablation on diabetic retinopathy. More convincing evidence will be produced if selective GH or IGF inhibition in patients with diabetic retinopathy, can result in similar dramatic effects. This point will be addressed in greater detail in chapters 9 and 10.

The lack of retinopathy in non-diabetic acromegalics, patients with the highest levels of biologically active GH, seems to present the strongest argument against the GH hypothesis. Apart from the absence of hypoinsulinaemia, a major difference in these patients compared to diabetics with retinopathy is the absence of retinal ischaemia. Widespread capillary occlusion and retinal non-perfusion, as demonstrated by fluorescein angiography, characteristically precedes the development of retinal neovascularisation (Kohner et al., 1987a). This reduction of retinal perfusion usually proceeds gradually over many years. It is this local retinal ischaemia which is lacking in the acromegalic patient.

Only after sufficient ischaemia and endothelial cell damage has occurred (as suggested by the epidemiological data), does the presence of GH (directly or via IGF-I) appear to influence the process of new vessel formation.

1.7 Summary

Evidence that GH plays some part in the development of retinal new vessel formation derives principally from clinical studies showing elevated GH levels in diabetics with retinopathy, long term follow-up of diabetic GH-deficient dwarfs who do not develop retinopathy, and the effects of hypophysectomy or Yttrium pituitary implantation in reversing or halting the progression of proliferative lesions.

GH increases coagulability and changes blood vessel wall composition, but appears to have no direct action on retinal endothelial cells. IGF-I, by contrast, is a potent stimulator of these cells. Unfortunately, despite much effort, no animal model of proliferative diabetic retinopathy has been found. The evidence is thus both tantalising and at the same time inconclusive.

If GH is in some way playing a causal role in proliferative retinopathy, as Lundbaek suggested, it is likely that this comes about through interaction with local tissue factors produced or released in the ischaemic retina. In the following chapters, the mechanism of GH hypersecretion in diabetes (chapter 2) and the possible role of tissue growth factors in diabetic retinopathy (chapter 3) will be discussed.

Chapter 2: Mechanisms of excessive secretion of GH in diabetes

2.1 Introduction

The mechanisms mediating excessive GH secretion in diabetes have not yet been clarified. At the level of the pituitary somatotroph, hyper-responsiveness to GH-releasing hormone (GHRH), or relative resistance to somatostatin (SS), failure of negative feedback (GH or IGF-I), or the effect of other peptides (eg Galanin), or metabolites could all increase GH secretion (see Figure 2.1). At the hypothalamic level, decreased secretion of SS or excessive secretion of GHRH, abnormalities in neurotransmitter release or abnormalities in the glucose sensing mechanism could result in excessive or inappropriate GH secretion. In addition to hypersecretion, diminished clearance of GH, may cause elevated GH levels. Evidence for each of the above mechanisms will be discussed below.

2.2 The role of IGF-I in GH hypersecretion

(1) The somatomedin group

The importance of GH in the stimulation of tissue growth has been appreciated for many years (Evans and Long, 1921; Li and Evans, 1944). It was subsequently shown that hypophysectomy in the rat resulted in impaired cartilage growth, as assessed by sulphate incorporation.

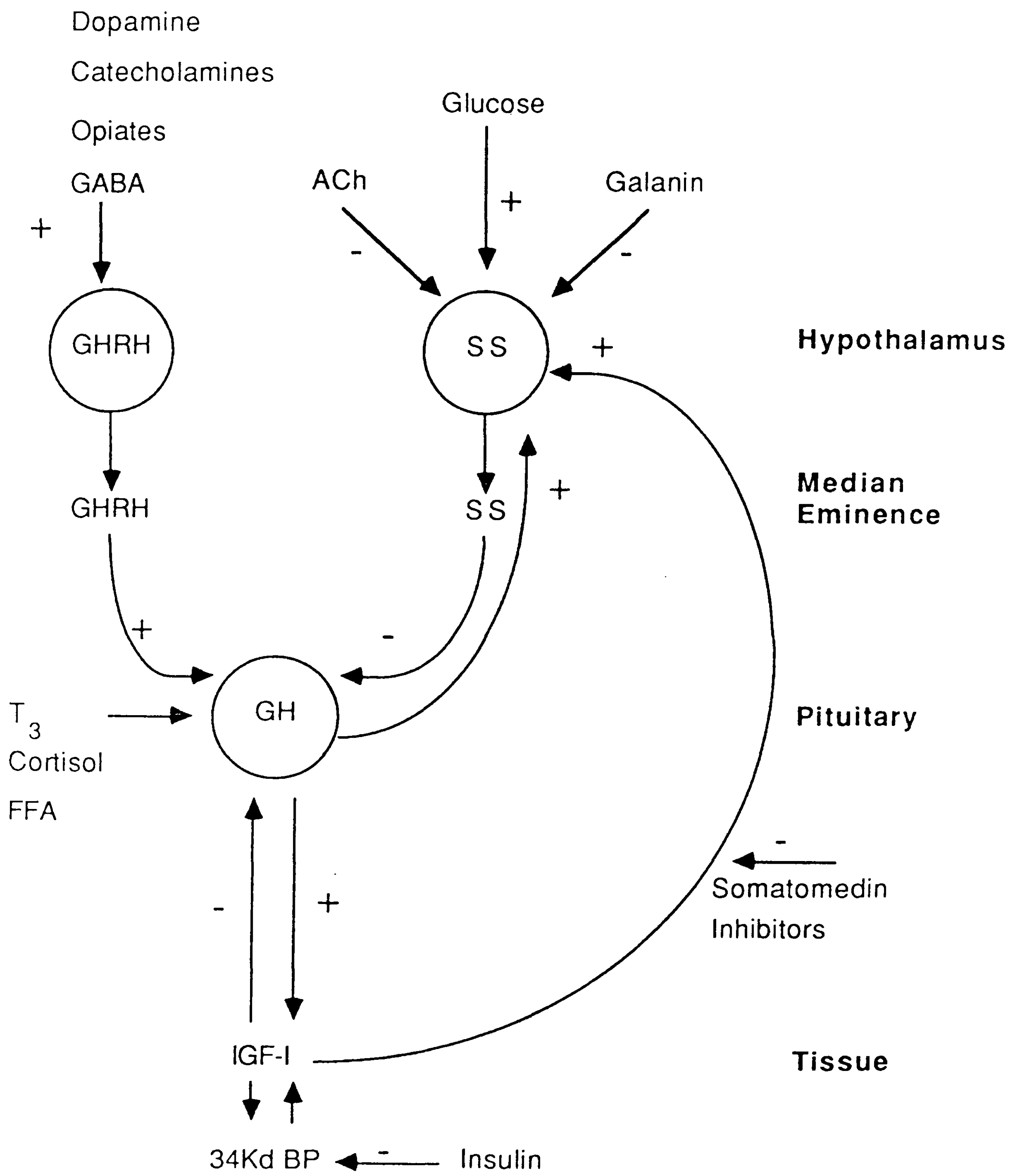


Figure 2.1 The control of GH synthesis and release

Suprisingly, growth could not be restored by the addition of GH (Salmon and Daughaday, 1957). Since serum from normal rats or GH-treated hypophysectomised rats was effective in restoring tissue activity, it was postulated that circulating factors (termed sulfation factors) mediated the growth promoting effects of GH. These were later renamed somatomedins (Daughaday et al., 1972).

Bioassays based on cartilage stimulation, insulin-like activity on fat and stimulation of cell multiplication resulted in the isolation of several factors known as somatomedin A, B, C and MSA (multiplication stimulating activity). Purification, sequencing and immunological characterisation has since reduced the known members of the somatomedin group to two peptides, known as insulin-like growth factor I (IGF-I), identical to somatomedin-C (Klapper et al., 1983), and IGF-II, homologous to at least some of the MSA peptides (Marquardt et al., 1981). Alternative techniques of purification may reveal further distinct insulin-like growth factors in the future.

IGF-I and IGF-II have now been fully sequenced and have primary and tertiary structure similar to that of proinsulin (Blundell et al., 1978). IGF-I is a basic peptide of molecular weight 7649 daltons, containing 70 amino acids in a single chain with three disulphide bridges. It is highly GH dependent and has potent growth promoting activity in a variety of in vitro systems (Van Wyk, 1984; Zapf et al., 1984).

Many of the growth promoting activities previously attributable to GH are now known to be mediated by IGF-I (Teale & Marks, 1986). In the circulation, two immunologically distinct proteins with molecular weights 150kDa and 34kDa bind IGF-I (Drop et al., 1984; Baxter and Martin, 1986). More than 90% of serum IGF-I is bound to the 150kDa binding protein, the production of which is regulated by GH (Wilkins and D'Ercole, 1985).

IGF-II is much less GH dependent and has less growth promoting activity than IGF-I; its physiological role is not known, although current evidence favours a role in foetal development (Underwood and D'Ercole, 1984).

(2) Feedback inhibition on GH secretion

Administration of IGF-I via an implanted cerebroventricular cannula dramatically inhibits spontaneous GH release in normal rats after a lag period of two hours (Tannenbaum et al., 1983; Abe et al., 83). IGF-I acts directly at pituitary level as it has been shown to inhibit GH release and suppress GH mRNA production in cultured rat pituitary cells (Yamashita & Melmud, 1986). In addition, IGF-I acts at hypothalamic level to both increase release of somatostatin (Berelowitz et al., 1981) and decrease GHRH release (Shibasaki et al., 1986).

A similar feedback relationship has not been directly demonstrated in humans. An intravenous injection of recombinant human IGF-I (100 micrograms [mcg]/kg) in normal volunteers induced both hypoglycaemia and a rise in GH similar to an intravenous bolus of insulin (0.15 IU/kg) (Guler et al., 1987). In this study, there was an acute rise in the serum level of free IGF-I capable of acting at either insulin or type-I IGF receptors. Further studies are needed assessing the effect of more prolonged treatment with a lower dose of IGF-I on diurnal GH levels.

(3) IGF-I levels in diabetes

Rats with streptozotocin induced diabetes have low circulating levels of both GH (Tannenbaum et al., 1983) and somatomedins (Baxter et al., 1979; Maes et al., 1983). Studies in human diabetes whilst showing elevated GH levels (see section 1.5) have produced conflicting results regarding circulating IGF-I concentration, reflecting differences in assay techniques employed, small numbers of patients, and the metabolic and nutritional state of the patients studied.

The majority of investigators have found either normal levels (Zapf et al., 1980; Blethen et al., 1981; Tamborlane et al., 1981; Horner et al., 1981; Merimee et al., 1983; Lamberton et al., 1984) or reduced IGF-I levels (Winter et al., 1979; Amiel et al., 1984; Tan & Baxter, 1986) in patients with diabetes.

It has been pointed out that a 'normal' IGF-I concentration is inappropriate for the elevated GH levels and suggests a blunted IGF-I response to GH (Horner et al., 1981, Salardi et al., 1986). Indeed, children with poorly controlled diabetes have been shown to produce lower levels of IGF-I to a standard dose of GH than children with better glycaemic control (Lanes et al., 1985). Impaired IGF-I generation would appear to be a feature of poor diabetic control.

Several studies have shown reduced IGF-I concentration when diabetes is poorly controlled (Winter et al., 1979; Blethen et al., 1981; Rieu & Binoux, 1985; Amiel et al., 1984; Tan & Baxter, 1986). Low levels of IGF-I would be expected to enhance GH release by a reduction of feedback inhibition, thus providing a mechanism for the increased GH secretion at times of poor diabetic control. As IGF-I suppresses the pituitary response to GHRH (Ceda et al., 1987), low IGF-I levels could also explain the exaggerated responses reported to this and other stimuli. As diabetic control is improved, GH responses to some stimuli (Tamborlane et al., 1979a; Topper et al., 1985) may be normalised.

However, it has not always been possible to demonstrate a relationship between glycaemic control (as reflected by glycosylated HbA_1 or mean serum glucose) and circulating IGF-I concentration (Horner et al., 1981; Merimee et al., 1984; Salardi et al., 1986).

Other changes during poor metabolic control could inhibit the functional activity of IGF-I resulting in a blunted feedback inhibition despite normal or only mildly reduced immunoassayable levels. These changes include the rise in the 34kDa binding protein with increasing insulin deficiency (Suikkari et al., 1988) and the increase in levels of 'somatomedin inhibitors' with increasing severity of diabetes (Phillips, 1986).

(4) Somatomedin inhibitors and the 34kDa binding protein

In rats given stepped increases in streptozotocin, somatomedin levels do not decrease appreciably until metabolic control has deterioriated markedly, whereas somatomedin inhibitory activity can be demonstrated with even the lowest dose of streptozotocin when glucose levels are only marginally elevated and ketone levels are normal (Phillips, 1986). Separate studies indicate that this inhibitory activity is due to peptides of apparent molecular weight 20 to 40kDa (Goldstein et al., 1985) but these remain to be fully characterised. It is not clear why GH levels in this diabetic animal model remain low despite markedly elevated levels of somatomedin inhibitors.

Whether such inhibitors operate in human diabetes is not known. If a similar increase in somatomedin inhibitors occurs in patients with only mild diabetes, this could explain the presence of elevated GH levels in diabetics with only mild carbohydrate intolerance and severe retinopathy (Barnes et al., 85).

In another study, however, somatomedin bioactivity was increased in sera from diabetics with retinopathy compared to normal control sera (Ashton et al., 1983). The relation of the somatomedins and in particular IGF-I to diabetic retinopathy will be further explored in chapter 3.

The finding of reduced IGF-I bioactivity in poorly controlled diabetes (Yde, 1969; Winter et al., 1979; Amiel et al., 1984) is consistent with an increase in somatomedin inhibitor levels although one study reported increased bioactivity in adult diabetics with long standing diabetes and high mean blood glucose levels (Cohen et al., 1977).

Recently, serum levels of the 34kDa IGF-binding protein have been investigated in diabetic patients (Suikkari et al., 1988). Compared with normal subjects, mean 34kDa binding protein levels were 4-fold higher in conventionally treated insulin-dependent diabetics and 2.5-fold higher in those treated with continuous subcutaneous insulin. Levels in patients with non-insulin-dependent diabetes were 2-fold higher than normal controls. The authors found no relation between the 34kDa binding protein and IGF-I levels measured by radioimmunoassay, as IGF-I concentration was virtually identical in patients and controls.

A striking inverse correlation between the 34kDa binding protein and insulin levels was demonstrated by clamp studies and by studying insulinoma patients before and after removal of their tumours.

Exactly how insulin influences the levels of the 34kDa binding protein is not understood. Current evidence suggests that insulin regulates the clearance of the binding protein (Brismar et al., 1987; Povoa, 1987) and this may account for the increase in serum levels in insulin-deficient states. Conversely, the acute administration of insulin sufficient to produce hypoglycaemia appears to raise binding protein levels (Yeoh & Baxter, 1988).

The physiological importance of the 34kDa binding protein is not known as both inhibitory and stimulatory effects on the action of IGF-I at the cellular level, have been described (Knauer & Smith, 1980; Ooi & Herington, 1986; Elgin et al., 1987; Rutanen et al., 1988; De Mellow & Baxter, 1988). An intriguing possibility is that the 34kDa binding protein is identical to, or the human equivalent of the serum somatomedin-inhibitor in the diabetic rat model, levels rising with increasing insulin deficiency. The molecular weight of the binding protein (34kDa) is in the same range as the rat somatomedin inhibitor. To date, there have been no reports of studies of the effect of this binding protein on IGF-I feedback inhibition at pituitary and hypothalamic levels.

Increased 34kDa binding protein levels, inhibiting IGF-I feedback mechanisms, could have been present in the patients with mild diabetes and elevated GH values referred to above (Barnes et al., 1985), as these patients also had significantly lower insulin levels compared to control groups.

2.3 Central mechanisms in the GH hypersecretion of diabetes

(1) Defects at the level of the somatotroph

The observation that good diabetic control can normalise 24h circulating GH profiles (Vigneri et al., 1976; Press et al., 1984b) and GH response to exercise (Hansen, 1972; Tamborlane et al., 1979a) implies that the hypothalamic-pituitary axis in diabetes is sensitive to the prevailing metabolic state. This may also imply that the GH abnormalities are secondary to poor glycaemic control. Alternatively, though less likely, improved diabetic control might activate inhibitory mechanisms on GH release, countering the effects of a primary GH abnormality.

No consistent ultrastructural change in the pituitary has been described in diabetes at autopsy (Legg & Harawi, 1985). Although circulating anterior pituitary cell surface antibodies have been reported in recent onset insulin-dependent diabetics (Vercammen et al., 1987), there is no histological evidence of immune destruction of the somatotroph in diabetes.

Pituitary resistance to somatostatin or increased sensitivity to GHRH could provide an explanation for the exaggerated GH responses and increased diurnal GH levels in diabetes. An exaggerated GH response to GHRH is reported in the streptozotocin-diabetic rat (Locatelli et al., 1984) although basal GH levels in these animals are depressed compared to controls.

The majority of clinical studies of GH responses to standard doses of GHRH have been normal (see section 1.5) and GHRH does not appear to mediate GH release to some of the stimuli provoking abnormal GH rises in diabetes such as arginine, (Burday et al., 1968; Page et al., 1988), insulin or clonidine (Tapanainen et al., 1988). Increased GHRH sensitivity alone is unlikely to account for GH abnormalities in diabetes.

Infusions of somatostatin (166 mcg/h) suppress GH responses to exercise, arginine and L-dopa as well as diurnal GH levels in both juvenile and maturity onset diabetic patients (Lundbaek & Hansen, 1980). In another study, GH levels were not completely suppressed by an infusion of somatostatin (150 mcg/h) in one young insulin-dependent patient (Ward et al., 1975). Nocturnal GH surges can be completely abolished in diabetics by intravenous somatostatin (350 mcg/h) (Campbell et al., 1985).

Relative pituitary resistance to somatostatin cannot be excluded from these studies as dose-response relationships were not examined. Very small doses of somatostatin (as little as 2 mcg intravenous pulses) can suppress GH levels in normal subjects and untreated acromegalic patients (LeBlanc et al., 1975). The question of somatostatin resistance will be further considered in chapter 10.

(2) Abnormalities in hypothalamic GH regulation

Evidence derived from measurements of GHRH and SS in rat hypophyseal-portal plasma, suggests that pulsatile GH release from the pituitary results from GHRH surges and decreases in SS secretion from the hypothalamus (Plotsky & Vale, 1985). Very frequent (10 min) blood sampling over 24h in insulin-dependent diabetics (Asplin et al., 1987) indicate that whilst both male and female diabetic patients have greater total GH secretion compared to non-diabetic control subjects, female diabetics produce more frequent GH pulses with similar pulse width and area, whereas male diabetics have normal GH pulse frequency but greater peak amplitude. These data suggest a disturbance at hypothalamic level in diabetes but no attempt was made to correlate observed changes with variation in glycaemic control.

Failure of glucose-mediated GH suppression in diabetic patients (Press et al., 1984a; Sharp et al., 1984b) may also be evidence of a hypothalamic defect in GH regulation. GH suppression by glucose is thought to be mediated by glucose stimulation of hypothalamic somatostatin secretion rather than a direct effect on the pituitary (Press et al., 1984a). The defect in diabetes could lie at the level of the glucose sensing cells or the somatostatinergic neurons leading to inadequate SS release.

The synthesis and secretion of the hypothalamic hormones GHRH and SS are in turn regulated by a number of neurotransmitters and neuropeptides (Dieguez et al., 1988b).

All the classical neurotransmitter pathways including catecholaminergic, serotoninergic and GABAergic have been implicated in GH control. Recently it has been appreciated that hypothalamic cholinergic pathways are of fundamental importance in the control of GH in man. Blockade of cholinergic muscarinic receptors with drugs such as atropine, pirenzepine or propanthelene virtually abolish the GH responses to many stimuli including L-dopa, apomorphine, arginine, physical exercise, slow wave sleep, clonidine, and GHRH (Delitala et al., 1983; Casanueva et al., 1984; 1986a; Massara et al., 1986; Davis & Davis, 1986; Jordan et al., 1986; Peters et al., 1986).

It is not clearly established if these drugs are acting at muscarinic receptors in the median eminence to block the inhibitory effect of acetylcholine on somatostatin release from the hypothalamus (Richardson et al., 1980; Casanueva et al., 1986b) or are exerting a direct effect at pituitary level (Young et al., 1979) since muscarinic receptors are present at both sites (Burt & Taylor, 1980; Casanueva et al., 1983). Supporting the concept that cholinergic pathways modulate SS release, antisomatostatin antiserum in normal rats abolishes the inhibitory effect of atropine on GHRH-stimulated GH release (Locatelli et al., 1986). Hypoglycaemia-induced GH release is not inhibited by cholinergic blockade (Blackard & Waddell, 1969; Evans et al., 1985) indicating that falling glucose concentrations are able to override the inhibitory cholinergic pathway.

An abnormality in the cholinergic control of GH release has not been demonstrated in diabetes, although to date, only acute studies have been reported. GH release during slow wave sleep is abolished in insulin-dependent diabetic patients by the acute administration of pirenzepine at the same doses as non-diabetic normal volunteers (Peters et al., 1986; Page et al., 1987). A single bedtime dose of propanthelene also significantly reduced nightime GH secretion in diabetic patients (Davis & Davis, 1986). GHRH-stimulated GH release in diabetics, as in normals, is inhibited by pretreatment with atropine (Pietschmann et al., 1986). In addition, the abnormal GH release by TRH in insulin-dependent diabetics, is abolished by atropine (Chiodera et al., 1984).

If the assumption is correct that treatment with cholinergic antagonists causes release of endogenous somatostatin resulting in suppression of GH secretion, then the above studies would suggest that the pituitary in diabetes is normally sensitive to somatostatin and that GH hypersecretion occurs because of failure of somatostatin release. It is possible that release of SS by these agents is large enough in acute studies to overcome relative pituitary insensitivity. GH suppression with atropine and propanthelene will be further discussed in chapter 9.

(3) Neuropeptides and GH regulation

A number of peptides, originally isolated from the gastro-interstinal tract and subsequently found in the hypothalamus, have been shown to modify GH secretion in some experimental models but their physiological role is unknown (Dieguez et al., 1988). Their action is usually not specific for GH since other hormones are also affected.

A notable exception is the 29 amino acid peptide Galanin, so called because it possesses a **g** lycine residue at the N-terminal end and **alanine** at the C-terminal end (Figure 2.2) (Tatemoto et al., 1983). Galanin bears very little resemblance to other peptides and seems to be the first member of a new peptide family. Galanin mRNA appears to encode a 123 amino acid precursor protein, known as preproGAL (Figure 2.2) (Rokaeus, 1987) which contains a pre-galanin message peptide and a galanin message-associated peptide. The production and secretion of these larger petides is not yet established and the regulation of the galanin message is at present unknown.

When infused into the rat third cerebral ventricle, galanin unlike other neuropeptides, elicited a dose-related selective rise in plasma GH (Ottlecz et al., 1986) with doses as low as 50 picomoles of galanin producing a significant rise in GH levels. Perifusion of dispersed anterior pituitary cells with galanin had no effect on GH release suggesting that it is acting at hypothalamic level.

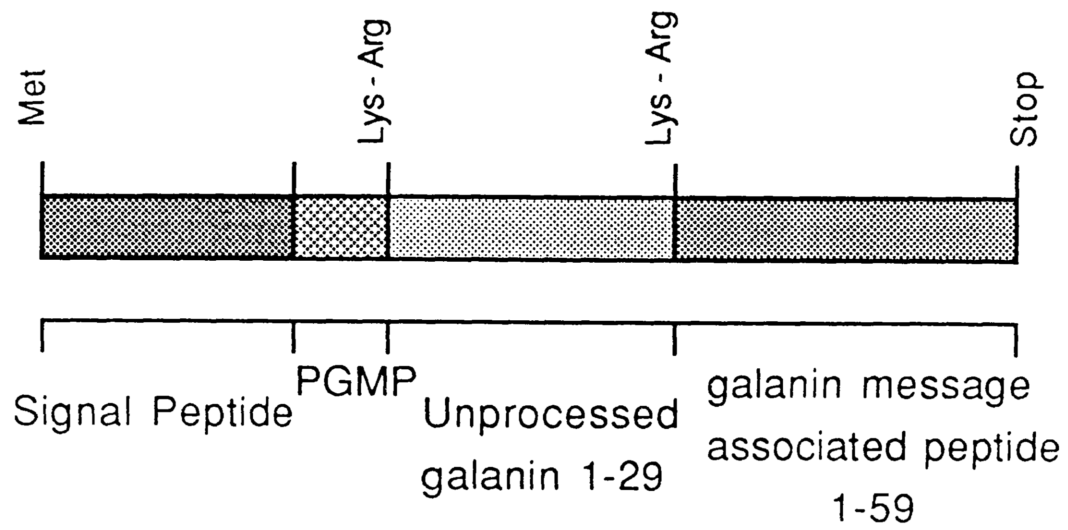


Figure 2.2 Schematic representation of the porcine galanin gene product.

High concentrations of galanin and specific galanin binding receptors have since been reported in the hypothalamus (Rokaeus et al., 1984; Servin et al., 1987) which is likely to be the principal site of action. Catecholamine pathways seem to be important as specific inhibitors of adrenaline synthesis abolish GH release by galanin (Cella et al., 1988). Recently, intraventricular injection in rats of a highly specific antiserum against galanin was shown to markedly reduce plasma GH levels suggesting that galanin stimulation of GH release is of physiological significance (Ottlecz et al., 1988).

Intravenous infusion of synthetic porcine galanin has been shown to stimulate GH release in normal male volunteers (Bauer et al., 1986a). Furthermore, galanin increases 3-fold the GH response to a supramaximal dose of GHRH (Davis et al., 1987). It therefore seems unlikely that GHRH alone mediates GH release by galanin in man. More recently high dose infusions of galanin have been shown to partially overcome suppression of GH release by atropine or pirenzepine (Chaterjee et al., 1988). These results could indicate that galanin reduces somatostatin release and that at high doses of galanin, the rise in somatostatin induced by cholinergic antagonists is insufficient to correct this reduction in somatostatin secretion.

It is conceivable that excessive galanin production or release could underly GH hypersecretion in diabetes perhaps by reducing somatostatin tone. The pituitary response to galanin in diabetics with retinopathy will be examined in chapter 12.

2.4 Abnormalities in circulating growth hormone forms and GH bioactivity

In addition to abnormalities in central regulation of GH and IGF-I feedback inhibition, circulating GH forms and GH biological activity have been examined in diabetic sera (MacFarlane et al., 1986a; MacFarlane et al., 1986b). It is possible that diabetic patients release biologically inactive GH which then fails to inhibit further GH secretion (see Figure 2.1) resulting in high immunoassayable GH levels. Diminished clearance of GH due to an excess of high molecular weight GH forms could also contribute to higher GH levels.

Circulating GH can be separated by Sephadex chromatography into three distinct forms known as 'little' or monomeric GH (molecular weight approx 20kDa), 'big' or dimeric GH (molecular weight approx 40kDa), and big-big or oligomeric GH (molecular weight greater than 60kDa) (Stoler et al., 1984).

The physiological importance of the different GH forms is not fully understood but in general, monomeric GH exhibits greater receptor binding activity and biological potency compared to the larger forms (Gorden et al., 1973, 1976). Monomeric GH is also cleared from the circulation at a faster rate than the larger GH forms (Hendricks et al., 1985). The proportion of circulating monomeric GH forms in poorly controlled diabetics is slightly higher than that found in stimulated normal subjects and slightly lower than that found in acromegalic subjects (MacFarlane et al., 1986a).

In the same study, there was no relationship between the proportions of GH forms and diabetic control (as judged by HbA_1 concentrations) or the presence of microvascular complications.

The discovery of specific GH binding proteins (Herington et al., 1986; Baumann et al., 1986, 1988) provides a possible explanation for the increase in monomeric GH in the diabetic and acromegalic patients, since the relative proportion of bound GH progressively falls and free GH rises, as GH concentration increases (Baumann et al., 1988). It is now thought likely that most if not all of the big-big GH represents GH bound to its binding protein, and the monomeric GH corresponds to the unbound fraction (Holly et al., 1988). Changes in binding protein levels in diabetes could influence GH binding to receptors and hence bioactivity but no such changes have yet been identified.

When bioactivity was assessed in a group of insulin-dependent diabetics using a pregnant rabbit liver radioreceptor assay (RRA), the majority of diabetics had undetectable GH by RRA (MacFarlane et al., 1986b). However four diabetics had RRA concentrations greatly in excess of the levels measured by RIA with no change in the proportions of the different GH forms. This finding prompted the authors to speculate that at times, highly bioactive GH is secreted in some diabetics. It is not possible to determine from this study whether bioactivity is reduced in the majority of patients compared to normal subjects, as the assay was insensitive to GH-RIA concentrations less than 20 mU/l.

Analysis of human pituitary gland extracts has shown that GH may also exist in a glycosylated form accounting for 3-5% of main GH protein (Sinha & Lewis, 1986). This GH form may be relatively bio-inactive (Sinha & Jacobsen, 1987). A greater proportion of GH in the glycosylated form might be expected in diabetes but this has not been reported.

Finally, whilst a reduction in GH clearance has been demonstrated in some studies in diabetic subjects by infusion of ^{131}I-labelled GH (Lipman et al., 1972; Sperling et al., 1973), although not in others (Navalesi et al., 1975), this would not explain the greater number of GH peaks in diabetes. Indeed, the increase in GH in diabetes appears to be in the fasted cleared GH form. The increase in GH and its responses to various stimuli are likely to reflect increased pituitary output (Holly et al., 1988).

2.5 Summary

Diabetic control greatly influences GH abnormalities in diabetes. Poorly controlled diabetic children show stunted growth despite high GH levels, suggesting that the GH released lacks growth promoting activity. Whilst no particular GH form is associated with poor glycaemic control, somatomedin generation is impaired and IGF-I levels are low when control is poor, possibly as a result of a reduction in GH receptors induced by insulin deficiency. Reduced IGF-I activity is likely to be an important stimulus to GH secretion. Other mechanisms such as increased sensitivity to GHRH and resistance to somatostatin have not been conclusively demonstrated. Failure of glucose mediated GH suppression in diabetes is suggestive of a hypothalamic defect in GH regulation, perhaps mediated by one of the newly discovered hypothalamic neuropeptides, such as galanin. This is not merely an academic question, since an understanding of these mechanisms is essential if successful suppression of GH levels is to be achieved in the treatment of diabetic retinopathy. Before discussing the results of clinical studies seeking to address some of the issues raised in this chapter, the role of growth factors in the development of retinal vascular proliferation will be considered.

Chapter 3: Tissue growth factors in diabetic proliferative retinopathy

3.1 Retinal ischaemia and release of a vasoproliferative factor

Although the stimulus for proliferation in diabetic retinopathy is not known, it has long been appreciated that large areas of capillary non-perfusion in the peripheral retina predispose to new vessel formation (Kohner et al., 1982). Widespread retinal capillary occlusion precedes the rapidly progressive 'florid' type of retinopathy as well as the more common slowly progressive form of diabetic proliferative retinopathy (Kohner et al., 1982). The association of new vessel formation to areas of capillary closure lead to the hypothesis that the non-perfused ischaemic retina liberates an angiogenic factor which stimulates the normally quiescent retinal vasculature to proliferate (Michaelson, 1948, Wise, 1956). The nature of this putative factor (or factors) is the subject of much current research.

Several clinical observations provide indirect evidence for the presence of an angiogenic factor released from the diseased retina in diabetes. Thus new vessels on the optic disc or elsewhere in the retina will regress after laser photocoagulation directed not at the new vessels themselves but in the peripheral retina. Even new vessels on the iris (rubeosis iridis) will regress after panretinal laser therapy.

By contrast, direct laser treatment to proliferative lesions is unnecessary and not always successful (Frank, 1986). This suggests that photocoagulation produces its beneficial effect not by directly sealing the abnormal vessels but by an indirect effect on the diseased retina.

Extensive (panretinal) laser treatment could destroy new vessels by stopping the release of stimulatory growth factor(s) from large portions of the retina. Alternatively, cells destroyed or damaged by laser treatment may release one or more inhibitors of neovascularisation (Glaser, 1988). The forward diffusion of an angiogenic growth factor could account for neovascularisation behind the lens in patients with severe proliferative retinopathy, and for the growth of iris new vessels after lens removal and vitrectomy (Blankenship et al., 1979).

Lack of an appropriate animal model has hampered attempts to provide direct confirmation of the role of angiogenic factors in the pathogenesis of diabetic proliferative retinopathy. Attention has therefore turned to in vitro studies examining factors controlling growth and proliferation of the retinal microvascular endothelium.

3.2 Pericytes and endothelial cells in proliferative retinopathy

Retinal capillaries consist of an inner lining of endothelial cells and an outer cap of pericytes separated by a specialised basement membrane. In mature capillaries, the endothelial cells are normally stable with a very low turnover rate as shown by incorporation of ^{3}H-thymidine into fewer than 0.1% retinal vascular cells after intravitreal injection of label (Engerman et al., 1967; Archer, 1983). In diabetic retinopathy, there is early loss of capillary pericytes long before proliferative retinopathy appears (Cogan et al., 1961; Speiser et al., 1968). Widespread capillary closure precedes endothelial cell proliferation; the proliferating endothelial cells form fragile new vessels which lack connective tissue support and are liable to bleed (Kohner, 1982).

Later, fibrous tissue appears around the new vessels as a result of fibroglial proliferation and may eventually obliterate the new vessels. Vision may be threatened by haemorrhage from the new vessels, or by traction retinal detachment as the fibrous tissue contracts. Proliferative retinopathy therefore involves loss of capillary pericytes, and proliferation of retinal endothelial cells and later, fibroblasts.

Because of its contractile properties, the pericyte has long been regarded as a regulator of microvascular flow as well as being important in the maintenance of the structural integrity of the capillary (Sims, 1986; Kelley et al., 1987).

More recently, in co-culture experiments in which endothelial cells were allowed to make contact with pericytes, endothelial growth was totally inhibited (Orlidge & D'Amore, 1987; D'Amore & Orlidge, 1988). Pericytes inhibited endothelial cell growth at endothelial cell to pericyte ratios of 1:1 up to 1:10. This effect was cell-specific in that fibroblasts, epithelial cells and 3T3 cells did not inhibit endothelial cell proliferation. In another set of experiments, pericytes and endothelial cells were co-cultured in a system that prevented contact but allowed the exchange of diffusable factors. Under these conditions, there was no inhibition of endothelial cell growth (D'Amore & Orlidge, 1988). These observations indicate that pericytes suppress endothelial cell growth by contact inhibition although the mechanism of this inhibition is still not clear. In diabetic retinopathy, the early loss of pericytes will encourage endothelial cell proliferation.

3.3 Competence and progression factors

Cellular proliferation requires a cell to progress from a quiescent (G_0) state through the first growth phase (G_1) and phase of DNA synthesis (S phase) to a second growth phase (G_2) and hence to cell division (Figure 3.1). During G_1 and G_2 phases, there is synthesis of structural and catalytic proteins. In recent years, several growth factors have been identified with differing effects on the cell cycle. Those factors which allow the cell to enter G_1 are known as competence factors (Van Wyk et al., 1981).

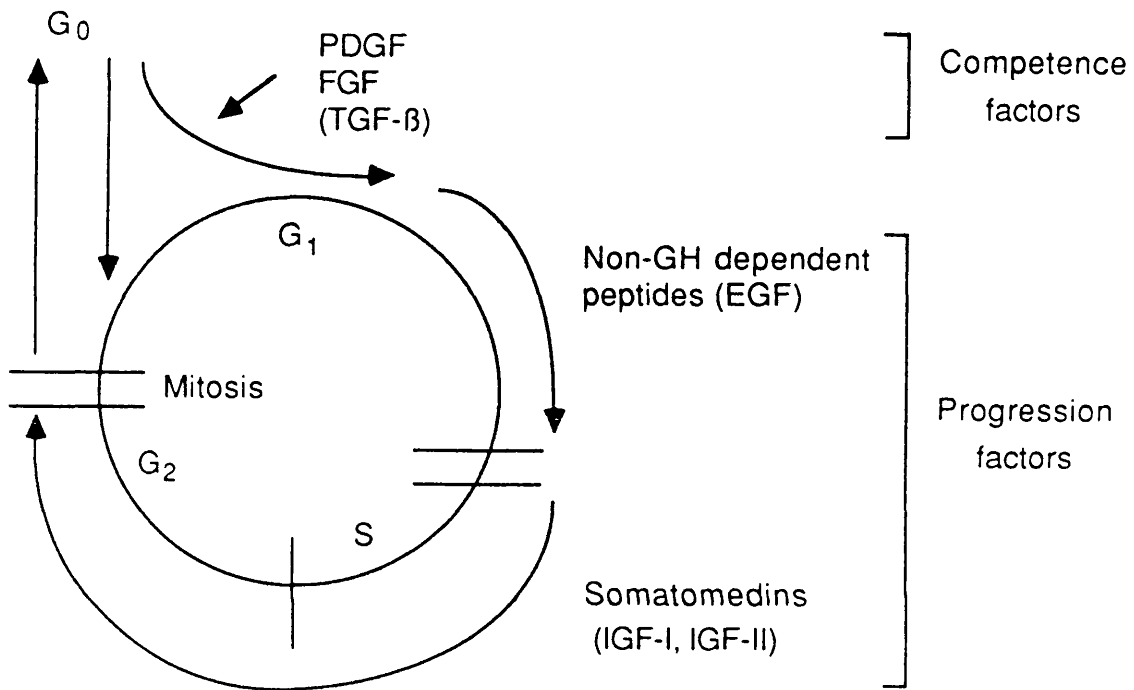


Figure 3.1 The cell cycle: site of action of tissue growth factors.

PDGF: Platelet derived growth factor **FGF**: Fibroblast growth factor

TGF-B: Transforming growth factor-B **EGF**: Epidermal growth factor

IGF: Insulin-like growth factor

By themselves, they are incapable of allowing the cell to synthesize DNA and divide. Futher advance through the cell cycle requires the addition of 'progression factors.' Cells that are not competent cannot respond to the progression factors and therefore cannot complete the cycle to cell division (Pledger et al., 1978). It must be borne in mind that the same growth factor can act as either a competence or a progression factor depending on the cell type. The BALB/c 3T3 mouse fibroblast has been extensively used for studying the cell cycle in relation to the various growth factors (Van Wyk et al., 1981) and it is not known whether the retinal endothelial cell behaves in the same way.

3.4 IGF-I and endothelial cell proliferation

IGF-I is capable of stimulating DNA synthesis and cell proliferation in a wide variety of cell types including retinal endothelial cells (King et al., 1985) and human fibroblasts (Clemmons and Van Wyk, 1981). It is therefore of great interest in the pathogenesis of the vascular and fibrous proliferation in diabetic retinopathy. The stimulatory effect of serum-free IGF-I on DNA synthesis in retinal endothelial cells was evident at concentrations greater than 1 nM, (equivalent to greater than 7.5 mcg/l), with a half maximal response of 75 mcg/l (King et al., 1985)

Free IGF-I levels in normal serum (26 $\pm$ 8 mcg/l) (Guler et al., 1987) would therefore be capable of stimulating these cells albeit at the lower end of the dose-response curve. Vitreous levels of IGF-I in diabetics with proliferative retinopathy (6.3 $\pm$ 0.93 mcg/l; Grant et al., 1986) are less than the minimum concentration required for cell stimulation in tissue culture. However, the predicted vitreous levels in patients with rapid accelerating retinopathy (20-30 mcg/l) (Grant et al., 1986a) would certainly be stimulatory, if the IGF was largely in the free form.

There are several possible reasons why the situation in vivo will be different. At circulatory level, several other progression factors in serum may potentiate the effect of IGF-I on endothelial cell proliferation such as low density lipoproteins or transferrin. The effect of serum added to a suboptimal dose of IGF-I was not tested in King's study (King et al., 1985). In addition, serum IGF-I levels in diabetics with proliferative retinopathy may be increased (Merimee et al., 1983) or may rise transiently at the time of proliferation. This point will be discussed further in chapter 5.

At the level of the endothelial cell, retinal derived growth factors such as fibroblast growth factor (FGF; see below) may enhance IGF-I effects. In rat aortic cells, binding of IGF-I to cell membrane receptors and the growth promoting effects of IGF-I are increased after cells are made competent with FGF (Pfeifle et al., 1987).

It is not known if this also applies to retinal endothelial cells. Human capillary endothelial cells are also able to synthesize IGF-I binding proteins which may modulate their response to exogenous IGF (Bar et al., 1987a).

3.5 IGF-I and the process of angiogenesis

In addition to endothelial cell proliferation, another important component of new vessel formation is endothelial cell migration. Proliferation of cells occurs only after active migration has begun (Ausprunk & Folkman, 1977). Endothelial cells are able to migrate through vessel walls following degradation of the basement membrane by proteases (Folkman & Klagsbrun, 1987).

It is therefore of great interest that IGF-I concentrations as low as 4 mcg/l were chemotactic to retinal capillary endothelial cells and that this effect of IGF-I was potentiated by serum (Grant et al., 1987). The same group have also shown that IGF-I increases collagenase production by retinal endothelial cells (Grant et al., 1986b) which is important in the degradation of the basal lamina. IGF-I also influences the synthesis of proteoglycans which are important components of vessel walls and extracellular matrix. In cultured capillary endothelial cells, IGF-I appeared to preferentially increase synthesis of heparan sulfate proteoglycans (Bar et al., 1987b).

Although the significance of this observation is not known, increased heparan sulfate proteoglycans within basement membranes will bind a group of growth factors along the cell membrane which may promote cell proliferation. This group of growth factors which exhibit a high affinity for heparin, appear to play a central role in the control of new vessel growth.

3.6 The heparin-binding growth factors

(1) Retinal derived growth factor and its relation to fibroblast growth factors

In 1980, Glaser and colleagues (Glaser et al., 1980) demonstrated that an extract of human or animal retina, made by simply soaking freshly dissected retina in a balanced salt solution, followed by filtration and centrifugation of the resulting suspension, was able to stimulate bovine endothelial cells in a dose-dependent manner, and produced a neovascular response on the chick chorioallantoic membrane. Moreover, the extract had some cell specificity, since it stimulated endothelial cells and fibroblasts but not smooth muscle cells.

The same group partially purified a 50-100kDa protein from bovine retina that exhibited angiogenic activity (D'Amore et al., 1981). The discovery that many endothelial cell growth factors had a strong affinity for heparin made it possible to increase purification several thousandfold using heparin-sepharose affinity chromatography.

By this means, the growth factor in the retinal extract, known as retinal derived growth factor (RDGF), was further purified to a protein of molecular weight 18K daltons (D'Amore & Klagsbrun, 1984). It appeared to be an anionic polypeptide which stimulated human and bovine endothelial cells but not smooth muscle cells. Examination of its molecular weight, isoelectric point and cell specificity led the authors to propose that it was identical to another endothelial cell mitogen derived from hypothalamus, known as either hypothalamic endothelial cell growth factor (ECGF) (Maciag et al., 1979) or brain acidic fibroblast growth factor (acidic FGF) (Gospodarowicz, 1975).

When retinal extracts were made by homogenising bovine retinae and purifying the extract before applying to a heparin-sepharose affinity column, two growth factors (aRDGF and bRDGF) were isolated from retina (Baird et al., 1985a). By studying cross-reactivity to specific antisera and amino-acid sequence analysis, the anionic growth factor (aRDGF) was again found to be identical to acidic fibroblast growth factor but this contributed to less than 0.15% of the bioactivity of the retinal extract. The majority of the bioactivity of the original extract was found to be due to the cationic bRDGF, identical to basic fibroblast growth factor (bFGF). It is possible that acidic fibroblast growth factor (aFGF) is present in retina in a more releasable form so that it accounts for most of the bioactivity of RDGF when prepared from retinae left soaking in a physiological buffer. It is also likely that the fractionation procedures used in the earlier studies precluded the isolation of the cationic growth factor.

(2) The structure of basic FGF

It is now appreciated that all the heparin-binding endothelial cell growth factors can be subdivided into two classes; (1) the anionic Class I Heparin Binding Growth Factors which are identical to, or extended/truncated forms of acidic FGF and (2) the cationic Class II Heparin Binding Growth Factors which are identical to, or different forms of basic FGF (Gospodarowicz et al., 1987a; D'Amore & Thompson, 1987). Both FGFs are single chain polypeptides with molecular weights of approximately 16kDa. The widespread distribution and wide range of cell types on which it acts, have given rise to at least 16 synonyms for bFGF (Gospodarowicz et al., 1987a). There is a 55% sequence homology between the acidic and basic forms of FGF.

The FGF genes have now been cloned and the genomic organisation has been described (Abraham et al., 1986). As shown in Figure 3.2, the bFGF gene localised on chromosome 4, contains 3 exons giving rise to 2 mRNAs (Gospodarowicz et al., 1987b). Interestingly, although high concentrations of bFGF can be extracted from many tissues, the level of mRNA in these tissues is very low (Abraham et al., 1986). It is suggested that although synthesis of bFGF is low, the peptide is very stable and accumulates in the tissues bound to matrix components.

The gene product is a 155 single chain amino-acid precursor which is cleaved further to the mature protein (146 amino-acids) or a truncated form (131 amino-acids) (Figure 3.2). The physiological importance of the various forms of bFGF found in different tissues is not known.

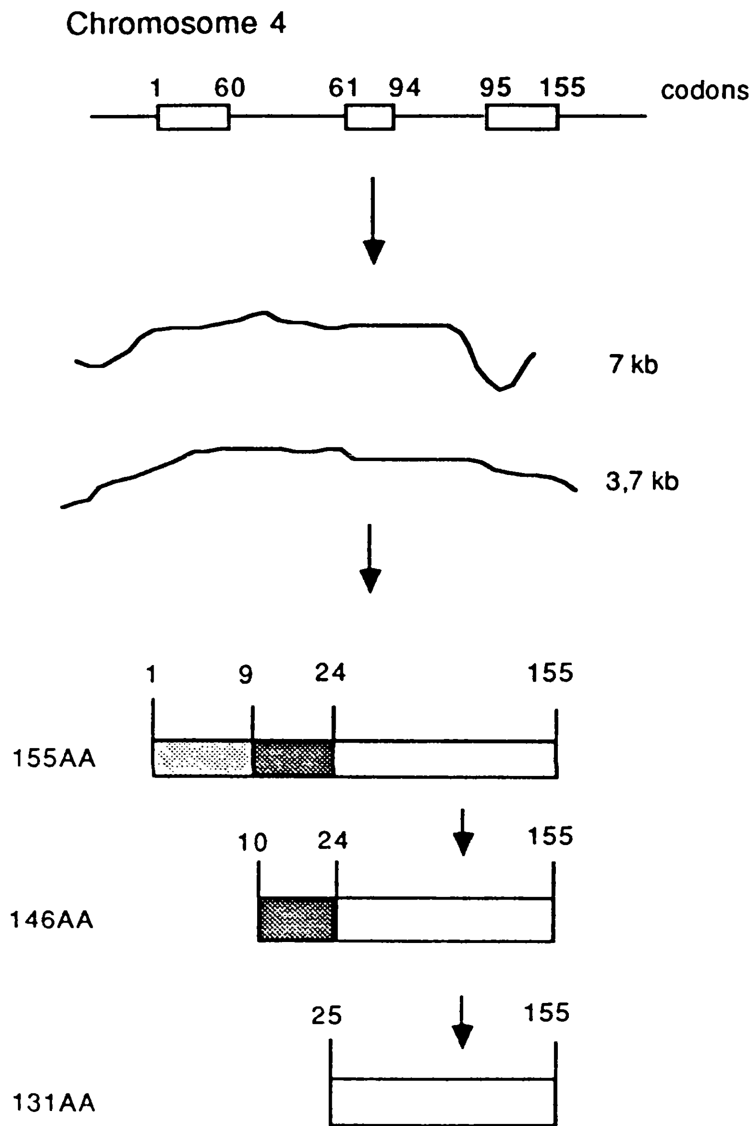


Figure 3.2 Schematic representation of the bFGF gene and gene products

(3) Basic FGF and endothelial cell proliferation

In contrast to aFGF which has only been reported in neural tissue, bFGF is ubiquitous having been found in virtually all normal tissues (Gospodarowicz et al., 1987a). Both growth factors are potent mitogens to many cell types including capillary endothelial cells and fibroblasts.

Retina-derived bFGF in the presence of serum was shown to cause proliferation in retinal capillary endothelial cells with a half maximal stimulation at a concentration of only 13 pg/ml (Gospodarowicz et al., 1986). It appeared a hundredfold more potent in stimulating these cells than aFGF. In this and other studies, bFGF acts as a competence factor, being unable to stimulate DNA synthesis without the addition of serum or plasma (Gospodarowicz et al., 1984). High density lipoproteins and transferrin can act as progression factors with retinal capillary cells made competent by bFGF (Gospodarowicz et al., 1986). It is likely that IGF-I could also substitute for the serum requirement in FGF-primed endothelial cells, but this experiment does not appear to have been tried.

Using an antiserum raised against a synthetic peptide (Tyr^{10}) FGF (1-10), Baird et al. (1985b) reported FGF levels in samples of intraocular fluid from 4 patients undergoing fluid exchange procedures for retinal detachment.

Levels in the 3 non-diabetic elderly patients were 0.6-1 pmol/ml whilst patient 4 who was a 57 yr old male diabetic with advanced proliferative retinopathy had a level of 8.9 pmol/ml equivalent to 148 ng/ml. Clearly, if this reflected the concentration of free bFGF and if the results of cultured retinal endothelial cells can be applied to the in vivo situation, then this concentration would produce a supramaximal stimulation of endothelial cells. However, there are now doubts about the validity of this assay due to the presence of aminopeptidase activity in vitreous, serum and retinal extracts which degrades ^{125}I (Tyr^{10}) FGF (1-10) (Gauthier et al., 1987; Brooks & Burrin, 1988). Studies on vitreous with a newly developed bFGF assay are described in chapter 8.

(4) Basic FGF and angiogenesis

Apart from stimulating vascular endothelial cells to proliferate, bFGF stimulates endothelial cell migration (Gospodarowicz et al., 1987a; Sato & Rifkin, 1988) and also appears to be a chemo-attractant for endothelial cells (Connolly et al., 1987). Furthermore, bFGF induces capillary endothelial cells to invade a three dimensional collagen matrix and organise into tubules that resemble blood capillaries (Montesano et al., 1986). It also stimulates the endothelial cells to produce a protease (plasminogen activator) which plays an important part in the degradation of the basal lamina at the onset of neovascularisation (Montesano et al., 1986).

Recently, bFGF has been shown to overcome pericyte-mediated endothelial cell growth inhibition (D'Amore & Orlidge, 1988) and hence further enhance cell proliferation and angiogenesis (section 3.2). In bioassay systems such as the chick chorioallantoic membrane assay or rabbit corneal pocket assay, bFGF is a potent angiogenic factor (Gospodoarowicz et al., 1987b).

The mechanism and control of bFGF release is not known. Bovine and human bFGF genes lack a classical signal sequence known to be required for extracellular transport and secretion of proteins (Abraham et al., 1986). It is suggested that bFGF is not secreted in vivo (Vlodavsky et al., 1987). Initial reports from in vitro studies showed no secretion of bFGF by cells in culture (Klagsbrun et al., 1986) but more recently, using a highly sensitive radiometric assay, it was possible to show that a small fraction of synthesized bFGF was released into the conditioned medium (Sato & Sato, 1988). The majority of bFGF remains either within the cell or bound to heparan sulphate glycosaminoglycans in the extracellular matrix (Baird & Ling, 1987; Vlodavsky et al., 1987). Under normal conditions, bFGF is inaccessible to stimulate cell growth. Active bFGF could be mobilised from the extracellular pool by heparinase enzymes liberated from damaged tissue or activated macrophages (Baird & Ling, 1987). In addition, cell injury could release intracellular bFGF. Both of these mechanisms may be important in mobilising bFGF in the ischaemic diabetic retina and providing a stimulus for cell proliferation. This will be further discussed in chapter 8.

(5) Basic FGF and pituitary ablation

The finding of high concentrations of bFGF in the pituitary gland and the demonstration that FGF purified from pituitary is identical to retinal-derived bFGF (Esch et al., 1985, Gospodarowicz et al., 1987a), raises some tantalising possibilities regarding the mechanism of benefit of pituitary ablation in the treatment of diabetic retinopathy (see section 1.4). Pituitary bFGF stimulates endothelial cell proliferation at concentrations as low as 5 pg/ml (Gospodarowicz et al., 1984).

It is tempting to speculate that removal of this source of angiogenic growth factors may have been responsible for regression of new vessels. The effects of hypophysectomy on immunorective FGF levels in the rat were reported by Mormede (Mormede et al., 1985), but retinal or eye tissue was not examined. In that study, serum FGF level concentration showed a significant rise after pituitary removal (11.9 pmol/l vs 14.6 pmol/l) as did extractable FGF from the spleen. The physiological relevance of these findings is not known, since changes in bioactivity were not assessed and because of doubts about the FGF assay employed (see 3.6 (3)). It is not known if physiologically important levels of bFGF are present in the circulation and if so, whether the pituitary gland is the major source.

Current evidence would favour a paracrine rather than endocrine role for bFGF related with the development and maintenance of microvessels within the pituitary (Gospodarowicz et al., 1987a). Pituitary FGF may also modify the response of the pituitary cells to hypothalamic releasing factors. Preincubation of rat anterior pituitary cells with pituitary bFGF increased their response to thyrotropin-releasing hormone but had no effect on the response to other releasing factors such as GH-releasing hormone (Baird et al., 1985c). An association of pituitary bFGF and GH was suggested by a recent report of a very high concentration of large molecular weight forms of bFGF extracted from a very extensive GH-producing pituitary tumour (Prysor-Jones et al., 1988). It is not known if this finding is unique to tumours secreting GH. FGF appears to exist in both normal and tumorous human pituitaries either bound to extracellular matrix or intracellularly. There is no evidence, to date, that FGF is released by the pituitary into the circulation.

3.7 Summary

The discovery of potent angiogenic factors in the retina, holds great promise in the understanding of the process of new vessel formation. Basic FGF accounts for the majority of angiogenic activity in homogenised retinal extracts. Levels of bFGF in serum and vitreous need reassessment because of degradation of the label in the original FGF radioimmunoassay and because of the low sensitivity of previous assays.

On its own, bFGF is incapable of causing DNA synthesis and cell proliferation, requiring progression factor(s) present in serum for its biological effects. One such factor is likely to be IGF-I which is a potent stimulator of capillary rather than large vessel endothelial cells. However, IGF-I in nanomol concentrations also stimulates pericytes to proliferate (King et al., 1985) yet these cells are lost early in diabetic retinopathy. Some other process such as hyperglycaemia (Lorenzi et al., 1985) or sorbitol accumulation (Frank, 1984) must be invoked to explain pericyte death. Proliferation of endothelial cells is inhibited by co-culture with pericytes on cell contact (Orlidge & D'Amore, 1986). Therefore, pericyte dropout would encourage uninhibited endothelial cell proliferation in response to stimulation by growth factors such as bFGF and IGF-I.

In the following chapters, some of these issues will be addressed, beginning with studies measuring circulating IGF-I levels in diabetic patients with varying grades of retinopathy.

Chapter 4: Serum IGF-I concentration in patients with diabetic retinopathy

4.1 Introduction

As discussed in chapter 2 (section 2.2), IGF-I is now known to mediate the growth promoting effects previously attributable to GH. It is therefore well placed to act as the link between GH and proliferative retinopathy. In addition, IGF-I but not GH, has been shown to stimulate both migration and proliferation in cultured retinal endothelial cells (section 3.5).

As the first step in investigating the role of GH and GH-dependent growth factors in the pathogenesis of diabetic retinopathy, circulating levels of IGF-I were measured in diabetic patients with varying grades of retinopathy and compared with diabetics with no detectable retinopathy and normal volunteers. The study sought to answer two questions; (1) Is there any difference between serum IGF-I concentration in diabetics with proliferative retinopathy compared to other groups? and (2) Is there any evidence that patients with the poorest diabetic control have reduced serum IGF-I levels? Low circulating IGF-I levels, by reducing feedback inhibition, would provide a stimulus for GH release (section 2.2 (3)).

4.2 Measurement of IGF-I concentration

The somatomedins were originally measured in terms of cartilage-stimulating activity, and the concentration expressed in international units (U)/ml, where 1 U was arbitrarily defined as the somatomedin activity in 1 ml of a pooled plasma from healthy adults. With the availability of purified IGF-I, radioimmunoassays were developed using pure synthetic peptide to define a standard curve for quantification of results. IGF-I concentration could now be expressed in a mass units as ng/ml or mcg/l; 240 mcg/l IGF-I is equivalent to 1 U/ml (Yeoh & Baxter, 1988) .

Before measurement of IGF-I levels, somatomedin binding proteins were removed from the serum sample by acid-ethanol extraction (Daughaday et al., 1980). This was done for two reasons; firstly because IGF-I is not accessable to the monoclonal antibody used in the assay when it is bound to its carrier protein, so that unextracted serum has virtually no measurable IGF-I (Baxter et al., 1982a). Secondly, on a more theoretical basis, unsaturated binding proteins may interfere in the assay by removing radiolabelled IGF-I tracer from the reaction mixture (Baxter, 1986).

The assay therefore measured total IGF-I concentration which has been shown to provide clinically meaningful results, levels being high in patients with acromegaly (Furlanetto et al., 1977; Baxter et al, 1982b; Burrin et al., 1987) and below normal in patients who have undegone pituitary ablation (Burrin et al., 1987).

Acid-ethanol extraction removes nearly all binding protein from human serum (Daughaday et al., 1987) but is not effective in uraemic serum as proteins remain which interfere with the assay (Powell et al., 1986). For this reason, patients with renal impairment (defined by serum creatinine >120 micromol/l) were excluded from the study.

The antiserum used in this study was kindly donated by Dr R.C. Baxter (Sydney, Australia) and distributed through the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases. It is a monoclonal antibody, highly specific for human IGF-I, having little or no cross-reactivity with human insulin or C-peptide and 7% cross-reactivity with IGF-II (Baxter et al., 1982a). The standard curve was made up using a recombinant analogue of human IGF-I with threonine substituted for methionine at position 59 (Amersham International PLC, Amersham, Buckinghamshire). The iodinated analogue (Amersham International PLC, Amersham) was used as label. Bound reactivity was precipitated by a polyethylene glycol assisted double antibody technique using donkey anti-mouse antiserum and normal mouse serum.

Inter- and intra-assay coefficients of variation (CV) were assessed by the use of two quality control samples; a normal adult with a value of approximately 200 mcg/l and an acromegalic patient with a value of about 700 mcg/l. At a concentration of 200 mcg/l, the intra-assay CV was 5.3% and the between-assay CV was 10.2% The sensitivity of the assay was 80 mcg/l with an assay range (CV <10%) of 80-700 mcg/l.

For measurement of very low IGF-I values such as in cell-conditioned medium or vitreous (chapter 8) or in samples from pituitary ablated patients (chapter 9), polyclonal antiserum kindly donated by Drs L. Underwood and J.J.Van Wyck (University of North Carolina, Chapel Hill, NC) was used. Radioimmunoassay of IGF-I with polyclonal antibody is more sensitive than with the monoclonal antibody (Burrin et al., 1987). The lower detection limit of IGF-I using the polyclonal antiserum was 5 mcg/l. The intra- and inter-assay CV using this assay were 5.0% and 8.0% respectively.

4.3 Measurement of serum GH

GH was measured with a specific double antibody radioimmunoassay (RIA) by an automated system (Kemtek, Burgess Hill, Sussex) (Burrin et al., 1985) using the WHO International Reference preparation for human growth hormone (66/217; 350 mU = 175 mcg) as standard and antiserum RD16 (Wellcome Diagnostics, Beckenham, UK) and antiserum M153 (Immunoassay Section, University of Edinburgh).

Lypho-Chek I-III were used, appropriately diluted, as quality control pools. The within and between assay coefficients of variation were 4.7% and 11.4% at a GH concentration of 14.3 mU/l. The detection limit of this assay was 0.5 mU/l.

4.4 Patients

Seventy three non-diabetic normal volunteers chosen from amongst staff members and 371 diabetic patients attending the Diabetic Retinopathy clinic at Hammersmith Hospital were studied. Patients with serum creatinine greater than 120 micromol/l were excluded from the study to eliminate overt renal failure as a variable. Clinical characteristics of the subjects are shown in Table 4.1.

Diabetic patients were divided into the following subgroups: (i) no retinopathy (84 patients); (ii) background retinopathy defined as the presence of microaneurysms only or microaneurysms and haemorrhages or small hard exudates (92 patients); (iii) preproliferative changes defined as the appearance of multiple haemorrhages associated with venous changes, cotton wool spots and intraretinal microvascular abnormalities (58 patients); (iv) active proliferative retinopathy defined by the appearance of retinal new vessels with no fibrosis (65 patients) and (v) previously active but now quiescent proliferative retinopathy (72 patients). These were patients successfully treated by photocoagulation. The appearance of the retina in subgroup (v) was that of inactive new vessels or an effectively treated retina without new vessels. Patients in subgroup (iv) were currently receiving laser therapy. Retinopathy was assessed by an ophthalmologist on the basis of direct and indirect ophthalmoscopy, colour photography and fluorescein angiography. Assessment was made without knowledge of the IGF-I values.

Table 4.1 Characteristics of patients and control subjects studied

Group	Male/ female	Age (yrs)	BMI (kg/m^2)	Insulin/ no insulin	Duration diabetes (years)	HbA_1 (%)	Proteinuria (yes/no)	Plasma glucose (mmol/l)
Control	41/32	37 ± 1	22.9 ± 2.4	-	-	5.0-8.0	-	3.0-5.0
All diabetics	195/13	49 ± 1	27.5 ± 0.9	219/127	16.1 ± 0.5	10.3 ± 0.1	57/175	12.5 ± 0.2
No Retinopathy	50/34	39 ± 2^A	25.6 ± 0.5	44/17	7.8 ± 0.7^B	10.3 ± 0.1	$0/46^B$	12.9 ± 0.4
Background retinopathy	42/35	50 ± 2	30.0 ± 2.6	66/25	19.8 ± 0.9	10.1 ± 0.1	15/47	11.7 ± 0.4
Preproliferative retinopathy	38/18	54 ± 2	27.3 ± 0.9	25/33	14.8 ± 1.1	10.8 ± 0.3	$17/20^A$	13.4 ± 0.8
Proliferative retinopathy (active)	25/18	48 ± 2	27.3 ± 0.5	38/25	17.0 ± 0.9	9.7 ± 0.2^A	$11/19^A$	13.1 ± 0.6
Proliferative retinopathy (inactive)	40/33	54 ± 2	7.0 ± 0.5	46/27	21.3 ± 1.2^A	10.4 ± 0.2	14/43	11.5 ± 0.6

Mean $\pm$ SE. $^A p<0.05$. $^B p<0.001$. compared with other diabetic subgroups.

Patient and control subjects were similar in respect of male:female ratio and body mass index. The non-diabetic normal volunteers were significantly younger than the diabetic subjects ($p < 0.05$). Within the diabetic group, those with no retinopathy were significantly younger than the other subgroups ($p < 0.001$). They also had a shorter duration of diabetes than the other patients ($p < 0.001$).

4.5 Methods

Non-fasting blood samples were taken from subjects between 0900 and 1200h during clinic time for measurement of IGF-I, blood glucose and glycosylated haemoglobin (HbA_1). A single urine sample was obtained in 232 patients for detection of protein by Albustix. Four patients with active proliferative retinopathy and four normal volunteers were admitted to the Metabolic Unit for 24h profiles of growth hormone (GH) and IGF-I. The purpose of this was to assess the variability of levels over 24h and the validity of measuring single samples as representative of 24h IGF-I levels, particularly in subjects with GH hypersecretion.

Plasma glucose was measured by the glucose oxidase method using the Beckman Glucose Analyser (Beckman, Fullerton, California, USA). Glycosylated haemoglobin was measured by cellulose acetate membrane electrophoresis (Corning, Palo Alto, California, USA); normal range 5.0-8.0 %.

4.6 Analysis

Statistical comparisons between multiple groups were made by analysis of variance (BMDP IV Software, Los Angeles, California, USA) while comparisons between two groups were made by unpaired, two-tailed Student's t-test. Analysis of covariance was used to adjust for differences in age, duration of diabetes and presence of proteinuria amongst the diabetic subjects. The relation of IGF-I levels to the clinical variables was determined by linear regression analysis and expressed as the correlation coefficient. Significance was taken at the 0.05 level. All group values are expressed as mean ± SE unless otherwise stated. Body mass index (BMI) was calculated as weight (kg)/ height $(m)^2$.

4.7 Results

The mean area under the GH curve (AUC) was higher in the 4 diabetic patients admitted for 24h profiles compared to the 4 normal volunteers (284 ± 24 vs 98.3 ± 28.3 $mU.l^{-1}h^{-1}$; $p < 0.05$). Despite a mean coefficient of variation (CV) for serum GH of 86% in the normal controls and 84% in the diabetic subjects, the mean CV of the IGF-I values was 14.9% (normals) and 19.7% (patients) (Figures 4.1 and 4.2).

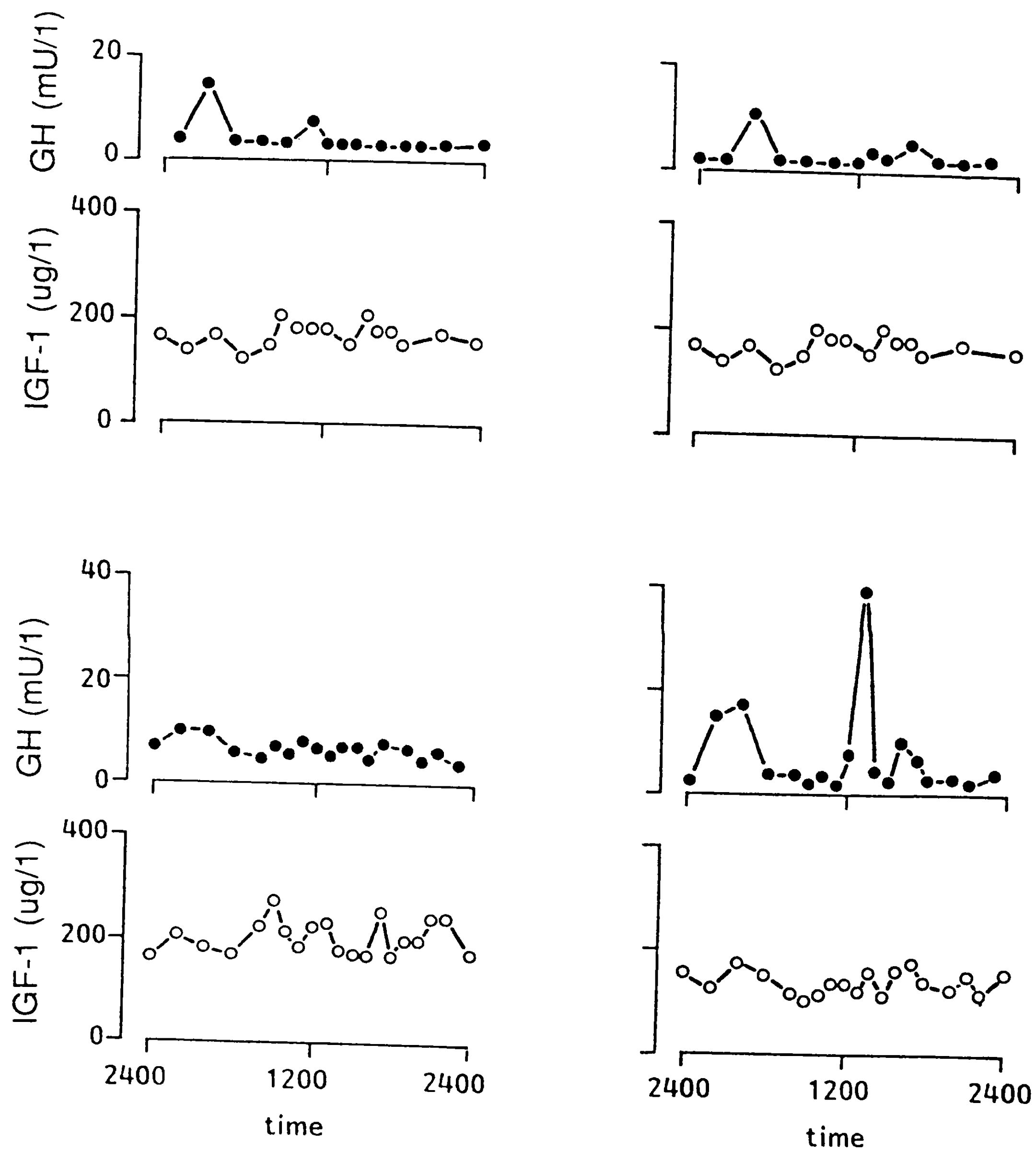


Figure 4.1 24 hour profiles of serum GH (closed circles) and IGF-I (open circles) in four normal subjects.

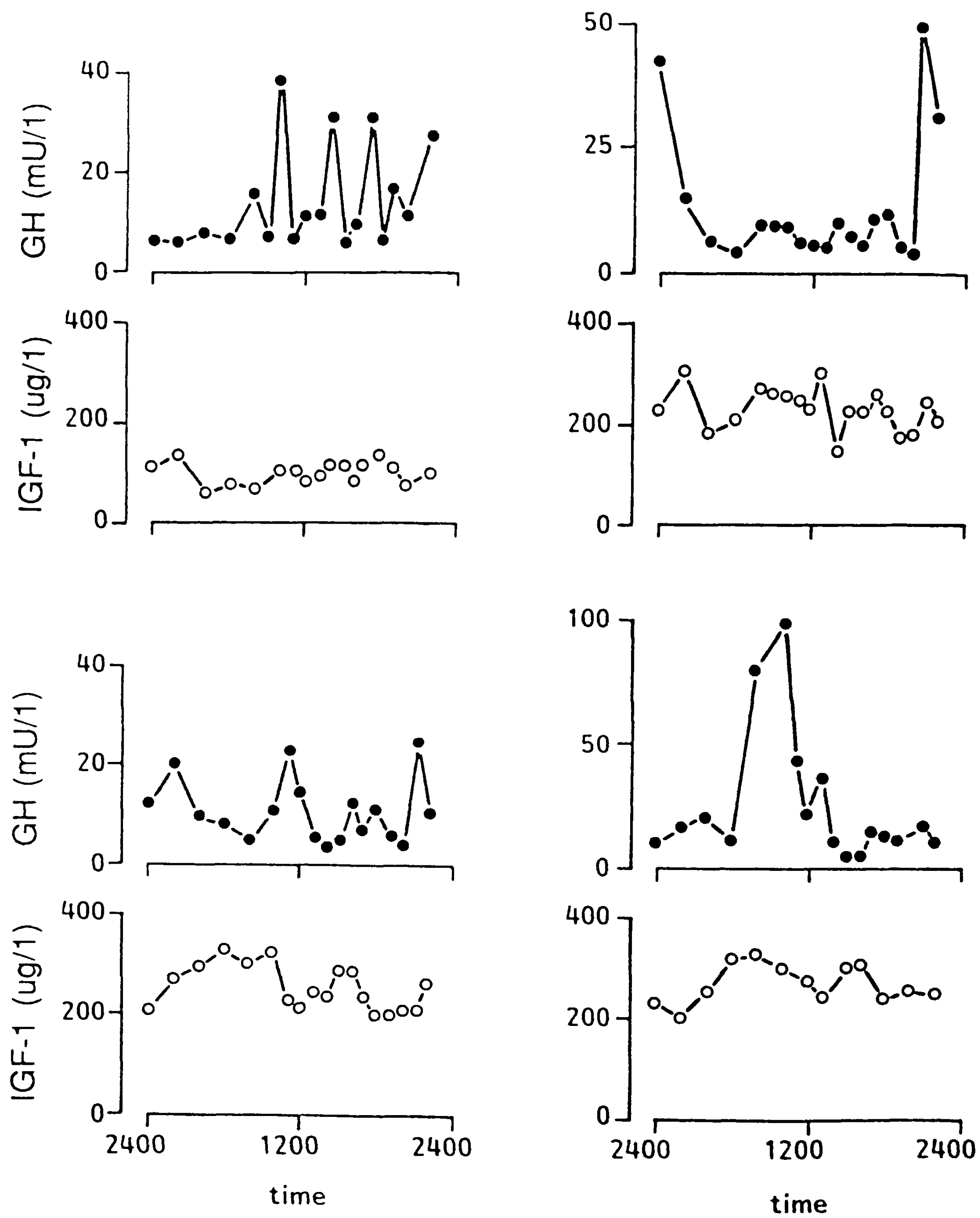


Figure 4.2 24 hour profiles of serum GH (closed circles and IGF-I (open circles) in four patients with proliferative retinopathy.

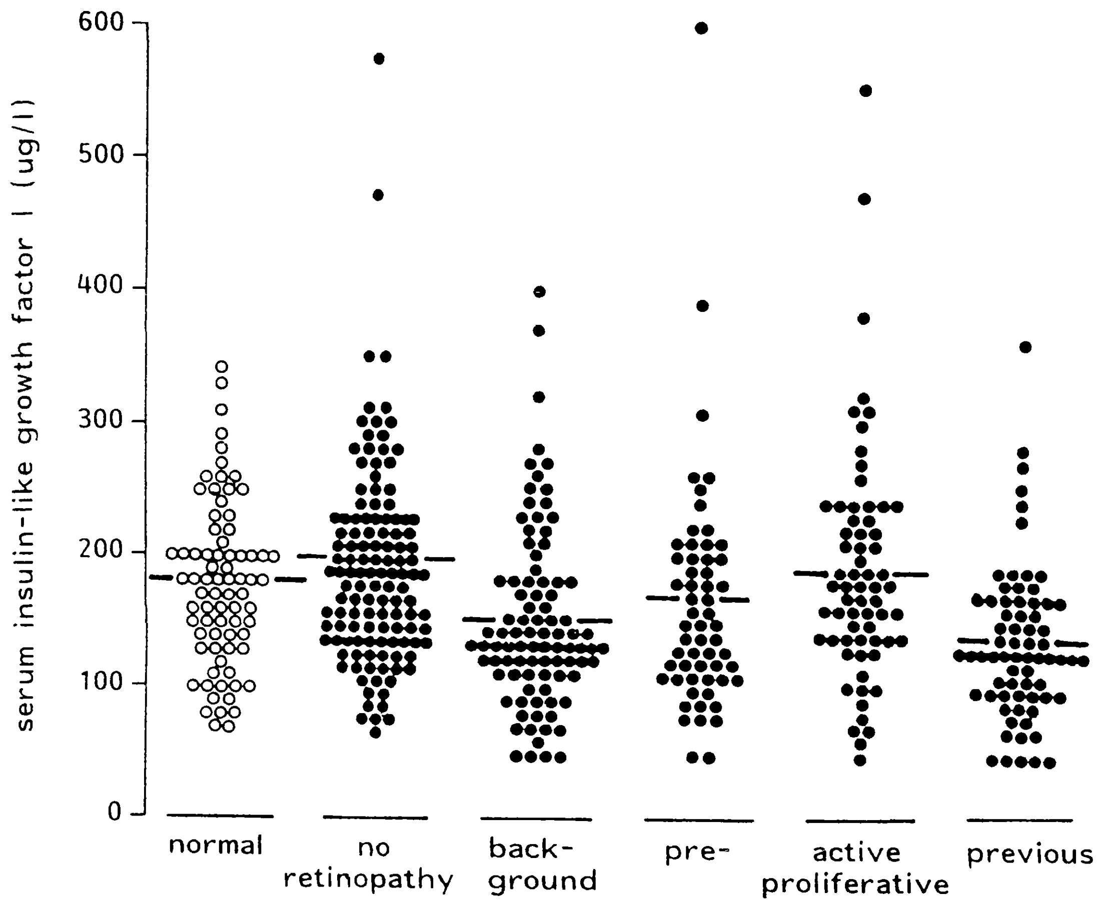


Figure 4.3 Individual serum IGF-I values for normal subjects and diabetic patients divided according to retinopathy type. The bar shows the mean for each group.

Individual values of IGF-I in the normal subjects and the diabetic subjects divided according to retinopathy appearance are shown in Figure 4.3. The normal range of serum IGF-I was 80-350 mcg/l with a mean value $\pm$ SE of 177 $\pm$ 7.4 mcg/l. No values were in the acromegalic range (> 700 mcg/l). The three individuals in the proliferative subgroup who had the highest levels [>400 mcg/l] were noted to be young diabetics (mean age 27 years) with very active and extensive new vessel formation.

The mean IGF-I level in the control and diabetic subjects did not differ significantly (177 $\pm$ 7.4 vs 168.4 $\pm$ 3.9 mcg/l; NS). There was no significant difference in mean IGF-I value between the diabetic patients with no retinopathy and those with active proliferative change (198.7 $\pm$ 8.8 vs 190.5 $\pm$ 11 mcg/l; NS), although both these subgroups had mean IGF-I values greater than the control group (198.7 $\pm$ 8.8, 190.5 $\pm$ 11 vs 177 $\pm$ 7.4 mcg/l; $p < 0.05$). This difference persisted after adjusting for age differences (Table 4.2) and is largely accounted for by several individuals with very high levels (Figure 4.3).

Diabetics without retinopathy and those with proliferative changes also had higher mean IGF-I values than the other diabetic groups. However, after adjusting for differences in age, duration of diabetes, HbA_1 and the presence of proteinuria, there was no significant difference between the patient subgroups apart from the inactive previously proliferative group which showed significantly lower mean values than the other subgroups (151.8 $\pm$ 11.5 mcg/l; $p < 0.05$). These results are summarised in Table 4.2.

Table 4.2 Serum IGF-I concentration (mcg/l) in control and diabetic subjects

Group	IGF-I	Age adjusted IGF-I	Age, duration, proteinuria adjusted IGF-I[A]
Control	177 ± 7.4	164 ± 9.1	n/a
All diabetics	168 ± 3.9	172 ± 9.1	n/a
No retinopathy	199 ± 8.8[B]	193 ± 8.3[C]	205 ± 16.3
Background retinopathy	149 ± 6.9	153 ± 8.2	164 ± 11.7
Pre-proliferative retinopathy	167 ± 12.6	178 ± 10.2	165 ± 13.1
Active proliferative retinopathy	190 ± 11.0[B]	191 ± 9.5[C]	181 ± 14.2
Inactive proliferative retinopathy	137 ± 6.7	148 ± 9.1	152 ± 11.5[B]

Mean ± SE

[A]:Adjusted for differences within diabetic group.

[B]$p < 0.05$, compared with other diabetic subgroups.

[C]$p < 0.05$, compared with control subjects.

Table 4.3 Correlation of IGF-I with clinical variables

Association	Group	p	r	Relation
IGF-I and age	Control	<0.001	-0.49	IGF= 266 - 2.50 x age
	Diabetic	<0.05	-0.23	IGF= 247 - 1.21 x age
IGF-I and sex	Control	NS		
	Diabetic	NS		
IGF-I and BMI	Control	NS		
	Diabetic	<0.05	-0.22	IGF= 332 - 4.99 x BMI
IGF-I and HbA_1	Diabetic	<0.05	-0.20	IGF= 108 - 8.76 x HbA_1
IGF-I and duration diabetes	Diabetic	NS		
IGF-I and insulin treatment	Diabetic	NS		
IGF-I and proteinuria	Diabetic	<0.001	+ 0.42	IGF= 138 + 44.8 x Prot
IGF-I and random plasma glucose	Diabetic	NS		

Age (years), BMI (kg/m^2), HbA_1 (%), proteinuria:prot (present = 1).

A significant negative correlation was noted between IGF-I and age; $r = -0.49$; $p < 0.001$ (normals); $r = -0.23$; $p < 0.05$ (patients). The presence of proteinuria was significantly associated with IGF-I level ($r = 0.42$, $p < 0.001$). Proteinuria was absent in the subgroup without retinopathy and most frequently present in the preproliferative and active proliferative patients (Table 4.1). Weaker associations of IGF-I were found with HbA_1 ($r = -0.20$; $p < 0.05$) and BMI ($r = -0.22$; $p < 0.05$ (patients) (Table 4.3). There was no significant correlation between IGF-I and sex, duration of diabetes, treatment with insulin, or random blood glucose.

4.8 Discussion

IGF-I values were similar in diabetic and normal subjects in accordance with previous studies employing radioimmunoassay methods (Zapf et al., 1980; Tamborlane et al., 1981; Lamberton et al., 1984). There was little diurnal variation in IGF-I level in contrast to the rapid fluctuations in GH levels, allowing single clinic samples to be used. The relatively long half-life (2-4h) of protein bound IGF-I (Clemmons & Van Wyk, 1984) and the delay of 18-36h between a rise in GH and a change in IGF-I concentration (Copeland et al., 1980) contribute to this lack of diurnal variation. Stable daytime IGF-I values were reported by Horner et al.(1981) and Minuto et al. (1981) although the latter found a decrease in levels after the onset of sleep. In another report (Yeoh & Baxter, 1988), IGF-I concentration remained stable over 24h in both fed and fasted normal subjects.

There was no significant difference in mean serum IGF-I concentration between the diabetic patients without retinopathy and those with active proliferative changes. This would argue against the hypothesis that serum IGF-I concentration is causally related to the development of proliferative retinopathy. Furthermore, there was no significant difference in mean IGF-I values between patients with preproliferative retinopathy (in whom extensive vascular occlusion was present on fluorescein angiography) and diabetic patients with no retinopathy. However this does not exclude differences in local concentration of IGF-I at the level of the vitreous or retina. Tissue levels will be examined in chapter 8. These observations are consistent with other studies (Cohen et al., 1977; Lamberton et al., 1984; Salardi et al., 1986) in which diabetic patients with and without retinopathy had similar IGF-I levels.

In this study, there was a highly significant correlation between serum IGF-I concentration and the presence of proteinuria, suggesting that nephropathy before overt renal failure, may increase IGF-I levels. The kidney is an important site of IGF-I degradation (D'Ercole et al., 1977) and an increase in IGF-I level would not be unexpected, analagous to the rise in serum insulin during the development of renal failure (Rabkin et al., 1970).

The expected negative association between age and serum IGF-I was confirmed and as in previous studies (Tan & Baxter, 1986), the relationship was less marked in the diabetic group. This presumably arises since other confounding factors are present in the patient group such as differences in energy intake which may influence IGF levels (Isley et al., 1983). The weak negative association with BMI is consistent with reduced GH secretion in obesity (Jung, 1984).

In this study, the highest values in the proliferative group (400-500 mcg/l) were in young diabetic patients with very active retinopathy. Merimee et al. (1983) found very high IGF-I levels only in a subgroup of seven patients with rapidly accelerating retinopathy. However, there is no information on the age of these patients or the presence of proteinuria. If these patients were younger (as in this study), this would have contributed to their higher IGF-I values. In addition, patients with proliferative retinopathy had significantly more severe and more frequent proteinuria in this study. It is likely, therefore, that subclinical nephropathy was present in many of the patients with rapidly accelerating retinopathy, and this too would have contributed to higher serum levels.

Ashton et al. (1983) reported that mean somatomedin activity as measured by a rabbit chondrocyte bioassay was higher in insulin dependent diabetic patients with retinopathy (background or proliferative) compared to normal control subjects although the differences were not large (1.42 ± 0.65 vs 1.05 ± 0.22 U/ml; $p < 0.05$).

However, there was no significant difference in somatomedin activity when diabetics with retinopathy were compared to those without retinopathy (Ashton, 1984), suggesting that the increased bioactivity in these patients may not have been related to the presence of retinopathy. Since the assay was also GH sensitive, it is possible that the differences found were due to the increased GH secretion in the diabetic patients.

The negative correlation between IGF-I concentration and HbA_1 value, although not strong, is in accordance with several previous reports. Thus a significant correlation coefficient of -0.39 was found in the study of Winter et al. (1979) and -0.48 in young insulin-dependent diabetic patients (Tan & Baxter, 1986). IGF-I levels will therefore be lower when diabetic control is poor, and this could provide the stimulus for excessive GH secretion at this time (section 2.2). The relation of IGF-I concentration to intensified insulin treatment will be examined in chapter 6.

Interestingly, patients with inactive previously proliferative retinopathy had the lowest mean IGF-I levels, significantly lower than other patient groups. Whether this was the result of laser treatment or whether these patients also had lower IGF-I values when they had active proliferative retinopathy, can only be resolved by prospective studies.

4.9 Summary

The results presented here demonstrate that while IGF-I levels are elevated in some patients with proliferative retinopathy, mean levels are very similar to diabetics without retinopathy or those with background and preproliferative changes. The finding of increased IGF-I levels in some patients with active proliferative retinopathy and decreased levels in the group with previously proliferative but now quiescent retinopathy is suggestive of a transient elevation in serum IGF-I during an early active phase of new vessel formation with a subsequent fall in serum concentration as the process becomes inactive. A prospective study is required to examine this possibility with serial measurements of serum IGF-I before and after the development of retinal neovascularisation. Such a study will be described in the following chapter.

Chapter 5: A 2 year follow-up study of serum IGF-I in diabetics with retinopathy

5.1 Introduction

In the previous chapter, the possibility was raised that serum IGF-I concentration rises transiently during an early active phase of neovascularisation. In this chapter, this possibility will be examined. A transient rise in serum IGF-I may explain the discrepancy between reports of increased levels of serum IGF-I (Merimee et al., 1983, Ashton et al., 1983) or normal levels (Lamberton et al., 1984, Salardi et al., 1986) in patients with retinopathy. A rise in IGF-I concentration, if occuring early enough and large enough, may be of clinical value as a predictor of new vessel formation as well as having implications for the pathogenesis of the proliferative lesions.

In this study, serum IGF-I levels were measured in diabetics with severe background and preproliferative retinopathy (Group 1) followed prospectively over 2 years. A group of diabetics with mild background retinopathy (Group 2) were also followed for comparison. The aim of the study was to assess circulating IGF-I concentration before and after neovascularisation had occurred.

5.2 Patients and Methods

Fifty nine patients attending the Diabetic Retinopathy clinic at Hammersmith Hospital were studied with a minimum follow-up of 2 years. All patients had serum creatinine values in the normal range (< 120 micromol/l). Twelve normal volunteers (6 male, 6 female; aged 24-36 years) were also studied to assess the variability of serum IGF-I in normal subjects over 2 years.

Retinopathy was assessed by direct and indirect ophthalmoscopy, colour photography of both eyes and fluorescein angiography. At entry to the study, 35 patients had severe background and preproliferative retinopathy (multiple haemorrhages associated with venous beading, cotton wool spots, and intraretinal microvascular abnormalities) equivalent to levels 40/40 to 50/50 on the Wisconsin grading system (Klein et al., 1984b) (Group 1). Twenty four patients with mild background retinopathy (microaneurysms, few haemorrhages, small hard exudates) equivalent to level 30/30 or less, were also studied (Group 2).

Patients were seen at 2-4 monthly visits. Fasting blood samples were taken during the morning clinics for measurement of plasma glucose, HbA_1 concentration and serum IGF-I. Urine was also examined for proteinuria by Albustix testing.

Serum IGF-I was measured by double antibody radioimmunoassay after acid-ethanol extraction (see section 4.2). Glycosylated haemoglobin A_1 was measured by agar gel electrophoresis and is expressed as percentage of haemoglobin in the glycosylated form (normal range: 3.5-5.8%).

5.3 Analysis

The two patient groups were compared by an unpaired t test. A stepwise regression analysis was performed to examine the relation between changes in IGF-I and changes in HbA_1 in the two patient groups and the subgroup who developed proliferative retinopathy. The relation of IGF-I values and age, duration of diabetes, proteinuria, use of insulin and BMI was also examined in each group by regression analysis. The predictive value of changes in IGF-I and the onset of proliferation was assessed by discriminant analysis. Significance is taken as $p < 0.05$. Values in the text are expressed as mean $\pm$ SD unless otherwise stated. BMI was calculated as weight (kg)/height $(m)^2$.

5.4 Results

The patient groups were similar in respect of sex distribution, age, BMI, prevalence of proteinuria and use of insulin (Table 5.1). The patients in Group 1 had significantly shorter duration of diabetes when compared to those in Group 2; median (95% confidence limits): 15 (12-17) vs 20 (16-23) years; $p = 0.02$.

Table 5.1 Clinical details and investigations in the patient groups

	Severe background & Preproliferative (Group 1) (n=35)	Mild background (Group 2) (n=24)	p*
Age (years)	47.7 ± 16.4	40.8 ± 13	NS
Sex (M/F)	26/9	20/8	NS
Duration diabetes (years)	16 (2-31)	20 (7-36)	0.02
BMI (kg/m^2)	26.2 ± 5.0	27.2 ± 4.8	NS
Albustix +ve n (%)	21 (60%)	10 (36%)	NS
Insulin treated n (%)	25 (71%)	24 (85%)	NS
HbA_1 initial (%)	10.55 ± 2.36	10.88 ± 2.07	NS
HbA_1 1 year (%)	9.50 ± 2.47	10.68 ± 2.11	NS
HbA_1 2 years (%)	10.38 ± 3.75	9.50 ± 2.12	NS
IGF-I initial (mcg/l)	157.1 ± 71	167.8 ± 77	NS
IGF-I 1 year (mcg/l)	165.7 ± 78	158.9 ± 87.2	NS
IGF-I 2 years (mcg/l)	142.8 ± 58	158.9 ± 67	NS

Data given as mean ± SD and mean (range).

*p comparing Group 1 and Group 2.

The mean $\pm$ SD of the individual coefficients of variation of serum IGF-I over the two year period was 9.6% $\pm$ 6.2 in 12 normal volunteers, 22.6% $\pm$ 15.7 in the mild retinopathy group and 25.8% $\pm$ 17.3 in the severe background and preproliferative group ($p < 0.05$ normals vs patients). At entry, one year and two years, mean serum IGF-I was 181 $\pm$ 47, 188 $\pm$ 30, 221 $\pm$ 56 mcg/l in the normal volunteers.

Mean IGF-I concentration (mcg/l) at entry, one year and two years was similar in both groups of patients (157 $\pm$ 71 vs 168 $\pm$ 77 at entry; 166 $\pm$ 78 vs 160 $\pm$ 87 at 1 year; 143 $\pm$ 58 vs 160 $\pm$ 67 at 2 years; NS). These values were also not significantly different when compared with the mean yearly IGF-I concentrations in the normal subjects. Mean HbA_1 values were similar in both groups of patients at entry, one and two years (Table 5.1).

During the study period, 8 patients in Group 1 (and none in Group 2) developed proliferative changes; new vessels occured in all quadrants in 6 patients and in only one quadrant in 2 patients. Clinical details of these patients are shown in Table 5.2. Changes in serum IGF-I in the eight patients developing proliferative retinopathy are illustrated in Figure 5.1. Values before proliferation (median 3 months [range 1-4]) are at the time of the last visit before the recognition of proliferative lesions.

Table 5.2 Clinical details of patients developing proliferative retinopathy

Patient Number	Sex	Age (years)	Duration diabetes (years)	BMI (kg/m^2)	Type	Proteinuria (g/l)
1	F	24	22	22.3	IDDM	2
2	F	27	17	22.9	IDDM	1
3	M	29	21	23.6	IDDM	3
4	M	36	9	34.7	IDDM	3
5	M	33	18	23.0	IDDM	1
6	F	24	20	20.9	IDDM	2
7	M	68	6	23.7	NIDDM	0
8	M	54	5	28.2	NIDDM	0

Patients 1-6 developed new vessels in all quadrants; patients 7 and 8 in only one quadrant.

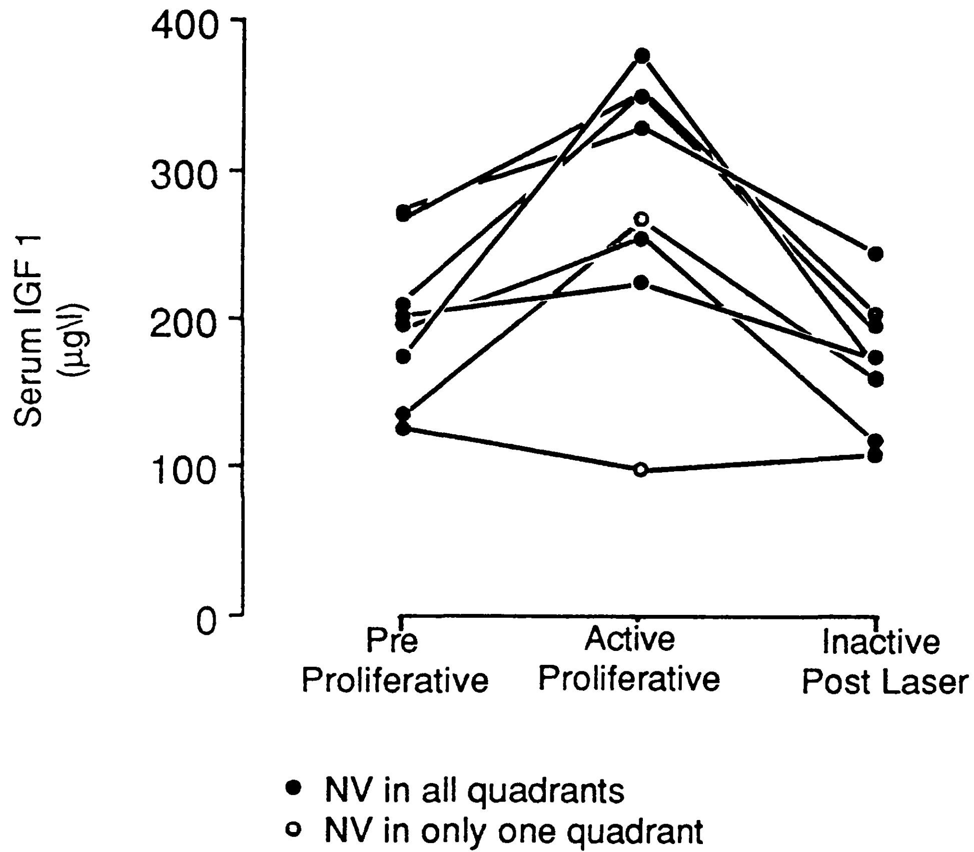


Figure 5.1 Changes in serum IGF-I concentration in eight patients developing proliferative retinopathy.

Mean IGF-I concentration at the first appearance of new vessels was significantly higher compaired to the value at the previous visit (281 $\pm$ 91 vs 196 $\pm$ 58 mcg/l; $p = 0.01$). The median rise in IGF-I values was 29.8% (95% confidence: 11.4-95.5%). The mean IGF-I value at time of proliferation was also significantly higher in the six patients with 4 quadrant new vessels (314 $\pm$ 60) compared to the 2 patients with single quadrant new vessels (181 $\pm$ 82); $p < 0.05$. In addition, the mean IGF-I value at the time of proliferation in all 8 patients (281 $\pm$ 91) was significantly greater than the mean for all Group I patients at entry (157 $\pm$ 71), 1 year (165.7 $\pm$ 78) or 2 years (142.8 $\pm$ 58) ($p < 0.001$).

The onset of proliferation could not be predicted by changes in IGF-I level. The combination of a rise in IGF-I and a fall in HbA_1 correctly identified 6 out of the 8 patients who developed proliferation but falsely classified 10 patients in Group 1 and 4 in Group 2 into the proliferative group.

No significant correlation was observed between serum IGF-I values and age, sex, BMI, HbA_1 concentration, duration of diabetes, insulin treatment or prevalence of proteinuria in either group of patients. Changes in HbA_1 concentration in the subgroup developing proliferative retinopathy are shown in Table 5.3. There was no significant correlation between changes in IGF-I values before and after proliferation and changes in HbA_1 levels ($p > 0.05$).

Table 5.3 Changes in serum IGF-I values (mcg/l) and HbA_1 concentration (%) in patients progressing from preproliferative to proliferative retinopathy.

Patient		Retinopathy Pre-proliferative	Active proliferative	Inactive post-laser
1.	IGF-I:	175	376	175
	HBA_1:	12.9	8.3	9.2
2.	IGF-I:	270	350	196
	HbA_1:	16.0	5.2	6.6
3.	IGF-I:	271	329	245
	HbA_1:	15.0	12.1	15.1
4.	IGF-I:	196	255	119
	HbA_1:	13.4	15.3	17.0
5.	IGF-I:	202	225	174
	HbA_1:	13.7	6.0	9.3
6.	IGF-I:	210	350	203
	HbA_1:	9.3	7.7	9.7
7.	IGF-I:	126	99	109
	HbA_1:	7.6	7.9	3.7
8.	IGF-I:	135	264	168
	HbA_1:	9.8	9.2	9.6
All patients				
	IGF-I:	198 ± 54	281 ± 91*	174 ± 44
	HbA_1:	12.2 ± 2.9	8.9 ± 3.3	10.0 ± 4.3

Values for all patients are given as mean ± SD.
*$p < 0.05$ compared to pre-proliferative and post-laser values

5.5 Discussion

In this study, a significant elevation in IGF-I concentration was observed at the time of proliferation especially in patients with new vessels in all quadrants. It should be noted, however, that only one patient had values outside the normal range. As in the previous study (section 4.7) younger patients with very active 4 quadrant (florid) proliferative retinopathy achieved the greatest IGF-I values. In this study, patients underwent laser treatment as soon as new vessels were detected. It is possible that mean IGF-I levels would have been even greater had the retinopathy been allowed to progress.

IGF-I values tended to be more variable in the diabetic group compared to the normal controls presumably reflecting the effect of fluctuations in diabetic control on serum IGF-I levels (section 2.2). It is likely that acid-ethanol extraction does not completely remove all IGF-I binding proteins (Mesiano et al., 1988) so that fluctuations in the insulin-dependent 34kDa binding protein could also have contributed to the greater variability of IGF-I values in the patients. With less variability, individual IGF-I levels may have been able to predict progression of retinopathy. However, the increase in IGF-I concentration was not early enough to be of clinical value as a predictor of deterioriation in retinopathy.

Although the rise in circulating IGF-I at the time of neovascularisation did not appear to relate to changes in glycaemic control, six out of the seven patients in whom IGF-I levels increased, showed a simultaneous reduction in HbA_1 values (mean reduction in HbA_1 of 33.5%), indicating improved glycaemic control.

In one study (Merimee et al., 1984) improved glycaemic control as reflected by a 28% fall in mean HbA_1 values, produced a non-significant rise in serum IGF-I in adult diabetic patients. A more dramatic fall in HbA_1 levels (40%) after the institution of continuous subcutaneous insulin pump treatment in 8 adult diabetics, resulted in a 70-75% increase in plasma somatomedin values (Tamborlane et al., 1981) and a fall in previously elevated GH concentrations.

Improved metabolic control would seem to correct the impaired IGF-I production in response to GH, that is reported in patients with poor diabetic control (Lanes et al., 1985). Indeed, IGF-I levels were disproportionately high relative to GH levels in some patients with proliferative retinopathy (Merimee et al., 1983), although it is not known if this followed an improvement in diabetic control. The rise in serum IGF-I as metabolic control is improved, may be analagous to the increase in circulating levels occuring after refeeding malnourished children (Hintz et al., 1978) and presumably reflects an improved use of metabolic substrates.

Whilst improved metabolic control is clearly important, it would not account for the rise in IGF-I in all patients as one patient (patient 4) showed a 30% rise in serum IGF-I at the same time as a 12% rise in HbA_1 levels. In addition, the magnitude of the rise in IGF-I values bore no relation to the extent of the improvement in diabetic control as judged by the fall in HbA_1 values, although this may be due to individual variability in the IGF-I response to improvements in metabolic control.

In two patients (Patients 2 and 5), a dramatic improvement in diabetic control (associated with falls in in HbA_1 level of 67% and 56% respectively) preceded the development of retinal new vessels. Rapid worsening of retinopathy associated with the sudden institution of tight glycaemic control has been reported during continuous subcutaneous insulin infusion (CSII) therapy (Lawson et al., 1982; Kroc Collaborative Study Group, 1984; Dahl-Jorgensen et al., 1985) and after pancreas transplantation (Ramsay et al., 1988). The mechanism of this deterioriation in retinopathy is not known.

Increased IGF-I production and/or decreased clearance could account for the rise in serum concentration found in this study. Traditionally IGF-I was considered to originate in the liver, and travel in the circulation to promote growth at distant sites.

Although the liver is still considered an important source of circulating IGF-I, accounting for at least 55% of the peptide concentration in blood (Underwood et al., 1986), peripheral tissues contribute the remaining 45%, at least in the rat. Little is known regarding the amount of IGF-I produced by the eye, but it is conceivable that the increase in serum concentration resulted from excessive production and release of IGF-I from the proliferating retina. Significant amounts of intact IGF-I are retained within capillary endothelial cells for extended periods of time (Bar et al., 1986) and may raise serum levels if released into the circulation.

The fall in serum levels after retinal photocoagulation would be consistent with the hypothesis that the eye was the source of the increase in serum IGF-I. If this were the case, vitreous IGF-I levels might be expected to exceed serum levels, but this has not been found (Grant et al., 1986a). However, IGF-I may be transported more readily into the bloodstream than into vitreous.

Diminished renal clearance of the peptide, is also likely to have contributed to increased IGF-I values. In chapter 4, a positive correlation was demonstrated between the presence of proteinuria and serum IGF-I value. Proteinuria, often heavy, was present in 6 of the patients developing proliferative retinopathy.

Whether there was a similar increase in retinal IGF-I concentration at the time of proliferation is not known. Since all the patients developing retinal vascular proliferation showed extensive leakage of fluorescein at angiography, it is possible that vitreous levels were even higher than serum levels would suggest. Indeed, a positive correlation between serum and vitreous IGF-I levels was demonstrated in diabetic patients with proliferative retinopathy but not non-diabetic controls (Grant et al., 1986a). However, it cannot be assumed that leakage of fluorescein (molecular weight 376Da) correlates with leakage of IGF-I (molecular weight 7,500Da), particularly as IGF-I circulates bound to carrier proteins.

Since local concentration of IGF-I is chemoattractant to endothelial cells (Grant et al., 1987) a rise in vitreous levels could attract new vessel growth. An increase in both circulating and local IGF-I concentration in a patient with an already ischaemic retina, could promote the development of retinal new vessels by acting in association with retinal derived growth factors (section 3.6). This relationship will be further explored in chapter 8.

5.6 Summary

In this longitudinal study, eight patients progressed from preproliferative to proliferative retinopathy. There was a significant rise in serum IGF-I at about the time when new vessels were first noted. Younger patients with more extensive retinopathy achieved the greatest IGF-I levels.

Although the magnitude of the increase in IGF-I values bore no relation to the change in HbA_1 level, all but one patient showing a rise in IGF-I, had a simultaneous improvement in diabetic control. Values at the time of proliferation were significantly greater than the mean value of all the patients with preproliferative retinopathy but the rise in serum concentration was not great enough or early enough, to be of clinical value as a predictor for the development of of proliferative lesions. Nevertheless, this increase in IGF-I concentration is likely to be important in promoting new vessel formation in these patients.

It is quite possible that levels would have been even higher if the retinopathy had been allowed to progress. Increased circulating IGF-I concentration with improved diabetic control could also contribute to the transient deterioriation in retinopathy commonly occuring during CSII therapy. The relation of changes in retinopathy during insulin pump treatment to serum IGF-I concentration will be examined in the following chapter.

Chapter 6. Progression of diabetic retinopathy and changes in serum IGF-I during continuous subcutaneous insulin infusion (CSII)

6.1 Introduction

Transient deterioriation in retinopathy during insulin pump treatment may occur in the first few months of treatment and may occasionally progress to new vessel formation (Lawson et al., 1982; Lauritzen et al., 1983; Kroc Collaborative Study Group, 1984; Dahl-Jorgensen et al., 1985). More usually, the deterioriation is temporary, characterised by the appearance of a few cotton wool spots and increased numbers of haemorrhages and intraretinal microvascular abnormalities (IRMA) which subsequently disappear. By 24 months, there is either no difference or slight improvement in retinopathy outcome in pump treated versus conventionally treated patients (Lauritzen et al., 1985; Hanssen et al., 1986).

An increase in serum IGF-I levels occurs soon after commencing pump treatment (Tamborlane et al, 1981; Amiel et al., 1984), and may contribute to the retinal changes, especially in patients with more advanced retinopathy at the time of commencing CSII treatment. In such patients, progression of retinopathy during CSII is more common (Lawson et al., 1982). New vessel formation, in particular, may be promoted by the increase in IGF-I concentration interacting with retinal-derived growth factors from an already ischaemic retina (section 3.6).

If the worsening of retinopathy does relate to IGF-I levels, suppressing the rise in serum concentration may provide a means to prevent this complication. In this chapter, a twelve month prospective study is described aimed at investigating the relation between retinopathy appearance during CSII therapy and serum IGF-I levels. The rate at which euglycaemia was achieved was also studied for its effects on IGF-I concentration and retinopathy.

6.2 Patients and Methods

Twelve non-obese insulin-dependent diabetic patients of mean age 32.7 years (range: 22-41 years) were studied. Clinical details are shown in Table 6.1. All patients had normal blood pressure, normal serum creatinine concentration and mild to moderate background retinopathy defined as less than level 50 in the Wisconsin grading system (Klein et al., 1984b) (Table 6.2). The study protocol was approved by the Ethical Committee of the Hammersmith Hospital.

Commencement of CSII (Graseby MS-36, Graseby Medical, Watford, UK) occurred during an initial three day hospital admission during which time patients were shown how to perform blood glucose monitoring. Thereafter, they were seen in the Retinopathy Out-Patient clinic, Hammersmith Hospital. Initially, patients were in telephone contact at weekly intervals to report on their progress. Medical advice was available at all times throughout the study. Before transferring to CSII therapy, patients were receiving conventional insulin by twice daily subcutaneous injection and were taking no other medication.

Table 6.1 Clinical details of patients

Patient	Sex	Age (years)	Duration of diabetes (years)	Proteinuria (g/l)
1	M	39	15	0
2	M	42	9	0
3	F	30	16	0
4	M	35	11	1
5	M	30	8	0
6	F	23	20	3
7	M	36	17	0
8	M	32	20	0
9	M	41	20	0
10	M	25	18	0
11	M	22	8	0
12	M	27	16	0
Mean $\pm$ SD		33 $\pm$ 6.8	15 $\pm$ 4.7	

Patients were allocated on an alternating basis, to rapid reduction (Group 1) or slow reduction (Group 2) of blood glucose; target 2 hour post-prandial blood glucose 7-10 mmol/l to be achieved as soon as possible in Group 1, or over a period of 3-4 months in Group 2.

Patients were formally assessed at entry to the study, and then at one, four and twelve months. In four patients, assessments have also been made at twenty four months. At these times, a full ophthalmic examination was performed including visual acuity, direct and indirect ophthalmoscopy.

Thirty degree stereoscopic photographs of eight standard fields of each eye were taken and assessed by a trained grader, masked to the patient's identity, treatment group or time of visit. Assessments were made in comparison to standard retinal photographs. Levels of severity were assigned according to the criteria of the Wisconsin study (Klein et al, 1984c) by comparison with standard retinal photographs. A retinopathy score (detailed in Table 6.2) (Canny et al., 1985) was derived for each assessment. Fluoroscein angiograms were taken of the right eye at each visit and the areas of non-perfusion assessed.

Blood samples were obtained at each visit for determination of fasting serum IGF-I concentration and Haemoglobin A_1 (HbA_1) level (normal range: 5-8%). Urine protein was estimated by Albustix testing.

Table 6.2 Retinopathy Levels and Retinopathy Score

Level 10 - no retinopathy

Level 20 - microaneurysms only

Level 30 - microaneurysms and at least one of the following: haemorrhages, small hard exudates, venous loops, questionable cotton wool spots (CWS), questionable intraretinal microvascular abnormalities (IRMA), and questionable venous beading.

Level 40 - microaneurysms and at least one of the following: definite CWS, definite IRMA, definite venous beading, moderate or large hard exudates or severe haemorrhages.

Level 50 - combinations of severe haemorrhages, CWS, IRMA and venous beading in four peripheral fields.

Level 60 - neovascularisation or fibrous tissue associated with new vessels or photocoagulation for neovascularisation.

Retinopathy Score (Level R eye/L eye):

10 / 10 = 1; 20 /<20 = 2; 20 / 20 = 3;

30 /<30 = 4; 30 / 30 = 5; 40 /<40 = 6;

40 / 40 = 7; 50 /<50 = 8, 50 / 50 = 9;

60 /<60 = 10; 60 / 60 = 11.

Note: The lesions are in comparison with standard retinal photographs.

6.3 Analysis

The relation between changes in serum IGF-I concentration and changes in the retinopathy score was examined by analysis of variance. This method was also used to examine the relation between HbA_1 values and serum IGF-I concentration or retinopathy score. Comparisons between groups were made by unpaired Student's t tests. Values are expressed as mean $\pm$ SEM unless otherwise stated.

6.4 Results

(1) Serum IGF-I concentration

Individual changes in serum IGF-I during 12 months of CSII treatment are shown in Tables 6.3.1 and 6.3.2. Mean serum IGF-I concentration (mcg/l) $\pm$ SEM in the 12 patients rose from an initial value of 155 $\pm$ 17.7 to 199 $\pm$ 23.1 at 4 months ($p > 0.05$) and thereafter declined to 163 $\pm$ 17.4 at 12 months (Table 6.4). The rise at 4 months was greater in those who retinopathy deterioriated but this did not achieve statistical significance (212 $\pm$ 35.5 vs 180 $\pm$ 27.1; $p > 0.05$). By 12 months, serum IGF-I levels were similar to the values at entry. Two year IGF-I values in 4 patients were little changed from the 12 month figures (182.5 $\pm$ 30.4 at 2 years vs 195 $\pm$ 44.8).

Table 6.3.1 Individual values of serum IGF-I (mcg/l), HbA_1 (%) and retinopathy scores during CSII - Group 1

Patient		Initial	1 month	4 months	12 months
1.	Serum IGF-I	87	50	117	81
	HbA_1	10.4	9.4	8.2	9.8
	Retinopathy score	3	4	3	4
2.	Serum IGF-I	199	152	255	239
	HbA_1	10.1	8.5	7.3	7.8
	Retinopathy score	5	5	5	6
3.*	Serum IGF-I	241	282	223	205
	HbA_1	12.5	7.5	8.0	9.9
	Retinopathy score	7	7	8	8
4.*	Serum IGF-I	244	203	245	108
	HbA_1	9.5	9.3	9.9	10.6
	Retinopathy score	6	7	7	7
5.	Serum IGF-I	110	161	202	227
	HbA_1	10.4	10.1	12.9	10.0
	Retinopathy score	6	5	5	5
6.*	Serum IGF-I	165	175	376	175
	HbA_1	15.6	12.9	8.3	9.2
	Retinopathy score	7	8	11	11
7.*	Serum IGF-I	176	146	129	164
	HbA_1	14.0	9.2	9.9	9.3
	Retinopathy score	6	7	6	7

* Indicates patients who developed CWS, IRMA or NV.

Table 6.3.2 Individual values of IGF-I (mcg/l), HbA_1 (%), and retinopathy scores during CSII - Group 2

Patient		Initial	1 month	4 months	12 months
8.*	Serum IGF-I	50	50	111	54
	HbA_1	10.3	11.4	9.8	8.9
	Retinopathy score	6	7	6	7
9.	Serum IGF-I	137	174	118	149
	HbA_1	10.4	11.2	12.2	9.9
	Retinopathy score	7	6	7	7
10.*	Serum IGF-I	108	50	139	-
	HbA_1	8.7	8.7	8.0	-
	Retinopathy score	6	6	7	-
11.	Serum IGF-I	208	256	207	184
	HbA_1	10.3	10.6	8.1	9.3
	Retinopathy score	3	3	3	3
12.*	Serum IGF-I	133	169	262	210
	HbA_1	10.3	10.6	5.8	9.2
	Retinopathy score	5	5	7	5

* Indicates patients who developed CWS, IRMA, or NV.

Patient 10 left the country after the 4 month visit.

Table 6.4 Changes in serum IGF-I (mcg/l) in patients developing CWS, IRMA or NV during CSII compared with patients showing no retinal deterioriation

Group	Initial	1 month	4 months	12 months
All patients	155 ±17.7	156 ±21.8	199 ±23.1	163 ±17.4
Patients developing CWS, IRMA or NV	160 ±26.5	154 ±31.3	212 ±35.5	153 ±24.7
Patients with no deterioration	148 ±23.9	159 ±32.8	180 ±27.1	176 ±28.6

Mean ± SEM

Table 6.5 Serum IGF-I (mcg/l) and HbA_1 (%) values in patients with rapid or slow correction of hyperglycaemia

Group		Initial	1 month	4 months	12 months
Rapid correction (Group 1) (n=7)	IGF-I	175 ± 22.8	167 ± 26.3	221 ± 32.9	171 ± 22.4
	HbA_1	11.8 ± 0.9	9.6 ± 0.6*	9.2 ± 0.7	9.5 ± 0.3
Slow correction (Group 2) (n=5)	IGF-I	127 ± 25.5	140 ± 39.7	167 ± 29.1	149 ± 34.1
	HbA_1	10.0 ± 0.3	10.5 ± 0.5	8.8 ± 1.1	9.3 ± 0.2

Mean ± SEM

*$p < 0.05$ Group 1 vs Group 2, for change in HbA_1 from initial to 1 month values

One patient (patient 6), described in greater detail in section 6.5, showed a marked rise in serum IGF-I at 4 months as shown in Figure 6.1, corresponding to the time of development of florid proliferative retinopathy.

There was a weak inverse correlation between the rise in IGF-I concentration for all patients over the first 4 months and HbA_1 levels (r = -0.32) which failed to reach statistical significance. However, subsequent changes in IGF-I values from 4 to 12 months were significantly inversely correlated with HbA_1 concentration (r= -0.58; p <0.05). Changes in mean IGF-I in patients divided according to the rate of correction of hyperglycaemia are shown in Table 6.5. Although by 4 months, mean IGF-I in Group 1 (221 $\pm$ 32.9) was greater than in Group 2 (167 $\pm$ 29.1), this difference did not achieve statistical significance.

(2) Retinopathy score

A total of 8 patients had deteriorioriorated by at least one level of severity in at least one eye by 4 months; 7 patients (4 in Group 1, 3 in Group 2) had developed new retinal cotton wool spots (CWS), intraretinal microvascular abnormalities (IRMA) or neovascularisation (NV), and one had developed a new retinal haemorrhage. The remaining 4 patients showed no deterioriation in retinopathy throughout the study.

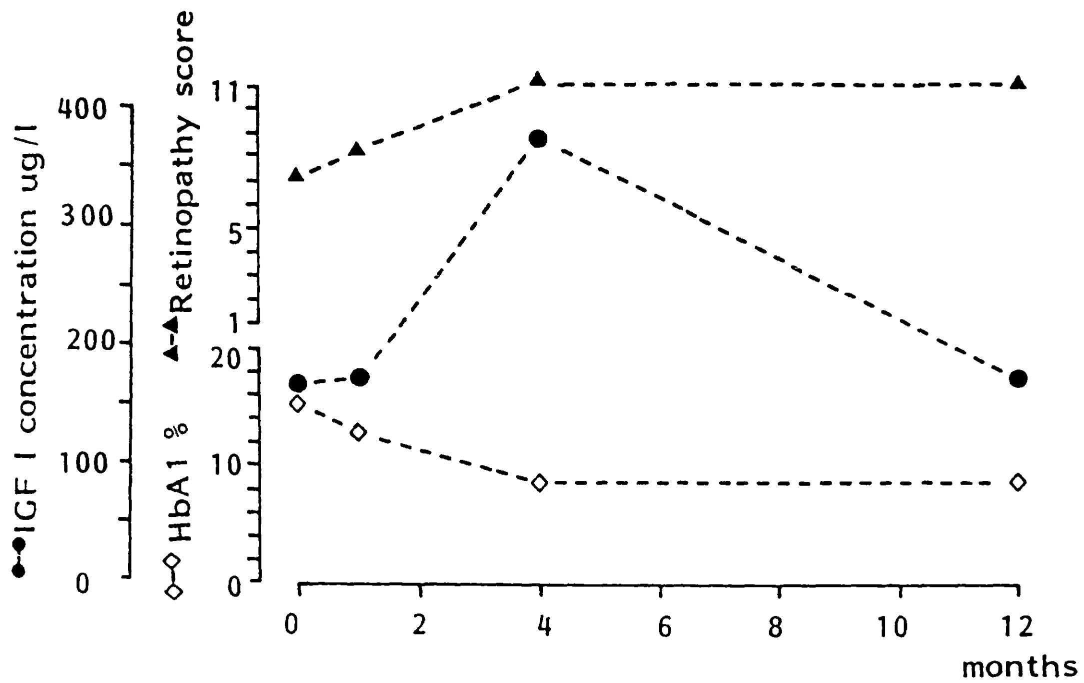


Figure 6.1 Changes in serum IGF-I, HbA_1, and retinopathy score in a patient who developed neovascularisation after four months of CSII treatment.

Fluorescein angiography in the 7 patients developing CWS, IRMA or NV showed extensive capillary closure and increased areas of non-perfusion. There was no change in visual acuity over the study period. There was no significant correlation between serum IGF-I and retinopathy score by analysis of variance either over the 12 month study period or during any time interval. Retinopathy score was not related to changes in HbA_1 nor were there any significant differences in deterioriation in retinopathy between Groups 1 and 2 when analysed on an intention to treat basis.

(3) Haemoglobin A_1

At one month, patients in Group 1 compared to Group 2 had achieved a significantly greater fall in HbA_1 from initial values (11.8 $\pm$ 0.9 vs 9.6 $\pm$ 0.6 [Group 1]; 10.0 $\pm$ 0.3 vs 10.5 $\pm$ 0.5 [Group 2]; $p < 0.05$) (Table 6.5). Thereafter, mean HbA_1 levels were similar in both groups. Despite intensified treatment, at no time during the study period, was mean HbA_1 concentration in the normal range (5-8%).

6.5 Changes in serum IGF-I, retinal blood flow and permeability index in a patient with rapidly accelerating retinopathy, occurring during CSII treatment.

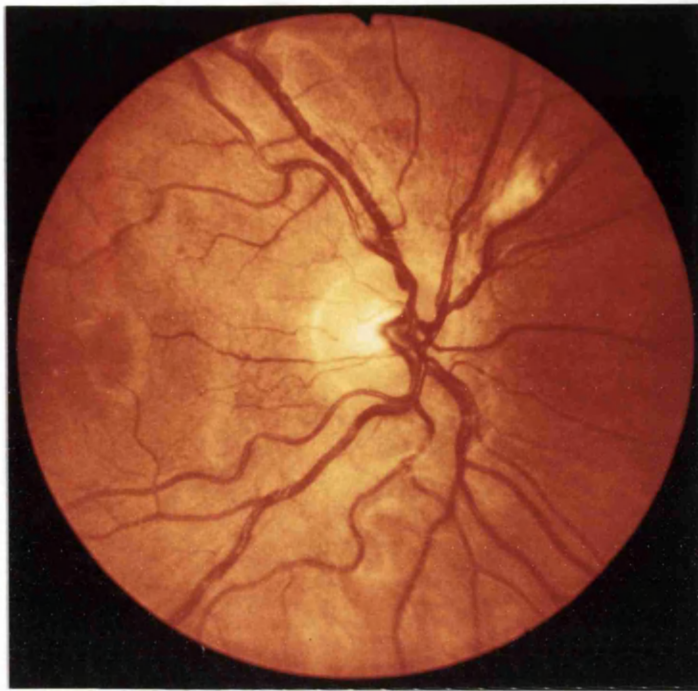
The patient (patient 6; Table 6.1, Table 6.3.1), a 23 year old nurse, had been diabetic since childhood. She had required frequent hospital admissions with diabetic ketoacidosis from the time of adolescence, and had not attended any clinic on a regular basis. She presented to the Casualty department with an acute iritis affecting the right eye and was subsequently referred to the Diabetic Retinopathy clinic. She was found to have some loss of visual acuity in the right eye (6/9 [right eye] 6/6 [left eye]). Background diabetic retinopathy was present in both eyes consisting of microaneurysms and 1-2 cotton wool spots in either eye (level 40/40). The appearances of the right disc at presentation are shown in Figure 6.2 (A) (colour photography) and Figure 6.2 (B) (fluorescein angiography).

Her diabetic control was very poor (HbA_1 at presentation: 15.6%) and she already had heavy proteinuria (3g/l). Serum creatinine was normal (71 micromol/l) as were serum lipids. In an effort to improve her erratic control, she was commenced on continuous subcutaneous insulin via a portable insulin (Graseby) pump. Within a month, the HbA_1 concentration had fallen by almost 3% to 12.9% and by 4 months, it was just outside the normal range (8.3%) (normal range 5-8%).

Her retinopathy, however, deterioriated. Two months after commencement of CSII she had developed preproliferative changes with venous beading, retinal haemorrhages and intraretinal microvascular abnormalities (Figure 6.2 (C) and (D)). By 4 months, there were retinal new vessels in all quadrants (florid proliferative retinopathy) (Figure 6.2 (E) and (F)). She was treated with bilateral panretinal laser photocoagulation (Professor Kohner) which caused regression of the new vessels. She has subsequently undergone vitrectomy in the right eye (Mr Schulenberg), because of repeated vitreous haemorrhages.

A marked increase in her serum IGF-I levels occurred at 4 months which corresponded to the time when proliferative retinopathy was first noted (Figure 6.1). IGF-I levels subsequently returned towards baseline values following treatment (Table 6.3.1).

As part of a separate study investigating changes in retinal blood flow and the blood-retinal barrier during CSII (Kohner et al., 1987c; Plehwe et al., 1988), these parameters were also measured in this patient. Retinal blood flow was asssessed by the blue light entoptic technique (Fallon et al., 1986). No significant change in blood flow measured as blue light entoptoscopy (BLE) speed occurred in this patient during CSII treatment (Figure 6.3).



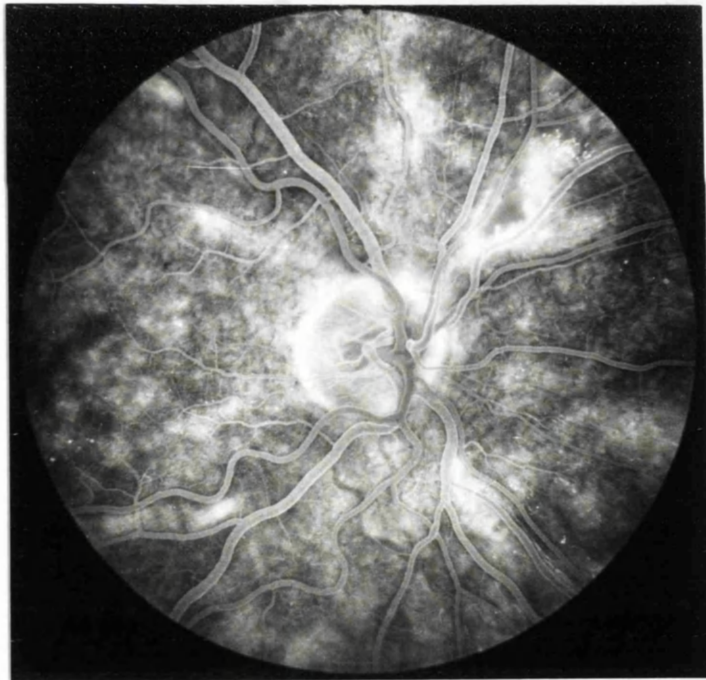
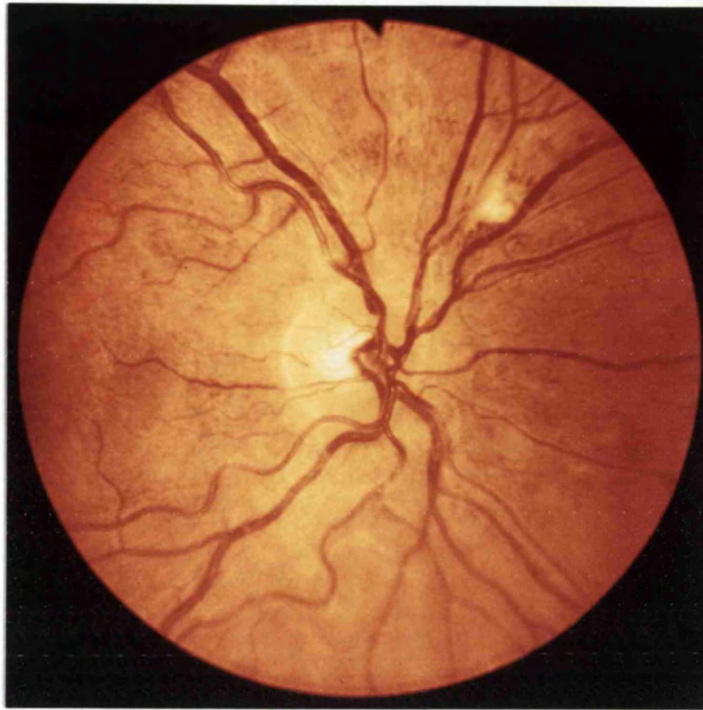


Figure 6.2 Right disc view at presentation in patient 6. Colour photograph (A) shows markedly dilated veins and a cotton wool spot (CWS). Fluorescein angiogram (B) shows areas of capillary non-perfusion.



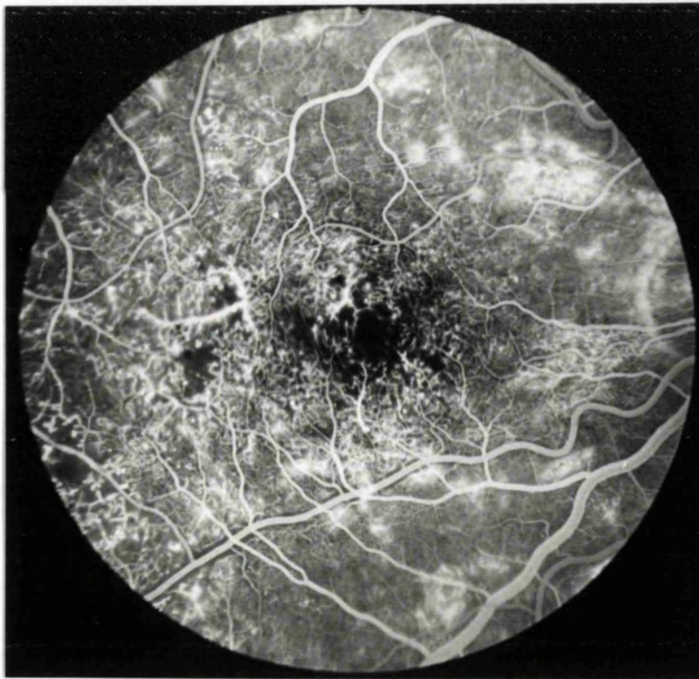
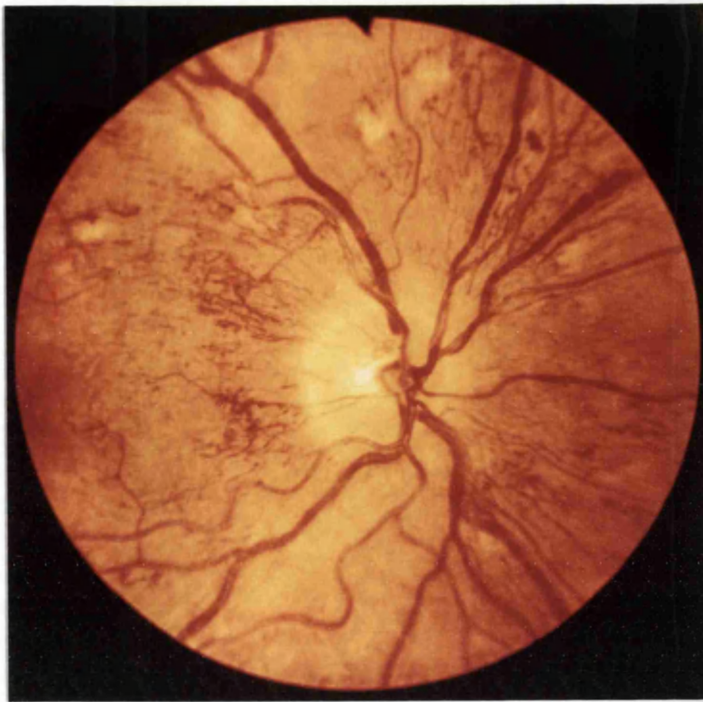


Figure 6.2 Appearances after 2 months of CSII. Colour photograph (C) shows early intraretinal microvascular abnormalities (IRMA) around the disc and in the periphery. Venous beading is present. Fluorescein angiogram of the right macular region (D) showing focal leakage, capillary non-perfusion and abnormal perifoveal vessels.



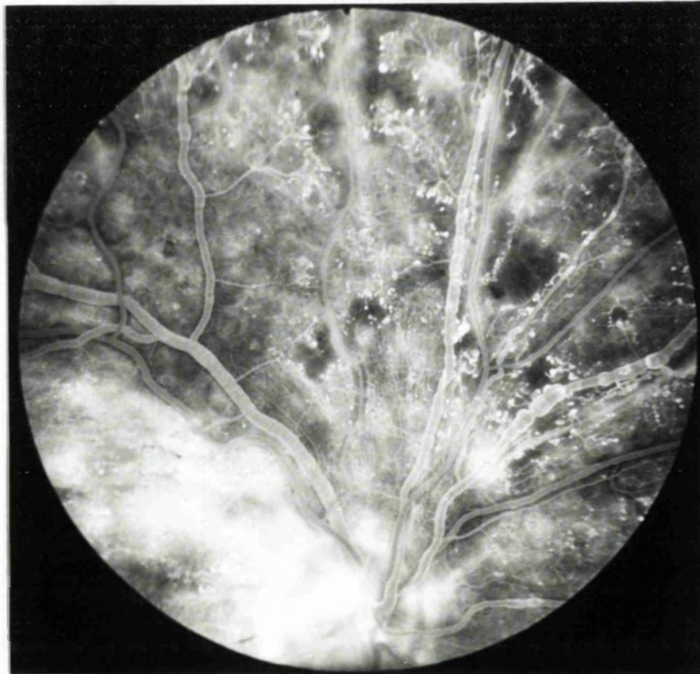


Figure 6.2 Appearances 4 months after CSII. There are now (E) new vessels around the disc and supranasally as well as increased numbers of IRMAs and CWS. At angiography (F), there are large areas of capillary non-perfusion, and extensive leakage of dye from new vessels at the disc. Marked venous beading is present.

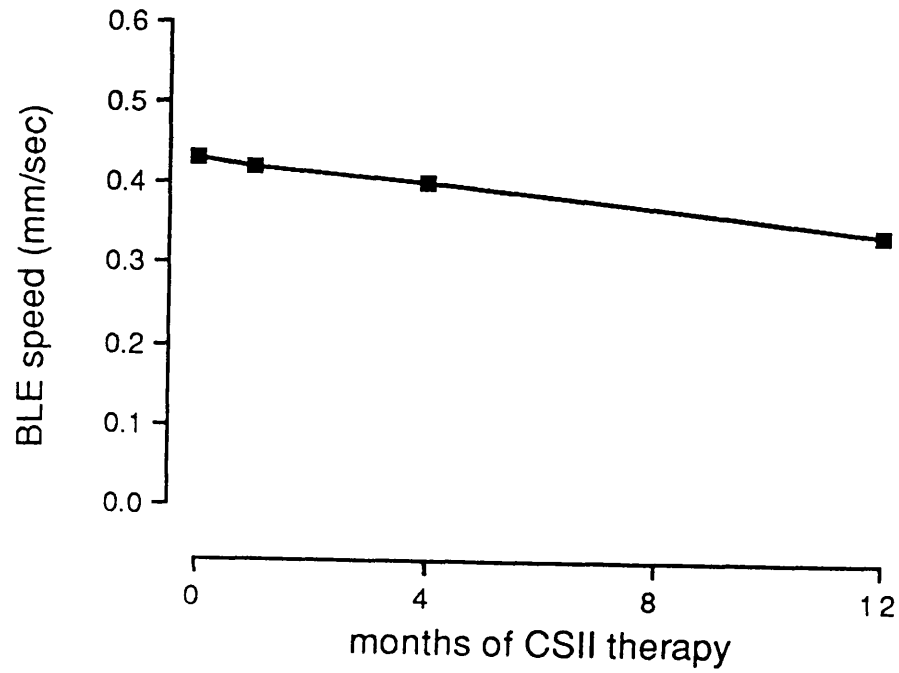


Figure 6.3 Change in BLE speed during CSII treatment. The patient developed proliferative retinopathy after 4 months of treatment.

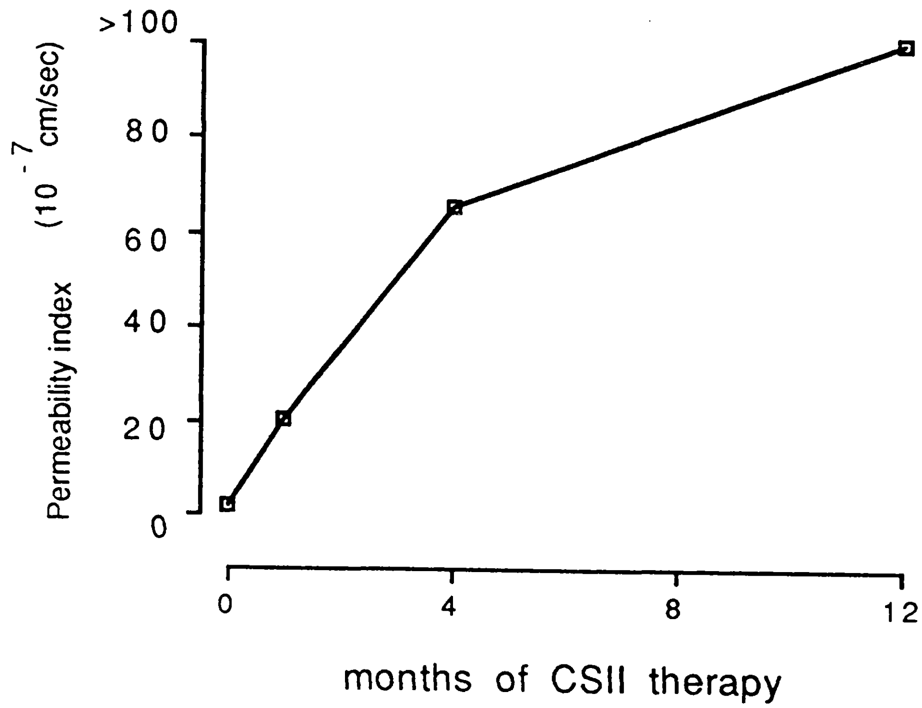


Figure 6.4 Change in Permeability Index during CSII in a patient who developed proliferative retinopathy after 4 months of treatment.

By contrast, there was a marked increase in the Permeability Index, an index of blood-retinal barrier permeability derived from analysis of vitreous fluorophotometry data (Chahal et al., 1985) (Figure 6.4). This increase in permeability corresponds to the extensive leakage of fluorescein from retinal new vessels seen at fluorescein angiography.

6.6 Discussion

Improvement in diabetic control by CSII resulted in a rise in serum IGF-I concentration in all but one patient. Nevertheless, values remained in the normal range apart from the one patient who developed proliferative retinopathy. Similar results were reported by Tamborlane (Tamborlane et al., 1981) who also noted a concomitant decrease in initially raised resting and exercise-induced GH levels. This latter finding suggests that the pump-induced increase in IGF-I results in feedback suppression of GH hypersecretion.

Although an inverse relation between serum IGF-I and glycaemic control (assessed by HbA_1) was demonstrated for the patients when considered together, it was not possible to show a significant difference in IGF-I values when comparing patients whose hyperglycaemia was corrected rapidly with those with slower correction of blood glucose. It proved difficult, however, to achieve the desired rates of reduction in glucose levels in the 2 patient groups and mean HbA_1 concentrations, although improved from initial values, remained outside the normal range throughout the study.

A difference may have been demonstrable with greater separation of the groups with respect to the rate of fall of blood glucose. Some patients with dramatic improvements in glycaemic control showed considerable corresponding rises in serum IGF-I. Patient 12, for example, showed a doubling of serum IGF-I during the first 4 months of CSII, when HbA_1 values had fallen from 10.3% to 5.8%. Similarly, IGF-I values more than doubled over 4 months in patient 6, associated with a 47% reduction in initial HbA_1 level. The rise in IGF-I in Tamborlane's study (Tamborlane et al., 1981) was associated with a mean fall in HBA_1 levels of 40%.

The mechanism of the rise in serum IGF-I during continuous insulin pump treatment is not known. There is in vitro evidence that insulin stimulates IGF-I production in the isolated perfused rat liver (Daughaday et al., 1976). In addition to reducing blood glucose levels, insulin pump treatment normalises many other metabolic abnormalities in diabetic patients including the restoration of normal lipid and amino-acid metabolism (Tamborlane et al., 1979b), normalisation of glucagon levels (Raskin et al., 1979), and reversal of urinary calcium and phosphate losses (Gertner et al., 1980). There may be an overall improvement in utilisation of metabolic substrates analagous to the rise in IGF-I seen after refeeding malnourished children (Hintz et al., 1978).

As regards the association of changes in retinopathy and serum IGF-I levels, no relation was observed in the patients when considered together, or when comparing patients with deterioriation of retinopathy with those who showed no such deterioriation. It would therefore seem unlikely that the rise in serum IGF-I was causally related to progression of background retinopathy. In some patients, for example patient 8, retinopathy score increased at times of no change or falling IGF-I values.

In other patients, for example patient 5, IGF-I levels doubled whilst retinopathy score improved. Only one patient, however, developed proliferative retinopathy, and in this patient (patient 6) the rise in serum IGF-I was striking and corresponded to the appearance of new vessels. It therefore remains possible that a sharp increase in IGF-I in an already ischaemic retina (cotton wool spots were evident at presentation) promotes retinal new vessel formation.

Two other patients had similar levels of retinopathy at entry to the study (patients 3 and 9). Patient 3, despite a 36% reduction in initial HbA_1 value over 4 months, failed to show a significant rise in IGF-I levels, and although experiencing a one stage deterioriation in retinopathy, has not developed proliferative changes at 2 years follow-up. Patient 9, has shown little overall improvement in diabetic control, and his retinopathy has remained stable. A combination of a high retinopathy score at entry and a marked rise in IGF-I was only found in patient 6.

Clearly other mechanisms could account for the deterioriation in retinopathy during CSII. The Oslo study group, for example (Dahl-Jorgensen et al., 1985), suggested that a sudden fall in retinal blood flow might initiate deterioriation of retinopathy when diabetic control is improved. Other investigators have also suggested that haemodynamic factors are important in the genesis of diabetic complications (Parving et al., 1983). Since hyperglycaemia appears to enhance retinal blood flow (Kohner et al., 1987c), rapid correction of hyperglycaemia would be expected to reduce flow and this may increase retinal ischaemia.

No such fall in retinal blood flow, as assessed by BLE speed, was detected in the diabetic subject developing new vessels. Indeed a small rise in BLE speed occurred 2 days after commencement of CSII (0.01 cm/sec). However, transient haemodynamic changes in the retinal circulation, particularly at times of rapid fluctuations in blood glucose levels, cannot be excluded in this or other patients.

A marked increase in vascular leakage as assessed by vitreous fluorophotometry, occurred in the patient with neovascularisation. In a parallel study (Plehwe et al., 1988), an increase in permeability index occurred in eyes that deterioriated after commencement of CSII. The implication from this vitreous fluorophotometry data is that there is disruption of the blood-retina barrier in those eyes that deterioriate, so that circulating free fluorescein leaks into the vitreous.

It is possible that other substances in the circulation may also leak from the vascular compartment into the vitreous and retina, such as IGF-I. Indeed, in a previous report (Grant et al., 1986a), vitreous IGF-I levels correlated with serum levels in diabetic patients with proliferative retinopathy but not in control subjects, suggesting that leakage is an important source of IGF-I in the vitreous of diabetic patients. If this was the case in the patient developing proliferative retinopathy, vitreous and retinal IGF-I concentrations would have been greatly increased, and this could then have promoted new vessel growth. However, the patients in Grant's study had suffered vitreous haemorrhages which probably explains the correlation between serum and vitreous levels.

As pointed out in chapter 5, it cannot be assumed that leakage at fluorophotometry correlates with leakage of IGF-I, given the fact that this method only detects leakage of free fluorescein which has a very much smaller molecular weight (376 Daltons) than IGF-I (7,500 Daltons) and the fact that the majority of circulating IGF-I is protein bound (Clemmons & Van Wyk, 1984).

6.7 Summary

An inverse relation between serum IGF-I and HbA_1 concentration, as found in the cross-sectional study in chapter 4, was again demonstrated, although it was not possible to correlate IGF-I values with the rate of correction of hyperglycaemia.

This relation may be relevant to the abnormalities of GH secretion associated with poor metabolic control. It could explain why, as control improves, GH levels return towards normal, since the rise in IGF-I will inhibit GH secretion. Feedback control mechanisms in diabetes will be further explored in chapter 11. No relation was found between progression of background retinopathy and serum IGF-I concentration, either when considering all patients together or when comparing patients whose retinopathy deterioriated with those who showed no such deterioration. This suggests that changes in circulating IGF-I during CSII do not play a major role in the development of preproliferative lesions. An increase in vascular leakage in those patients whose eyes deterioriate, could provide local increases in IGF-I but this is probably only important when vitreous haemorrhage has occurred.

A role for circulating IGF-I in promoting new vessel growth is suggested by the single patient who developed proliferative retinopathy during CSII. It is tempting to speculate that the dramatic increase in IGF-I levels in this patient promoted the formation of retinal new vessels in an already ischaemic retina. In order to further investigate the role of serum factors in diabetic retinopathy, the contribution of IGF-I and GH to the proliferative effect of diabetic serum on cultured endothelial cells, will next be examined.

Chapter 7. The relation between serum content of GH and IGF-I and the stimulatory effect of diabetic serum on cultured endothelial cells

7.1 Introduction

In chapter 5, diabetic patients were found to show raised serum IGF-I levels at the time when proliferative retinopathy was first recognised. The patient described in chapter 6 who developed new vessels during insulin pump treatment also demonstrated a dramatic rise in serum IGF-I. The effect of this increased serum concentration on endothelial cell proliferation will depend upon the proportion of IGF-I bound to carrier proteins compared to the proportion of the peptide in the free state. In addition, other serum factors will influence the action of IGF-I on cell growth (Heulin et al., 1987).

Recently, sera from diabetic patients with retinopathy have been shown to produce a mean threefold increase in ^{3}H-thymidine incorporation into human umbilical vein endothelial cells compared with that found with pooled non-diabetic sera (Petty et al., 1988a). Of particular interest, sera from diabetic patients with proliferative retinopathy stimulated endothelial cell growth significantly more than sera from patients with only background retinopathy (Petty et al., 1988a). Sera from patients with proliferative sickle retinopathy but no diabetes did not stimulate cell multiplication (Petty R, personal communication).

The stimulatory effect in that study showed cellular specificity, as the effects of sera from patients with retinopathy on human dermal fibroblasts or 3T3 cells were no different from controls.

In another study, Koh et al. (1985) demonstrated a stimulatory effect of diabetic serum on endothelial cells but did not attempt to characterise the stimulatory activity in terms of cell specificity and did not compare sera from patients with and without retinopathy. In preliminary studies, other investigators (Forrester, 1987), have reported that sera from patients with diabetic retinopathy stimulate retinal capillary endothelial cell migration more than sera from non-diabetics or diabetics without retinopathy.

The nature of the factor(s) responsible for the excessive stimulatory activity of diabetic serum is not known, but the fact that it was abolished by dialysis through a selective membrane with a 15kDa cut off suggests that the activity resides in a serum fraction with a molecular weight less than 15,000 (Petty et al., 1988a). This fraction might alone be responsible for cell stimulation, or might act by enhancing the effect of larger molecules present in excess in the diabetic sera, such as IGF-I or GH.

In order to investigate the possible relation of the stimulatory effect of diabetic serum to GH dependent growth factors, GH and IGF-I concentrations were measured in serum samples from patients divided into 3 groups (1) no retinopathy (2) background retinopathy or (3) proliferative retinopathy. These levels were then compared with the proliferative effect of the serum samples as assessed by ^{3}H-thymidine incorporation into actively replicating human umbilical vein endothelial cells (Petty, Hyer et al., 1989).

7.2 Patients and Methods

This work was carried out in collaboration with Dr R Petty and the Section of Vascular Biology, MRC Clinical Research Centre, Harrow. Sera was collected from 59 patients attending the Diabetic clinic at Northwick Park Hospital and the Diabetic Retinopathy Clinic, Hammersmith Hospital and stored at -30°C. Retinopathy was assessed by direct and indirect ophthalmoscopy, colour retinal photography and fluorescein angiography. Patients were divided into the following categories (1) no retinopathy (21 patients) (2) background retinopathy consisting of combinations of microaneurysms, haemorrhages, and hard exudates (16 patients) and (3) proliferative retinopathy defined by the presence of new vessels (22 patients). Age and sex matched normal controls were drawn from members of staff.

Human umbilical endothelial cells were isolated and cultured according to the method of Jaffe et al. (Jaffe et al., 1973) and were grown in medium 199 containing 10% fetal calf serum and 10% newborn calf serum. The cultured cells gave a positive immunofluorescent stain for von Willebrand factor when tested with a rabbit anti-human monoclonal antibody (CLBRAg) or an anti-human polyclonal antibody and they took up acetylated low density lipoprotein.

First passage endothelial cells pooled from more than one donor were seeded in medium 199 into 96 well microplates at a density of 10,000 cells per well. After 24h the medium was replaced with Ham's F12 medium containing the test serum, with 8-10 replicate wells for each test. 24 hours later, 1 microCi (1 microlitre) of ^{3}H-thymidine was added to each well. After a further 24h, the experiment was terminated and trichloroacetic acid-insoluble radioactivity in the cells was measured.

Serum GH and IGF-I concentrations were determined by radioimmunoassay as previously described (sections 4.2, 4.3). All samples were measured on the same GH or IGF-I assay.

7.3. Analysis

Results are expressed as percentages of ^{3}H-thymidine incorporation into cells exposed to 10% pooled human serum (reference serum). Data were analysed by a multiple means comparison (Peritz F) test.

The relation of ^{3}H-thymidine incorporation to serum concentration of GH or IGF-I was examined by linear regression analysis. Significance is taken as $p < 0.05$. Values are expressed as mean $\pm$ SD.

7.4 Results

^{3}H-thymidine incorporation into human umbilical endothelial cells expressed as percentage of reference serum, was significantly greater in patients with proliferative retinopathy compared with the other patient groups (370 $\pm$ 97.4 [proliferative retinopathy] vs 200.3 $\pm$ 78.2 [background retinopathy]; $p < 0.01$; 370 $\pm$ 97.4 vs 152.3 $\pm$ 70.8 [no retinopathy]; $p < 0.01$; Table 7.1). Mean serum GH values and IGF-I values were not significantly different in the three patient groups (Table 7.1).

The relation between serum GH and ^{3}H-thymidine incorporation in each patient group is shown in Figure 7.1. No significant correlation was found in any patient group or when all patients were considered together ($r = 0.78 \pm 3.41$; $p = 0.8$).

Figure 7.2 shows the relation between ^{3}H-thymidine incorporation and serum IGF-I in the three patient groups. Once again, no significant correlation was found in any patient group or when all patients were considered together ($r = -0.26 \pm 0.18$; $p = 0.16$).

Table 7.1 ^{3}H-thymidine incorporation into human umbilical endothelial cells by sera from diabetics with or without retinopathy compared with serum content of GH and IGF-I

Retinopathy	Number	$[^{3}H]$ Thymidine incorporation (% of control)	Serum IGF-I (mcg/l)	Serum GH (mU/l)
No retinopathy	21	152.3 ± 70.8	233 ± 69.1	2.78 ± 2.6
Background	16	200.3 ± 78.2	210 ± 70.2	4.14 ± 5.0
Proliferative	22	370.0 ± 97.4*	198 ± 60.9	3.38 ± 3.6

Mean ± SD

*$p < 0.05$ proliferative versus other groups

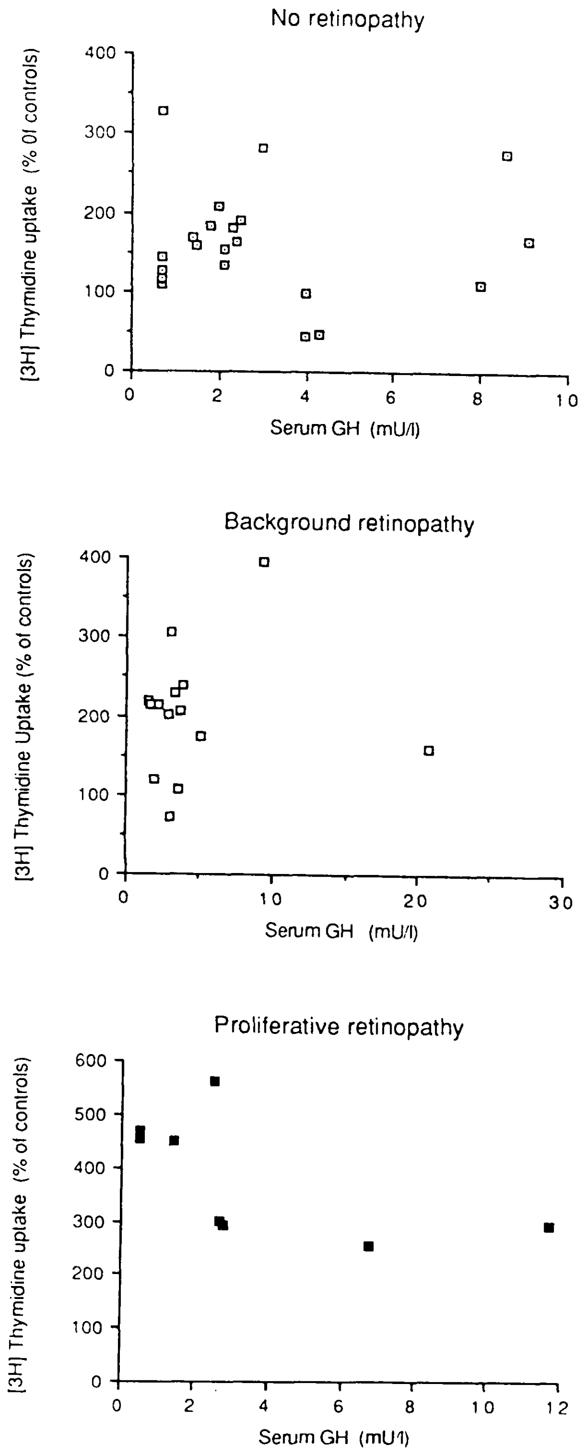
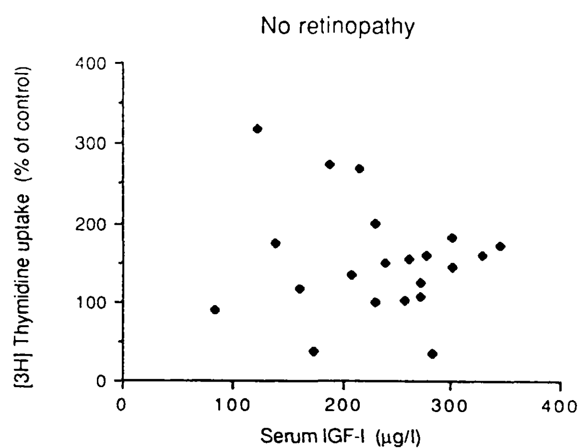
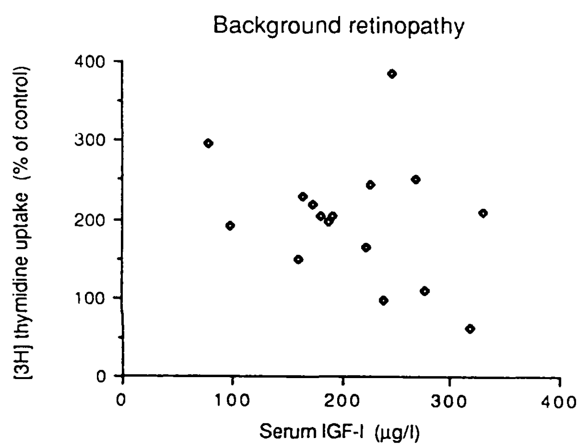


Figure 7.1 Stimulation of endothelial cell growth by sera from diabetic patients compared with serum GH concentration.





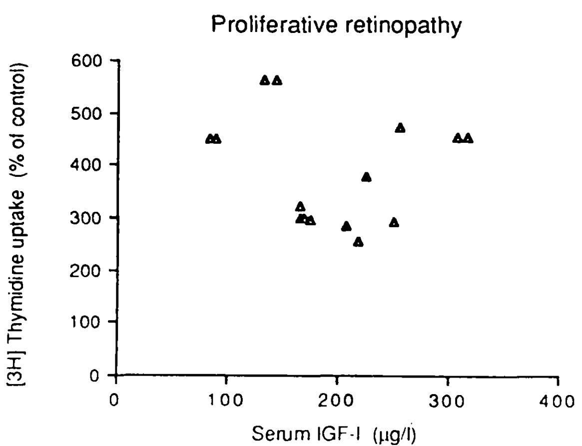


Figure 7.2 Stimulation of endothelial cell growth by sera from diabetic patients compared with serum content of IGF-I.

7.5 Discussion

In this study, no correlation was found between the stimulatory activity of serum from diabetic subjects with proliferative retinopathy and serum levels of IGF-I or GH. This, together with the cellular specificity of the stimulatory activity (Petty et al., 1988a), makes it unlikely that the activity is related to either of these growth factors. IGF-I, for example, is a potent stimulator of both dermal fibroblasts and 3T3 cells (Baxter, 1986). This does not discount a role for GH-dependent factors as permissive agents required for the maximal stimulation of endothelial cells induced by some other serum component(s).

A low molecular weight (300-600 Daltons) heat stable factor was isolated from normal human plasma by Heulin et al. (Heulin et al., 1987) which enhanced the growth promoting effects of IGF-I on a number of cell lines including chick embryo fibroblasts and human lymphocytes. Although it was not tested on similar cell lines as reported in the study of Petty et al. (Petty et al., 1988a), it clearly had broad spectrum mitogenic activity and was certainly not endothelial cell specific.

Lack of cell specificity also argues against the serum peptide reported by Koschinsky et al. (Koschinsky et al., 1981; Koschinsky et al., 1985) as being the factor responsible for the stimulatory activity. In addition, that serum peptide was only described in non-insulin dependent diabetics.

A small molecular weight growth factor (endothelial cell stimulating angiogenesis factor [ESAF]) which stimulated adrenal microvessel endothelial cells but not skin fibroblasts has been reported in the serum of patients with osteoarthritis (Odedra & Weiss, 1987). Its nature and structure are unknown and it has not yet been purified. Whether it is present in diabetic serum and its importance in angiogenesis remain to be determined. Others (West & Kumar, 1988) have suggested that the active component in the sera is a low molecular weight hyaluronate oligosaccharide which appears to exclusively stimulate endothelial cells.

The source of this low molecular weight factor in diabetic serum is not known but could be from the eye. Supporting this suggestion, preliminary work suggests that following successful panretinal photocoagulation for the treatment of proliferative retinopathy, previously increased serum stimulatory activity in these patients returns to normal (Dr R Petty, personal communication).

It should be noted that mean IGF-I levels were similar in all patient groups. It is possible that serum proliferative activity does relate to serum IGF-I in the early active phase of neovascularisation, at a time when IGF-I levels are elevated (section 5.4). Later, other serum factors may contribute to continuing cell stimulation or may be secondary factors, released by the proliferating tissue. Alternatively, it is possible that the low molecular weight factor has a permissive effect for cell stimulation by IGF-I or GH.

Preliminary data from diabetics with previously proliferative retinopathy who have subsequently undergone pituitary ablation indicates that the serum growth promoting activity is still present, which suggests that GH and IGF-I are not required for the serum stimulatory effects (Petty et al., 1988b). As yet, non-diabetic acromegalic serum has not been examined for its stimulatory activity, but the growth promoting effects on endothelial cells cannot be reproduced by adding GH to control serum.

Whilst no increase in mean serum GH was present in any of the patient groups, this does not imply that diurnal GH secretion was necessarily similar in the patient groups, as 24h GH secretion was not measured in this study. The results of 24h GH profiles in diabetics with retinopathy are described in chapters 9 and 10.

Finally, it is important to note that the results of these proliferation studies were obtained in human umbilical vein endothelial cells. Although similar growth stimulation was demonstrated with microvascular endothelial cells isolated from human omental fat (Petty et al., 1988a), it cannot be assumed that human retinal endothelial cells will respond in the same way to diabetic serum. Preliminary results using cultured bovine retinal endothelial cells, whilst showing that these cells are stimulated by retinal derived growth factor (bFGF), have not demonstated any excessive stimulation by sera from diabetics with proliferative retinopathy (Kinshuck et al., 1989).

This is unlikely to have been due simply to their bovine origin, as serum from diabetics with retinopathy affect migration of these cells to a greater extent than non-diabetics and diabetics without retinopathy (Forrester, 1987). Bovine retinal cells proliferate very rapidly in culture making it difficult to demonstrate a stimulatory response. It is possible, for example, that a stimulatory effect with diabetic sera may have been found with an alternative stain sensitive to small changes in cellular growth such as Page blue G90 (McIntosh et al., 1988).

7.6 Summary

In this study, no correlation was found between the excessive stimulation of human umbilical endothelial cells by sera from diabetics with proliferative retinopathy, and serum content of GH or IGF-I. The nature of the active component of serum responsible for the serum stimulatory activity is not known, but is unlikely to relate to either GH or IGF-I. However, this does not discount a role for either of these growth factors acting at a different phase in the process of neovascularisation.

Both circulating and local concentrations of growth factors are likely to be important in the formation of new vessels in diabetic retinopathy. In the following chapter, measurements of IGF-I and bFGF in vitreous, in retinal endothelial cell conditioned medium and in the extracellular matrix of the cultured cells will be examined.

Chapter 8: Levels of IGF-I and bFGF in vitreous and retinal extracts

8.1 Introduction

As discussed in chapter 3, an increase in concentration of IGF-I and bFGF at the level of the retina could play a major role in the process of angiogenesis in diabetic retinopathy. Such an increase could come about by excessive local production of growth factors in the retina or by leakage from the circulation.

Vitreous is a convenient ocular fluid in which to measure growth factors. IGF-I levels in the vitreous of diabetic patients with proliferative retinopathy are reported to be increased compared to non-diabetic control subjects (Grant et al., 1986a). Interestingly, vitreous IGF-I values in the diabetics but not the control subjects correlated with serum values, suggesting that vascular leakage was an important source of the increased IGF-I concentration in the vitreous of the diabetic group. A short report described an increase in bFGF in the vitreous of a patient with proliferative diabetic retinopathy (Baird et al., 1985b) but there are now doubts about the validity of the bFGF assay employed in that study (section 3.6 (3)).

In this chapter, the results of measurements of IGF-I and bFGF in the vitreous of diabetics with neovascularisation will be described and compared with levels in non-diabetic control subjects. In addition, local production and release of these factors by whole retina and cultured retinal endothelial cells will be examined.

8.2 Radioimmunoassay (RIA) for bFGF

An improved RIA has been developed (RA Brooks) using polyclonal antiserum directed against FGF 1-24. This antiserum was kindly donated by Dr A.Baird, Salk Institute for Biological Studies, California. Unlike the antiserum used in previous assays (Baird et al., 1985), this antibody no longer has N-terminal proline specificity and can measure other FGF forms (Baird & Ling, 1987). Purified recombinant whole FGF (1-146) (Amersham International) is used as standard and is iodinated by the chloramine-T method for use as radioactive tracer. This radiolabel is not susceptible to protease activity.

Bound reactivity is precipitated by a double antibody technique using goat anti-rabbit and non-immune rabbit serum. At concentrations of 8 mg/l (0.5 micromol/l), there is cross-reactivity with acidic FGF. The sensitivity of this assay is 0.3 mcg/l (18 pmol/l). The inter- and intra-assay coefficients of variation are 18% and 8% respectively.

8.3 Patients and Methods

Vitreous and serum samples were obtained from 7 patients at the time of vitrectomy at the Moorfield's Eye Hospital. Five patients were diabetic (mean age 44 years (range 20-63) in whom vitrectomy was indicated for persistent vitreous haemorrhage accompanied in 2 patients with retinal detachment. In the 2 non-diabetic subjects (aged 30 years and 44 years), one was undergoing vitrectomy because of heterochromic cyclitis with vitreous opacities and the other had suffered vitreous haemorrhage following branch retinal vein occlusion.

The sample of vitreous was immediately frozen at $-20^{O}C$. Samples for IGF-I measurement were acid-ethanol stripped prior to assay. IGF-I concentration was determined by RIA using polyclonal antibody as described in chapter 4 (section 4.2). The sensitivity of this assay was 5 mcg/l. Standards were made up in bovine vitreous or charcoal-stripped human serum devoid of growth factors, as appropriate.

For measurement of growth factors in whole retinal extracts, bovine retina was dissected, weighed, and placed in serum-free Dulbecco's Modified Eagle's Medium (DMEM) at $37^{O}C$ for 3 hours. Medium was then removed, centrifuged, and the supernatant assayed for IGF-I and bFGF.

Levels of IGF-I and bFGF were also examined in medium conditioned by bovine retinal endothelial cells (BREC). Second subculture cells were seeded at 10^5 cells/ml and cultured until the cells reached confluence.

The monolayers were then washed twice in Hank's buffered salt solution and then placed in serum free medium. The conditioned medium was harvested after 48 hours and levels of growth factors were assessed.

8.4 Results

Serum and vitreous measurements of IGF-I and bFGF are shown in Table 8.1. Mean $\pm$ SE serum IGF-I values (mcg/l) were similar in diabetic and control subjects (221 $\pm$ 37.6 vs 203 $\pm$ 17.5; $p > 0.05$). Mean $\pm$ SE IGF-I levels in vitreous (mcg/l) were also similar in all patients (27.4 $\pm$ 6.8 vs 23.3 $\pm$ 0.9; $p > 0.05$). One diabetic patient, however, was found to have a vitreous IGF-I concentration twice that of other subjects (52.8 mcg/l) (patient 1; Table 8.1). There was a history of recurrent vitreous haemorrhage in this patient. Basic FGF was undetectable in serum or vitreous samples.

Table 8.2 shows mean IGF-I and bFGF concentration in whole bovine retinal extracts and bovine retinal endothelial cell (BREC) conditioned media. No IGF-I was detected in the extract from whole retina, in contrast to bFGF which was readily detected after 3 hours incubation. bFGF was not found in the medium before this time. IGF-I was present in BREC conditioned medium (mean concentration 21.5 $\pm$ 2.8 mcg/l). bFGF could not be detected in cell conditioned medium after 48 hours. Even when the cells were incubated for a further 48 hours, bFGF remained undetectable in the cell conditioned medium.

Table 8.1 Serum and vitreous measurements of IGF-I and bFGF in diabetics with proliferative retinopathy and non-diabetic controls

Patient	IGF-I (mcg/l)		bFGF
	Serum	Vitreous	Serum or Vitreous
Diabetics			
1.	259	52.8	undetectable
2.	317	27.4	undetectable
3.	100	19.2	undetectable
4.	253	24.8	undetectable
5	176	12.8	undetectable
Non-diabetics			
1.	186	24.3	undetectable
2.	221	22.4	undetectable

Table 8.2 Mean concentration ± SD of IGF-I and bFGF in bovine retinal extracts and bovine retinal endothelial cell (BREC) conditioned media

Sample	IGF-I	bFGF
Whole retinal extract (ng/g wet weight retina)	Undetectable	12 ± 3.0
BREC conditioned medium (mcg/l)	21.5 ± 2.8	Undetectable

8.5 Discussion

Considerable difficulty was experienced in obtaining vitreous samples and hence the number of vitreous samples in this study was small. It is not possible to say from this data whether vitreous IGF-I levels are increased in diabetic compared to non-diabetic subjects. Nevertheless, evidence is provided that at least some diabetic patients have increased concentration of IGF-I in the vitreous. Values of IGF-I were higher in all subjects than those reported in a previous study (Grant et al., 1986a). Differences in extraction and processing of the samples probably account for the discrepancy. In this study, IGF-I was measured in pure vitreous whilst Grant and co-workers collected vitreous in buffer which was subsequently lyophilised and dialyzed - a process they found decreased IGF-I concentration by at least 50% compared to values obtained in pure vitreous.

In some patients, IGF-I presumably enters the vitreous at the time of vitreous haemorrhage. This would not account for all the IGF-I as vitreous haemorrhage was either absent (control subject) or occurred more than 6 months previously. Retinal endothelial cells may represent an additional source of IGF-I as, at least in vitro, they were able to release IGF-I into the surrounding medium. Other investigators have shown that human capillary endothelial cells are able to synthesize IGF-I binding proteins which may modulate their response to IGF-I (Bar et al., 1987a). It is likely that paracrine and/or autocrine mechanisms play an important role in the growth promoting actions of IGF-I (Underwood et al., 1986).

Normal vitreous has been found to inhibit angiogenesis in 2 animal models; tumour-induced neovascularisation in the rabbit corneal micropocket and retinal extract-induced angiogenesis in the chick chorioallantoic membrane assay (Patz et al., 1978; Lutty et al., 1983). The nature of the growth inhibitor in vitreous is not known. There is evidence that hyaluronate in normal vitreous inhibits endothelial cell proliferation (West and Kumar, 1988) and could therefore be important in controlling retinal vessel proliferation.

Vitreous also contains transforming growth factor beta (TGF-B) which is a potent inhibitor of bFGF-induced vascular endothelial cell proliferation (Frater-Schroeder et al., 1986; Heimark et al., 1986). bFGF stimulation of bovine retinal endothelial cells in vitro is completely inhibited by TGF-B at a concentration of 25 pg/ml (Bensaid et al., 1988) which is a concentration far below that found in the non-diabetic vitreous (3.6 ng/ml) as measured by a radioreceptor assay (Gaudric et al., 1988). Conversely, extracts of normal vitreous also possess mitogenic activity for a variety of cells, including fibroblasts, which is thought to be due to the presence of retinal derived growth factor (i.e. FGF) (Lutty et al., 1985).

In diabetics with proliferative retinopathy, the balance between stimulatory and inhibitory factors appears to be disturbed as vitreous from these patients has increased angiogenic activity (Hill et al., 1983; Lutty et al., 1986; Gaudric et al., 1988).

Increased concentration of IGF-I, bFGF and/or other growth factors or decreased inhibitor levels could contribute to this stimulatory activity present in diabetic vitreous. In this study, immunoreactive bFGF was not detected in any of the vitreous samples. This does not exclude a role for bFGF since concentrations as low as 0.08 mcg/l, below the sensitivity of the assay (0.3 mcg/l) still possess potent stimulatory effects on retinal capillary endothelial cells in vitro (Gospodarowicz et al., 1986).

In another study, both aFGF and bFGF were demonstrated in normal and diabetic vitreous by bioassay (Gaudric et al., 1988). In that study, no difference was found in bioactive bFGF levels in samples from diabetic and non-diabetic patients, although suprisingly there was increased TGF-B (6.72 $\pm$ 6.4 vs 3.6 $\pm$ 4.8 ng/ml; $p < 0.05$) in the diabetic group, albeit with much individual variability. The increased level of TGF-B in that study may have resulted from release by retinal pigment epithelial cells following previous laser treatment (Hayashi et al., 1988). The combination of increased TGF-B and increased angiogenic activity of the diabetic vitreous is not necessarily contradictory since under different circumstances, TGF-B can act as both a stimulator and an inhibitor of neovascularisation (Glaser, 1988).

Consistent with previous reports (Baird et al., 1985a; Klagsbrun et al.,1986), bFGF was present in extracts from whole retina but not in the conditioned medium of BREC. Unlike bFGF, IGF-I was not found in retinal extracts but was present in cell conditioned medium.

This suggests that the two growth factors are released by different mechanisms. Since the bFGF gene has no typical signal sequence (Abraham et al., 1986; Kurokawa et al., 1987), it is not thought to be released in the same way as normal secreted proteins. Endothelial cells appear to preferentially deposit bFGF into the subendothelial extracellular matrix rather than release it from the apical cell surface into the medium (Vlodavsky et al., 1987). Any bFGF that is released from the cell is likely to be rapidly bound to matrix components and to be undetectable in the cell medium (Sato and Rifkin, 1988).

bFGF has been localised by immunocytochemistry to the extracellular matrix and basement membrane of the capillaries of the inner nuclear layer and nerve fibre layer (Hanneken & Lutty, 1988) whilst aFGF is associated with the rod outer segments (Plouet et al., 1986). Microscopic examination of the retina at the time of removal of the medium showed disruption of the retinal layers, particularly at the level of the photoreceptors. This disruption and damage to the retina presumably resulted in FGF release.

These findings are consistent with the hypothesis that under normal conditions, retinal endothelial cells are maintained in a quiescent state because bFGF is sequestered within the cell or its basement membrane and is therefore inaccessible to stimulate proliferation or migration (D'Amore & Orlidge, 1988). Damage to the retina would then release bFGF from disrupted cells and associated cell membranes.

If ischaemic injury in diabetic retinopathy has a similar effect, the released bFGF will then be free to induce competence in surviving endothelial cells and fibroblasts. In addition, proteases liberated from the damaged retinal tissue and from macrophages attracted to the region of cell injury could release bFGF that has previously been sequestered in basement membranes (Vlodavsky et al., 1987).

The IGF-I pool does not appear to be as releasable from whole retina under these circumstances. On the other hand, actively growing and dividing endothelial cells are capable of secreting quantities of IGF-I which in other studies (King et al., 1985), have been shown to enhance their growth. The interaction of a high local concentration of bFGF in the microenvironment of the capillary space with IGF-I released from endothelial cells could then promote angiogenesis. Local IGF-I concentration will be enhanced by increased circulating GH whilst some IGF-I may arrive at the retina by leakage through a disrupted blood-retinal barrier.

The situation is likely to be far more complicated, however, as many other substances derived from serum or locally produced in the eye may influence the stimulatory activities of polypeptide growth factors. These include steroidal compounds, endothelial cell stimulating angiogenesis factor (ESAF), nucleotides, and heparinoids (Weiss et al., 1985; Folkman & Klagsbrun, 1987).

8.6 Summary

Although, IGF-I levels in samples of vitreous from diabetic and non-diabetic patients were similar, one diabetic patient was found to have an increased vitreous IGF-I concentration, twice that of other subjects. At least some patients with proliferative retinopathy may have increased vitreous IGF-I levels.

bFGF was not detected in diabetic or control vitreous although it may have been present at concentrations mitogenic to BREC but below the sensitivity of the assay. IGF-I but not bFGF, was demonstrated in BREC conditioned medium whereas bFGF and not IGF-I was present in an extract of retina in which the retina had become severely disrupted. bFGF appears to be released from within the cell or from the extracellular matrix by different mechanisms when compared to IGF-I. How this release comes about is not known but cell injury as may occur with ischaemic damage, is likely to be important. Local release of bFGF together with local production of IGF-I may promote angiogenesis in the ischaemic preproliferative retina.

Chapter 9: GH suppression by atropine and propanthelene in diabetics with proliferative retinopathy

9.1 Introduction

The beneficial effects of pituitary ablation or hypophysectomy in the treatment of diabetic retinopathy (Lundbaek et al., 1969; Kohner et al., 1972; Sharp et al., 1987) were thought to be due to removal of GH but as outlined in section 1.4, other factors released by the pituitary could also have been responsible for the improvement in retinopathy. More conclusive evidence of a role for GH in the process of neovascularisation would be provided by studying the effects of selective suppression of GH secretion on the growth of new vessels. Aside from theoretical considerations, pharmacological suppression of GH, if effective in inhibiting neovascularisation, would be a useful adjunct to photocoagulation in those patients who continue to develop retinal new vessels despite repeated laser treatment.

As previously discussed in section 2.3, cholinergic muscarinic receptor antagonists are potent and selective inhibitors of GH release under a variety of conditions including sleep (Peters et al., 1986), physical exercise (Casanueva et al., 1984) and stimulation by GHRH (Casanueva et al., 1986a).

Studies to date in diabetic subjects have shown that sleep related GH release is markedly reduced by the acute administation of pirenzepine (Page et al., 1987; Martina et al., 1987) or propanthelene (Davis and Davis, 1986) at bedtime. GHRH-stimulated GH release in diabetics, as in normal volunteers, has also been shown to be inhibited by pretreatment with atropine (Pietschmann et al., 1986). There is no information on the effect of these agents given for longer periods on 24h GH levels or on their effectiveness in diabetics with proliferative retinopathy.

The aim of this study was to assess the feasibility of GH suppression with two cholinergic antagonists, atropine and propanthelene, in patients with active proliferative retinopathy despite repeated laser therapy.

9.2 Patients and Methods

Seven patients with insulin-dependent diabetes and active proliferative retinopathy despite extensive and repeated photocoagulation were selected for the study. Serum creatinine was within the normal range in all patients; one patient had proteinuria by Albustix testing. The clinical details are shown in Table 9.1. Six non-diabetic normal volunteers (four males, two females; aged 24-33 years) were also investigated by the same procedure.

Table 9.1 Clinical characteristics of study patients

Patient	Sex	Age (years)	Body mass index (kg/m^2)	Duration of diabetes (years)	Proteinuria (g/l)	HbA_1 (%)
1	M	27	28.6	19	1	12.1
2	F	22	27.1	18	0	10.5
3	F	45	23.1	33	0	10.3
4	F	31	25.4	20	0	9.4
5	M	36	21.9	17	0	9.3
6	F	34	26.1	18	0	7.6
7	F	24	18.7	17	0	9.7

Informed written consent was obtained from all study subjects and the protocol approved by the Ethical Committee of the Royal Postgraduate Medical School and the Hammersmith Hospital. Baseline studies were proceeded by a full medical and eye examination including colour retinal photography and fluoroscein angiography. Blood was taken for measurement of glycosylated haemoglobin A_1 (HbA_1). Subjects were admitted to the Metabolic Unit for 24h studies of GH secretion after an overnight fast. An indwelling catheter was inserted into a forearm vein for the purpose of blood sampling.

Blood was drawn without disturbing the subjects for measurement of serum GH, IGF-I and plasma glucose at hourly (0600-2400) or 2-hourly intervals (2400-0600) with at least 21 samples over 24h. Subjects were encouraged to remain ambulant throughout the day and retired to bed at 2200.

Baseline 24h sampling was performed on 2 occasions separated by at least 2 weeks. Patients and control subjects then received, on separate occasions, in randomised order, oral atropine sulphate 1.2 mg taken from 0600 at 4h intervals or oral propanthelene bromide 30mg taken from 0600h at 6h intervals. After 2 weeks of treatment, 24h GH and IGF-I profiles were repeated. The laboratory analysis was performed masked to the subjects' current treatment.

Studies of stimulated GH release were performed at the end of the 24h baseline studies. Blood samples were drawn at 15 minute intervals for 30 minutes before and 90 minutes after a bolus of GH-releasing hormone 1-29 NH_2 (GHRH) 1 mcg/kg i.v. dissolved in 10ml of acidified saline and given over 2 minutes. This test was repeated after pretreatment with atropine 1.2 mg i.m. at -15 minutes or propanthelene 45mg p.o. at -30 minutes.

Serum was stored at $-20^{0}C$ until assayed. Serum GH and IGF-I were measured by specific double antibody radioimmunoassay as described in sections 4.2 and 4.3. HbA_1 was measured by cellulose acetate membrane electrophoresis; normal range 5.0-8.0 %.

9.3 Analysis

The 24h GH secretion was measured as the area under the GH curve calculated by the trapezoidal rule. Results were analysed non-parametrically; comparisons before and after treatments within the same group were made by the Wilcoxon test for paired data and between groups by the Mann-Whitney test. $P < 0.05$ was taken as the level of significance. Body mass index (BMI) was calculated as weight (kg)/ height $(m)^2$. Values in the text are expressed as mean $\pm$ SD.

9.4 Results

Atropine 1.2 mg by intramuscular injection, and propanthelene 45 mg by mouth, were effective in suppressing the GH response to GHRH in the patient and control groups (Figure 9.1). Table 9.2 gives the results of 24h studies. Baseline values on 2 control 24h periods did not differ significantly in either controls or diabetic subjects.

All subjects receiving atropine experienced an unpleasant dry mouth, difficulty in swallowing, blurring of vision and tachycardia. Symptoms were less severe with propanthelene. Two subjects (one patient and one control) could not tolerate oral atropine for more than a week; studies on them were performed at one week.

After treatment with oral atropine, the mean area under the GH curve (AUC) $\pm$ SD in the control subjects was reduced from 103 $\pm$ 53.1 (baseline) to 73 $\pm$ 83.6 $mU.l^{-1}.h^{-1}$, but this was not statistically significant ($p > 0.05$). Mean AUC after propanthelene treatment was 122 $\pm$ 71.6 $mU.l^{-1}.h^{-1}$, which was not significantly different from baseline values or from results after atropine ($p > 0.05$). There was no significant difference in numbers of GH peaks during either treatment compared to baseline.

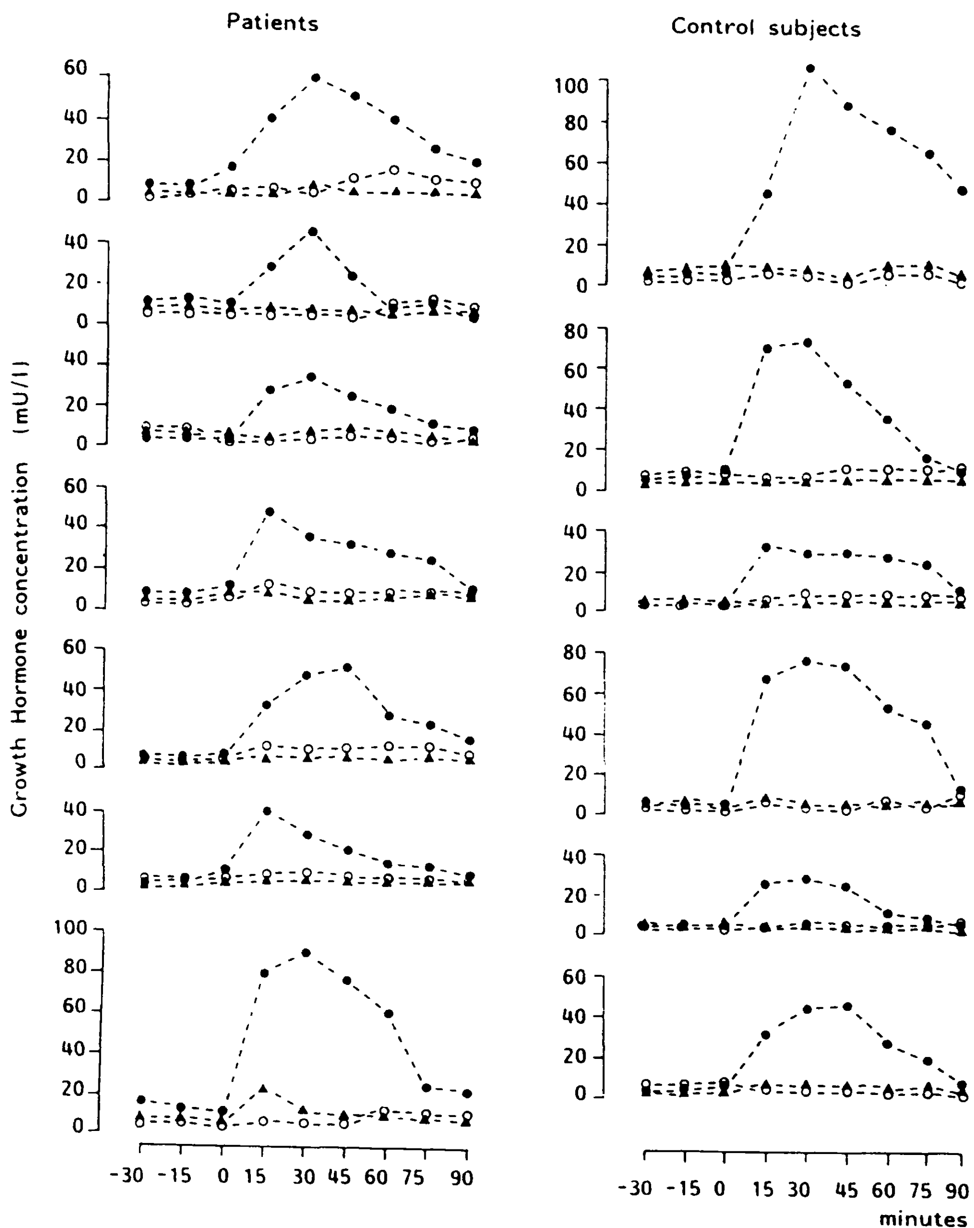


Figure 9.1 Serum GH responses to GHRH given at time 0 (closed circles) and after pretreatment with atropine 1.2mg i.m. at -15 mins (triangles) or propanthelene 45 mg p.o. at -30 mins (open circles).

Table 9.2 Area under the 24h GH curve (AUC) $mU.l^{-1}.h^{-1}$ before and after atropine and propanthelene

	Baseline*	Atropine 1.2 mg x 4h	Propanthelene 30mg x 6h
Control subjects			
1	47	20	71
2**	113	20	67
3	81	63	232
4	167	50	146
5	161	240	167
6	49	45	49
Mean ± SD AUC	103 ± 53.1	73 ± 83.57	122 ± 71.6
Patients			
1	428	161	145
2	247	145	236
3	138	68	110
4	258	104	107
5**	206	126	140
6	350	390	225
7	129	224	295
Mean ± SD AUC	251 ± 108.7	174 ± 106.9	180 ± 72.38

* Mean of two baseline profiles
** Indicates subjects who only tolerated atropine for 1 week

Baseline 24h GH secretion was greater in the 7 patients compared with the control subjects, although this did not achieve statistical significance (251 $\pm$ 108.7 vs 103 $\pm$ 53.1 $mU.l^{-1}.h^{-1}$; $p > 0.05$). Mean AUC compared with baseline values was not significantly reduced by either atropine (251 $\pm$ 108.7 vs 174 $\pm$ 106.9; $p > 0.05$) or propanthelene (251 $\pm$ 108.7 vs 180 $\pm$ 72.4; $p > 0.05$) in the patient group. The mean number of GH peaks during treatment compared with baseline values was not significantly reduced after atropine (4.14 $\pm$ 1.2 vs 2.7 $\pm$ 1.1; $p > 0.05$) or after propanthelene (4.14 $\pm$ 1.2 vs 2.86 $\pm$ 1.1; $p > 0.05$).

The pattern of GH secretion is illustrated in Figure 9.2 (control subjects) and Figure 9.3 (patients). GH peaks associated with hypoglycaemia (plasma glucose < 2.2 mmol/l) were not suppressed by atropine or propanthelene as illustrated by four representative patients shown in Figure 9.4. There was no difference in frequency of hypoglycaemic episodes during baseline measurements or during treatment with either drug.

Mean 24h serum IGF-I (mcg/l) was not significantly reduced after either drug in the normal volunteers (baseline 203 $\pm$ 47.9; after atropine 166 $\pm$ 34.3; after propanthelene 214 $\pm$ 57.4; $p > 0.05$) or in the patient group (baseline 279 $\pm$ 136.4; after atropine 195 $\pm$ 36.6; after propanthelene 180 $\pm$ 47.4; $p > 0.05$). Changes in mean serum IGF-I concentration are summarised in Table 9.3.

Table 9.3 Mean 24h serum IGF-I mcg/l ± SD at baseline, and after atropine and propanthelene

	Patients	Control subjects
Baseline*	279 ± 136.4	203 ± 47.9
Atropine	195 ± 36.6	166 ± 34.3
Propanthelene	180 ± 47.4	214 ± 57.4

* Mean of two baseline profiles

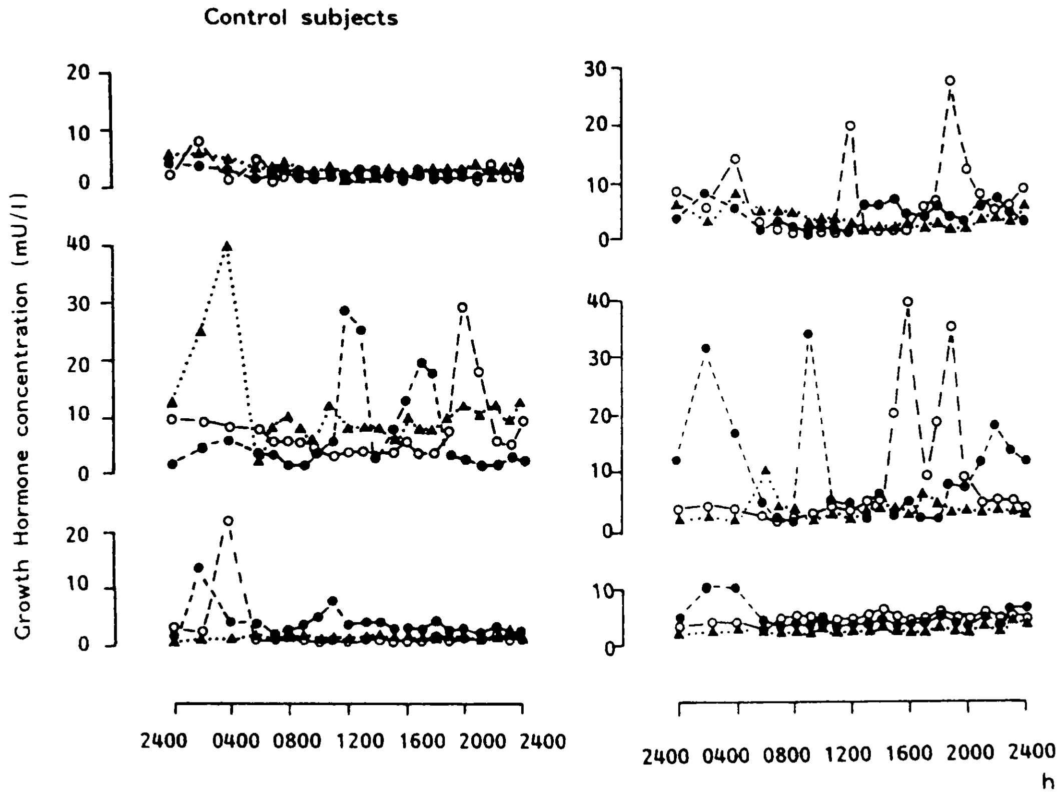


Figure 9.2 Twenty four hour serum GH profiles in six healthy control subjects at baseline (closed circles), after two weeks treatment with atropine 1.2mg orally x 6 daily (triangles) and after two weeks treatment with propanthelene 30mg x 4 daily (open circles).

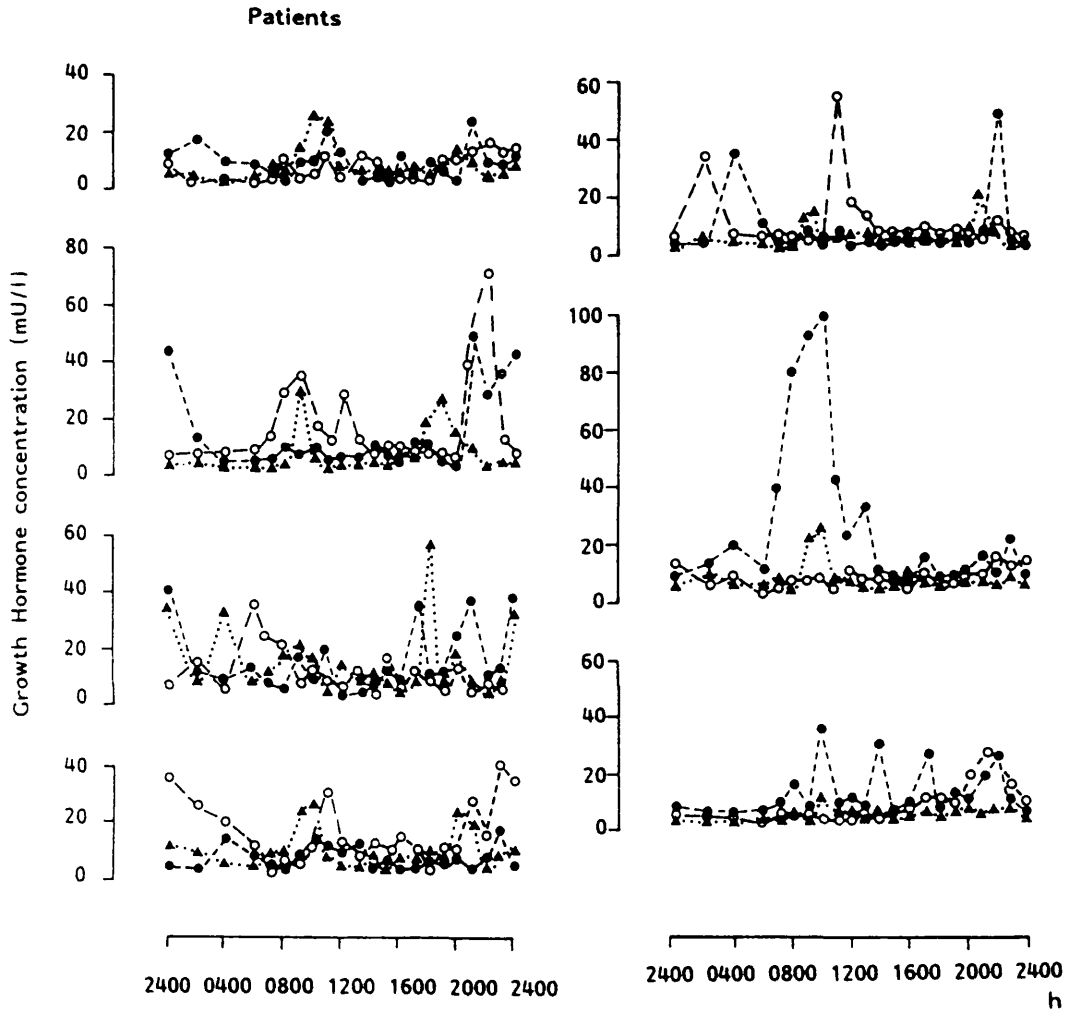


Figure 9.3 Twenty four hour serum GH profiles in seven diabetic patients at baseline (closed circles), after two weeks treatment with atropine 1.2mg orally x 6 daily (triangles) and after two weeks treatment with propanthelene 30mg orally x 4 daily (open circles).

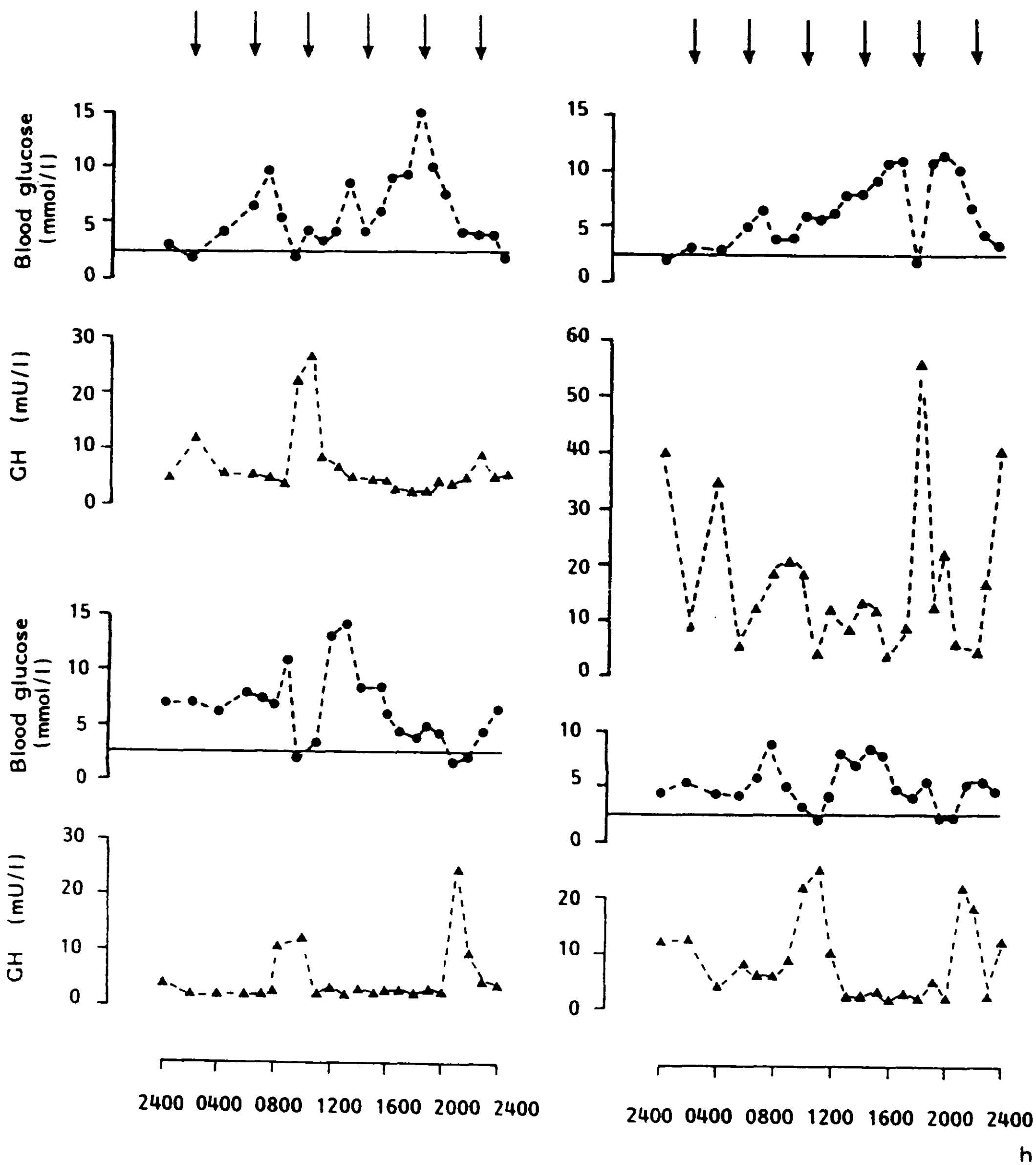


Figure 9.4 Twenty four hour GH and plasma glucose profiles in four diabetic patients taking atropine 1.2mg orally as indicated by the arrows. Plasma glucose values below the line shown are in the hypoglycaemic range (<2.2 mmol/l).

9.5 Discussion

Blunting of the GH response to GHRH in the presence of anticholinergic agents is well recognised (Casanueva et al., 1986; Pietschmann et al., 1986) and was demonstrated in both groups of subjects. There have been no previous reports of chronic treatment with these drugs on 24h GH levels.

In a previous study in normal volunteers (Taylor et al., 1985), daytime GH secretion was inhibited for 5h after the acute administration of atropine 0.6 mg orally, and was completely suppressed in two out of four subjects given 1.2 mg by mouth. Atropine 1.2 mg was therefore given at 4h intervals in an effort to abolish GH secretion throughout 24 hours. This dose was not effective in totally suppressing 24h GH levels in either normal controls or diabetic subjects.

With more frequent sampling, particularly at night, it may have been possible to demonstrate periods of complete GH suppression. Nevertheless, the finding of high GH levels in our samples demonstrates that GH secretion was not totally suppressed. This is disappointing as pituitary ablation was only really effective in the treatment of proliferative retinopathy when GH secretion was totally abolished (Wright et al., 1969; Adams et al., 1974). There was no discernible effect on retinopathy appearance over the study period.

As discussed in section 2.3, the site at which anticholinergic drugs exert their influence on GH secretion is not yet established. Data in animals suggest that the effect of cholinergic blockade on GH secretion is mediated through increased hypothalamic somatostatin secretion (Richardson et al., 1980; Locatelli et al., 1986). If an increase in somatostatin secretion is the principal mechanism by which these agents inhibit GH release, then the results of this study imply that the increased somatostatin production induced by atropine and propanthelene is not sufficient to suppress basal GH release in normal volunteers or diabetic subjects.

Inability to suppress the GH response to hypoglycaemia which has been previously reported (Blackard & Waddell, 1969; Evans et al., 1985), also implies that the induced somatostatin release is not sufficient to inhibit GH release under these circumstances. This sparing of the GH response to hypoglycaemia may be an advantage to the patient in avoiding the increased frequency of hypoglycaemia and brittleness of control that were major problems in patients after pituitary ablation (Sharp et al., 1987). Hypoglycaemic attacks were no more frequent or severe during treatment compared to baseline study days. On the other hand, this would make it difficult to maintain 24h GH suppression in patients liable to hypoglycaemic episodes.

Atropine would not be suitable for long-term use because of severe adverse effects. Propanthelene, a synthetic cholinergic antagonist was better tolerated than atropine. However, given at the maximally recommended daily dose of 120 mg (British National Formulary, 1988), it was not able to suppress 24h GH levels effectively in either group of subjects. A lower dose given more frequently may have been more effective.

It is not possible to exclude an acute suppressive effect of these drugs which subsequently became attenuated as tachyphylaxis to repeated doses developed. Nor is it possible to exclude rebound GH secretion if adequate drug levels were not maintained throughout the study days. However, larger doses given more frequently than 4-hourly would not have been acceptable to the subjects in this study.

The acute administration of a similar drug, pirenzepine, abolished sleep-related GH release in five diabetic patients (Page et al., 1987) and inhibited but did not totally suppress nocturnal GH secretion in eight insulin-dependent diabetics during a euglycaemic clamp (Martina et al., 1987). The effect of chronic administration of pirenzepine on 24h GH secretion in diabetic patients is not known, but there is evidence from studies of children with tall stature, that the effects of pirenzepine are transient and are accompanied by rebound GH secretion (Hindmarsh et al., 1987).

The patients in this study were chosen because of the presence of active proliferative retinopathy despite repeated laser therapy as they would be potential candidates for GH suppressive therapy. All patients had unsatisfactory glycaemic control as judged by HbA_1 results. Whether these drugs would be more successful in better controlled patients with milder grades of retinopathy is not known, but is unlikely as they were not effective in the normal control subjects.

9.6 Summary

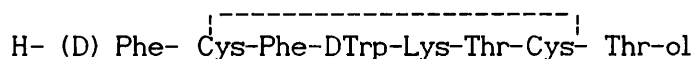
In this study, an attempt was made to suppress basal and GHRH-stimulated GH secretion in normal volunteers and diabetics with retinopathy. Both atropine and propanthelene completely inhibited the GH response to GHRH. By contrast, neither drug was effective in suppressing 24h GH levels. GH release at times of hypoglycaemia was not suppressed. Unpleasant side-effects were common with both drugs and more severe when taking atropine. It is probable that neither drug was able to maintain sufficiently high endogenous somatostatin release to completely suppress GH secretion over 24 hours. An alternative strategy, treating patients with continuous high doses of a somatostatin analogue, will be described in the following chapter.

Chapter 10: Effects of octreotide, a long acting somatostatin analogue, in diabetics with retinopathy

10.1 Introduction

Native somatostatin (SS), a 14 amino acid peptide, is a potent inhibitor of GH secretion and has been shown to suppress basal and stimulated GH release in diabetic patients (Hansen et al., 1973; Lundbaek & Hansen, 1980). However, its short half-life of a few minutes (Sheppard et al., 1979) and its relative inactivity after subcutaneous injection (Brazeau et al., 1974) requires it to be administered by continuous intravenous infusion. After administration, rebound hypersecretion of GH occurs (Besser et al., 1974). Furthermore, it is not GH selective, having multiple hormone suppressive effects (Gottesman et al., 1982). It therefore has little practical value for clinical use.

Recently, a somatostatin analogue has become available which has several advantages compared to native SS (Bauer et al., 1982). Octreotide, formerly known as SMS 201-995, is a synthetic cyclic octapeptide having the basic structure;



It has a longer half-life (113 minutes) than native SS because of its resistance to enzymatic degradation, and can be given by subcutaneous (s.c.) injection without rebound GH release (del Pozo et al., 1986).

Another advantage of the analogue is that, at least in animal studies, it is 45 times more potent than native SS in its inhibitory effect on GH secretion, whilst having less suppressive effects on glucagon and insulin release (Bauer et al., 1982). This raises the possibility that octreotide may be able to suppress GH secretion in diabetic patients without causing deterioriation in metabolic control.

Because its duration of GH inhibition in normal volunteers and diabetic patients is 3-6 hours (Davies et al., 1986a; Davies et al., 1986b), multiple daily injections are required to achieve 24h GH suppression. Alternatively, the analogue can be given by continuous subcutaneous infusion (CSI) (Christensen et al., 1987).

In this study, the effectiveness of octreotide in suppressing GHRH-stimulated GH release and 24h GH and IGF-I secretion was investigated in diabetics with retinopathy and healthy volunteers. The feasibility and effectiveness of long term treatment with the analogue administered by multiple injections or by continuous pump infusion was also examined in the diabetic patients. The effects of the drug on retinopathy appearance and metabolic control were also assessed in the patient group.

10.2 Patients and Methods

Nine patients with insulin-dependent diabetes and six age-matched normal control subjects were studied. Patients were selected if they had active proliferative diabetic retinopathy that had not responded adequately to photocoagulation (8 patients) or severe preproliferative changes in both eyes (1 patient) consisting of multiple haemorrhages, cotton wool spots and intraretinal microvascular abnormalities. No patient had significant fibrous proliferation at the time of the study. The clinical details of the patients are shown in Table 10.1. Informed written consent was obtained from all study subjects and the protocol approved by the Ethical Committee of the Royal Postgraduate Medical School and Hammersmith Hospital.

For studies of GHRH stimulation, serum GH levels were measured for 15 minutes before and every 15 minutes for 90 minutes after a bolus of GHRH 1-29 (Bachem) 100mcg i.v. dissolved in 10ml of acidified saline and given over 2 minutes. GHRH stimulated GH release was examined before and during an intravenous infusion of somatostatin (Sanofi) 2 $mcg.kg^{-1}.h^{-1}$ for 90 minutes (patients 1-4, 2 controls). Blood glucose in the patients was maintained in the normal range (3.5-5.5 mmol/l) by a variable i.v. infusion of insulin. In addition, GHRH-induced GH release in each subject was assessed before and after octreotide 50 mcg s.c. given as a single injection 30 minutes prior to the GHRH injection (patients 1-9, controls 1-6). Studies were performed after an overnight fast, with at least 2 weeks between each study.

Table 10.1 Clinical details of study patients

Patient	M/F	Visual Acuity R	Visual Acuity L	Age (years)	Duration diabetes (years)	BMI (kg/m^2)	HbA_1 (%) (a)	HbA_1 (%) (b)	Creatinine (micromol/l) (a)	Creatinine (micromol/l) (b)	Proteinuria (g/l)
1.	M	6/36	6/5	27	19	28.6	12.1	12.2	106	117	3
2.	F	6/12	6/36	22	18	27.1	10.5	12.0	106	80	2
3.	F	6/6	6/9	45	33	23.1	10.3	10.9	71	53	0
4.	F	6/9	6/9	31	20	25.4	9.4	9.1	72	80	1
5.	M	6/18	6/9	36	17	21.9	9.3	9.1	88	106	2
6.	F	HM	6/18	34	18	26.1	7.6	5.2	71	62	2
7.	F	6/18	CF	24	17	18.7	9.7	9.6	63	62	2
8.	M	6/6	6/6	28	25	24.8	11.8	9.8	88	97	2
9.	M	6/9	6/6	26	23	20.9	8.1	6.2	96	80	2

M/F: male/female R/L: right/left eye HM: Hand movements CF: Counting fingers BMI: Body mass index.

HbA_1 and serum creatinine are shown (a) before and (b) after treatment with octreotide.

All subjects were admitted to the Metabolic Unit for 24h investigations. They were also examined ophthalmologically which included colour retinal stereophotography and fluorescein angiography. Blood was taken for measurement of glycosylated haemoglobin A_1 (HbA_1). Two baseline 24h studies were performed during which blood was sampled at hourly intervals for serum GH and 6 hourly intervals for serum IGF-I. Subjects remained ambulant throughout the sampling day and retired to bed at about 2200.

In the first part of the study, four patients (patients 1-4; Table 10.1) and six control subjects received octreotide 50mcg by s.c. injection at 0600, 1400 and 2200 for 3 days and the 24h blood sampling was repeated.

In order to assess the feasibility of long term treatment with this drug, the above 4 patients were then instructed on self administration and continued treatment at home (median total duration of treatment 14 weeks; range 8-20 weeks). During this time, the daily dose of octreotide was increased at 2 weekly intervals from 100mcg x 3, to 200mcg x 3, to 500mcg x 3. All patients tolerated 200mcg x 3 for 2 weeks but only 3 patients agreed to receive the higher dose of 500mcg x 3 and continued it for 8, 12 and 16 weeks respectively. The fourth patient continued on 200mcg x 3 for 6 weeks. Treatment was stopped because of intolerable gastrointestinal symptoms particularly abdominal discomfort and steatorrhoea.

Blood sampling was performed during the last 24h of each treatment regimen. Plasma octreotide measurements were made immediately before and 1h after octreotide injections.

Six patients (patients 3, 5-9; Table 10.1) and four control subjects participated in the second part of the study investigating possible advantages of continuous s.c. pump delivery. Patient 3 entered this part of the study six months after completing the earlier section.

After the initial baseline measurements, continuous subcutaneous infusion (CSI) of octreotide by means of a Graseby MS-36 portable infusion pump was commenced. Subjects received 500mcg octreotide/24h which was continued for 3 days. At the end of this period, the GH profile was repeated. A futher 24h study was performed in subjects who appeared to show GH suppression during which the interval between blood samples from 2300 to 0800 was decreased to 20 minutes. Patients contined CSI treatment for median: 6 weeks (2-16 weeks) and were restudied in the last 24h of therapy. Plasma octreotide levels were measured in 2 samples from each 24h profile.

10.3 Assays

Serum was separated and stored at -20^{0}C until assayed. GH and IGF-I were measured by specific double antibody radioimmunoassay (sections 4.2 and 4.3).

Samples with values at the lower limit of detection of the IGF-I assay using monoclonal antibody, together with sera from pituitary ablated diabetic patients, were reassayed using polyclonal IGF-I antiserum (section 4.2). Both assays showed excellent correlation for IGF-I values in the 100-300 mcg/l range (Burrin et al., 1987) but the monoclonal assay was not suitable for values in the hypopituitary range as values lie below the sensitivity of this assay (80 mcg/l).

Octreotide was donated by Sandoz, Basel, Switzerland as was octreotide antibody and ^{125}I labelled antigen for RIA. HbA_1 was measured by cellulose acetate gel electrophoresis; normal range 5.0-8.0%.

10.4 Analysis

24h GH secretion was expressed as the area under the GH curve (AUC) calculated by the trapezoidal rule. Results were analysed non-parametrically; comparisons before and after treatment within the same group were made using the Wilcoxon test for paired data. The Mann-Whitney test was used to compare results between groups. Significance was taken as $p < 0.05$. Body mass index is calculated as weight (kg)/ height $(m)^2$. Duration of treatment is expressed as the median value together with the range of values. All other values in the text are expressed as mean $\pm$ SD.

10.5 Results

(1) Growth Hormone Concentration

The response to GHRH was similar in patients and control subjects. Somatostatin 2 $mcg.kg^{-1}.h^{-1}$ completely inhibited GHRH-induced GH release in the controls whilst GH levels were not totally suppressed in 2 of the 4 patients (patients 1 and 2; Table 10.1) (Figure 10.1). Rebound GH release occurred in all subjects on stopping the infusion. Octreotide 50mcg s.c. completely inhibited GHRH-induced GH release in both control and diabetic subjects (Figure 10.2).

Baseline 24h GH values were increased in the diabetic group compared to the non-diabetic normal controls (285 $\pm$ 99 vs 154 $\pm$ 72; $p < 0.05$). There was no significant suppression of 24h GH secretion after 3 days treatment with thrice daily octreotide injections in either the control subjects (Mean AUC $mU.l^{-1}.h^{-1}$ $\pm$ SD; baseline 154 $\pm$ 72 vs 94 $\pm$ 69 ; $p > 0.05$) or in the patients (baseline 285 $\pm$ 99 vs 213 $\pm$ 74; $p > 0.05$).

Continued injection treatment for a total of 8-20 weeks failed to suppress 24h GH secretion in the patients; mean AUC 205 $\pm$ 28 (200mcg x 3 for 2-6 weeks); 234 $\pm$ 83 (500mcg x 3 for 8-16 weeks) as shown in Figures 10.3 to 10.4.

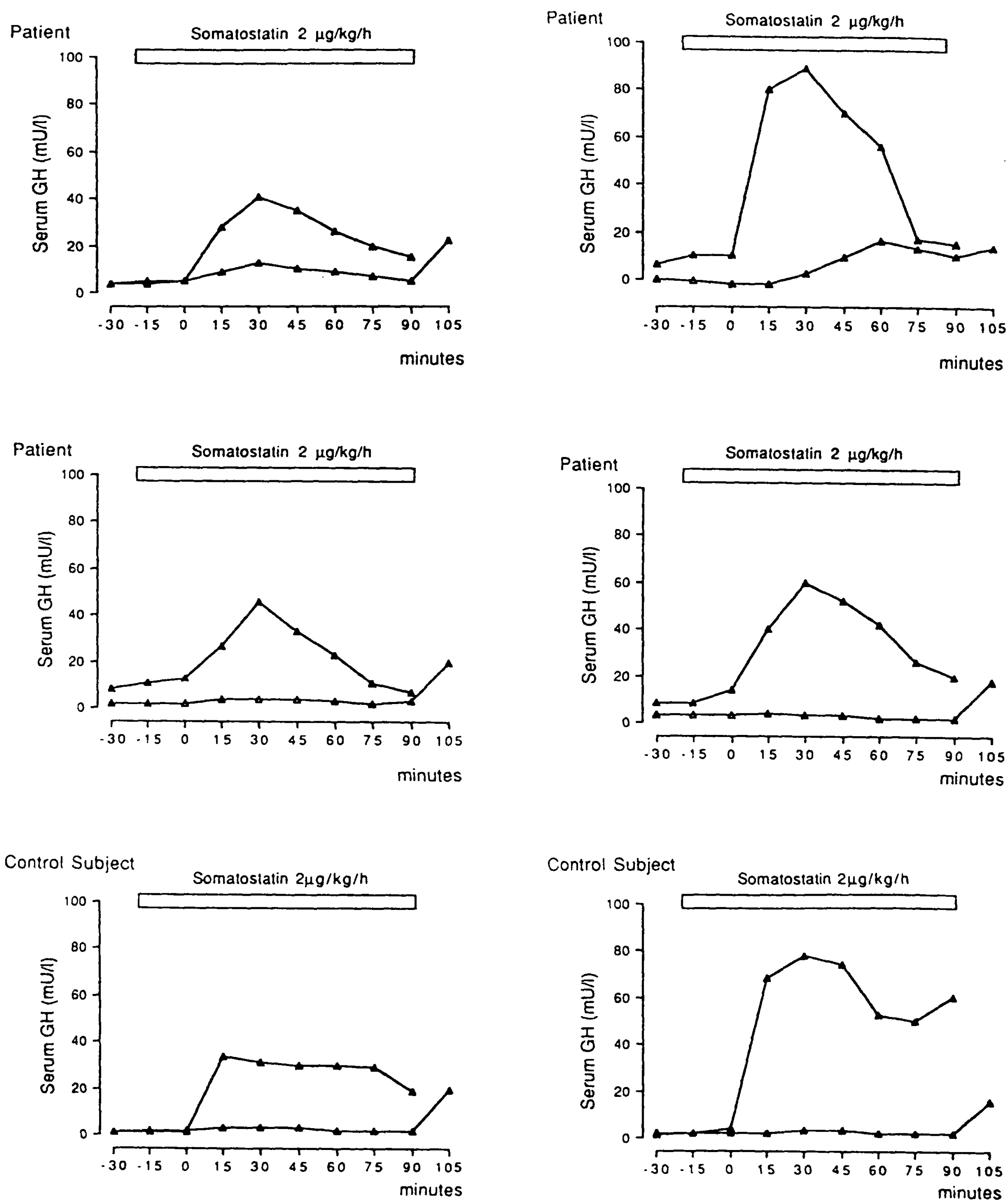


Figure 10.1 Serum GH responses to GHRH before (closed triangles) and during (open triangles) an infusion of native somatostatin in four patients and two control subjects.

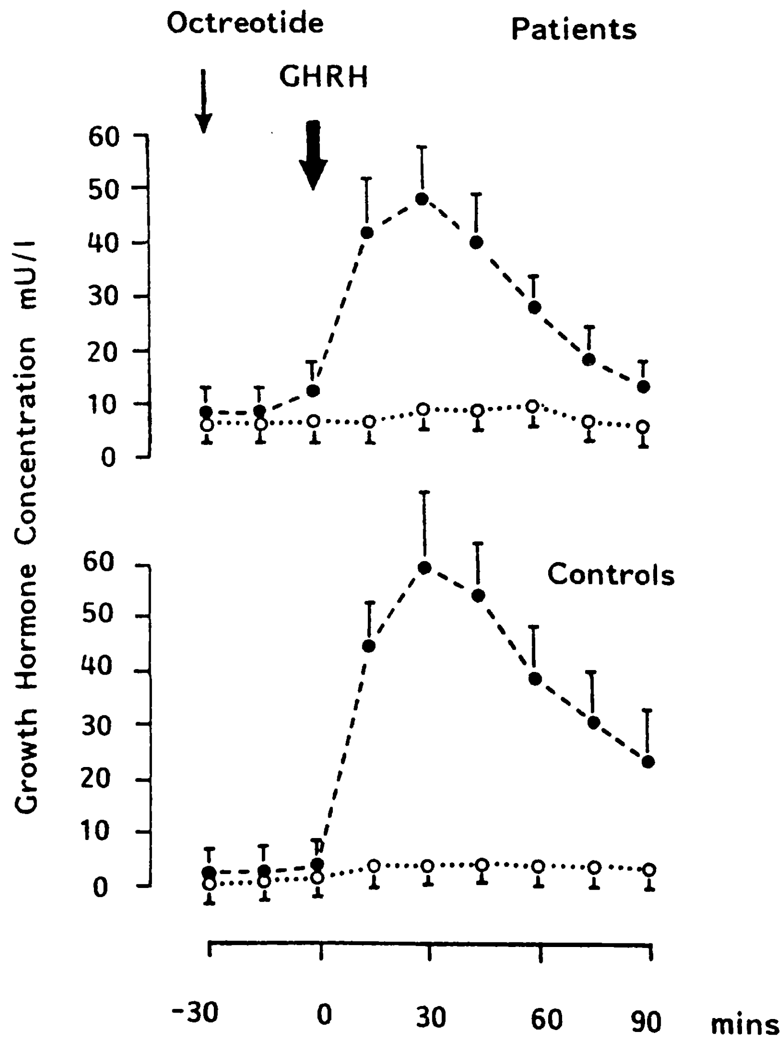


Figure 10.2 Serum GH responses to GHRH (open circles) and after (closed circles) pretreatment with octreotide 50mcg sc in four patients and six healthy volunteers.

Table 10.2 Mean $\pm$ SD 24h area under the GH curve (AUC) and IGF-I concentration in patients and normal volunteers before and after treatment with octreotide

Group	Octreotide treatment	Duration therapy median (range)	AUC ($mU.l^{-1}.h^{-1}$)	IGF-I (mcg/l)
Controls	Baseline	-	154 ± 72	211 ± 57
	50mcg 3 x daily	3 days	94 ± 69	$125 \pm 48^{*}$
	500mcg/24h CSI	3 days	$< 20^{*}$	$128 \pm 44.8^{*}$
Diabetics	Baseline	-	$285 \pm 99^{**}$	203 ± 62.3
	50mcg 3 x daily	3 days	213 ± 74	240 ± 58.4
	200mcg 3 x daily	14 (8-20)wks	205 ± 28	195 ± 72.8
	500mcg 3 x daily	14 (12-20)wks	234 ± 83	180 ± 74.8
	500mcg/24h CSI	3 days	$138 \pm 62^{*}$	$102 \pm 33.2^{*}$
	500mcg/24h CSI	6 (2-16)wks	$121 \pm 82^{*}$	$60 \pm 25.0^{*}$

$^{*}p < 0.05$ comparing values after treatment with baseline values

$^{**}p < 0.05$ baseline values patients vs baseline value controls

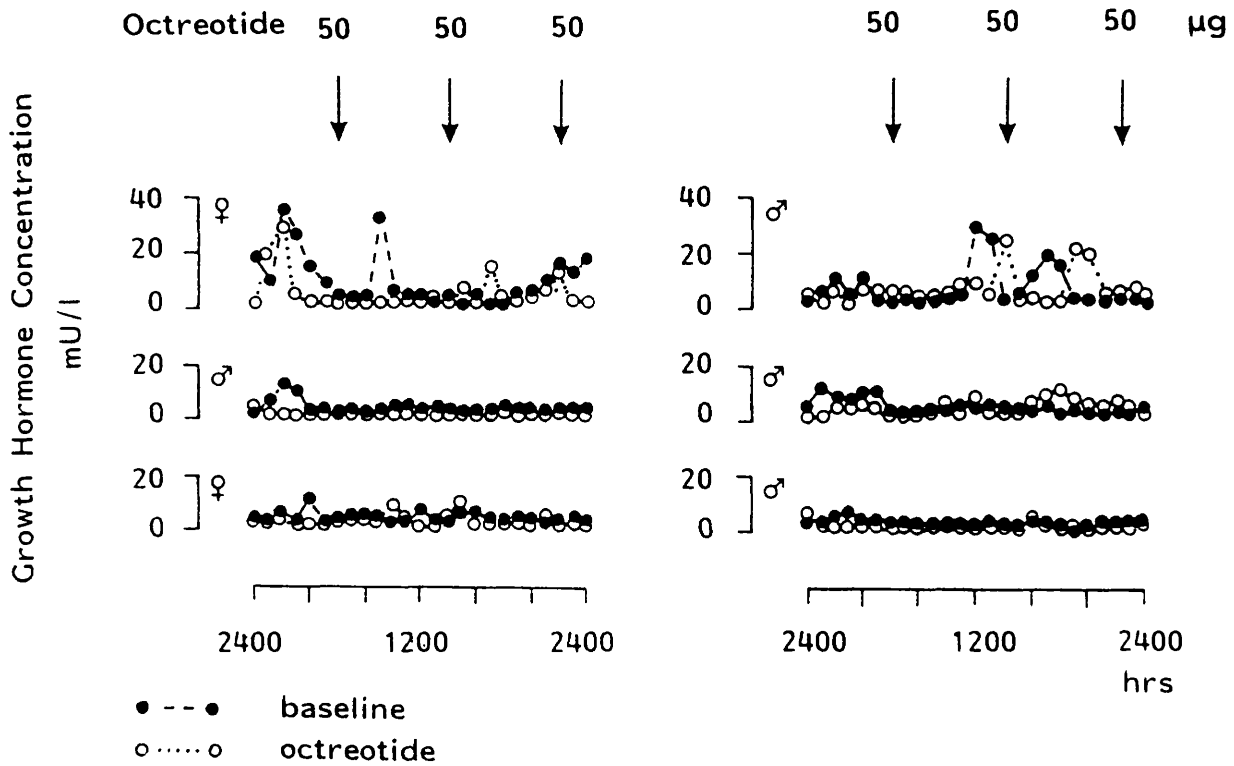


Figure 10.3 Twenty four hour serum GH profiles in six healthy volunteers before and after three days of octreotide 50mcg x 3 daily.

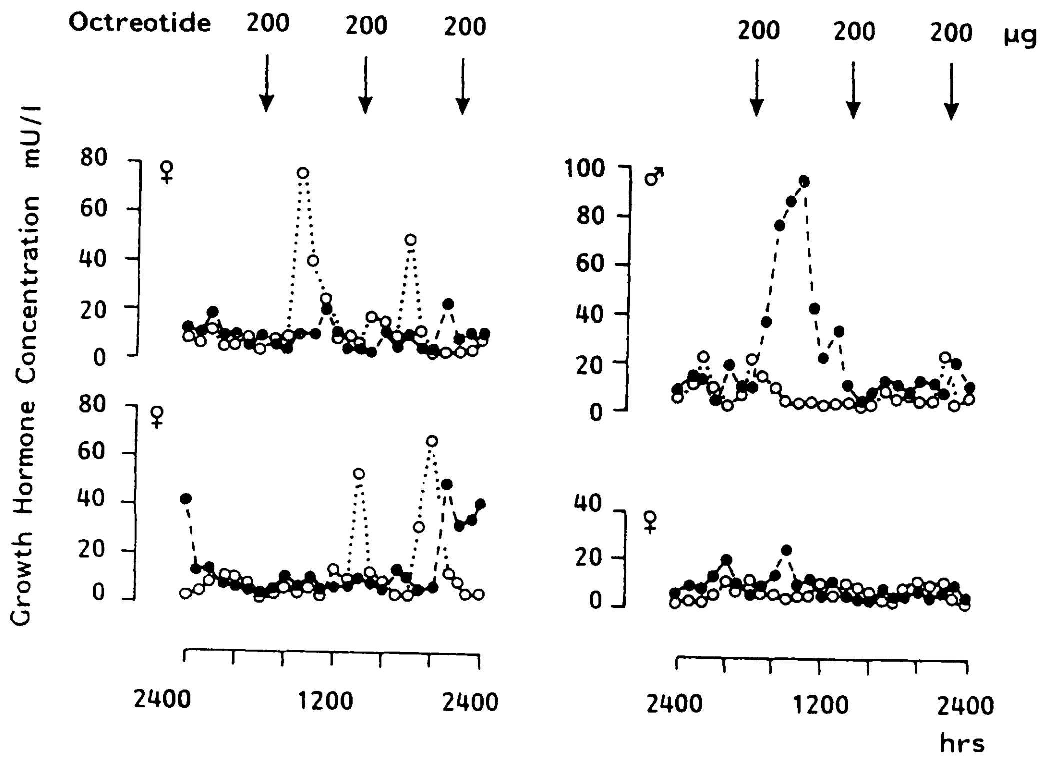


Figure 10.4 Twenty four hour serum GH profiles in four diabetic patients receiving octreotide 200mcg x 3 daily for 2 - 6 weeks. Baseline (closed circles); after treatment (open circles).

By contrast, continuous subcutaneous infusion (CSI) (500 mcg/24h) of the analogue for 3 days completely suppressed GH secretion in 4 control subjects confirmed by 20 minute sampling overnight (all GH values <0.5 mU/l). 24h GH concentration in 6 patients was reduced but not totally suppressed after 3 days (138 $\pm$ 62) and (2-16) weeks (121 $\pm$ 82); $p < 0.05$ compared to baseline values (Table 10.2). The pattern of GH secretion during octreotide treatment is illustrated in Figures 10.3 -10.6.

One patient (patient 5) with poor diabetic control received 6 weeks CSI octreotide on 2 occasions, initially (HbA_1: 9.3%) and again when control had been improved (HbA_1: 7.3%). The interval between the studies was 10 months. Baseline 24h GH measurements (AUC $mU.l^{-1}.h^{-1}$) were reduced by improved control from 193 to 101, associated with a rise in mean IGF-I from 121 mcg/l to 174 mcg/l. After continuous s.c. octreotide, 24h GH secretion (AUC) was 110 (poor control) compared to 80 (improved control); Figure 10.7.

(2) Plasma octreotide concentration

The relation of GH concentration to plasma octreotide levels is shown in Figure 10.8. Plasma octreotide immunoreactivity was consistently high during pump treatment in all patients and control subjects with levels greater than 2.5 $mcg.l^{-1}$ whilst levels immediately preceding injection treatment were variable in both groups of subjects.

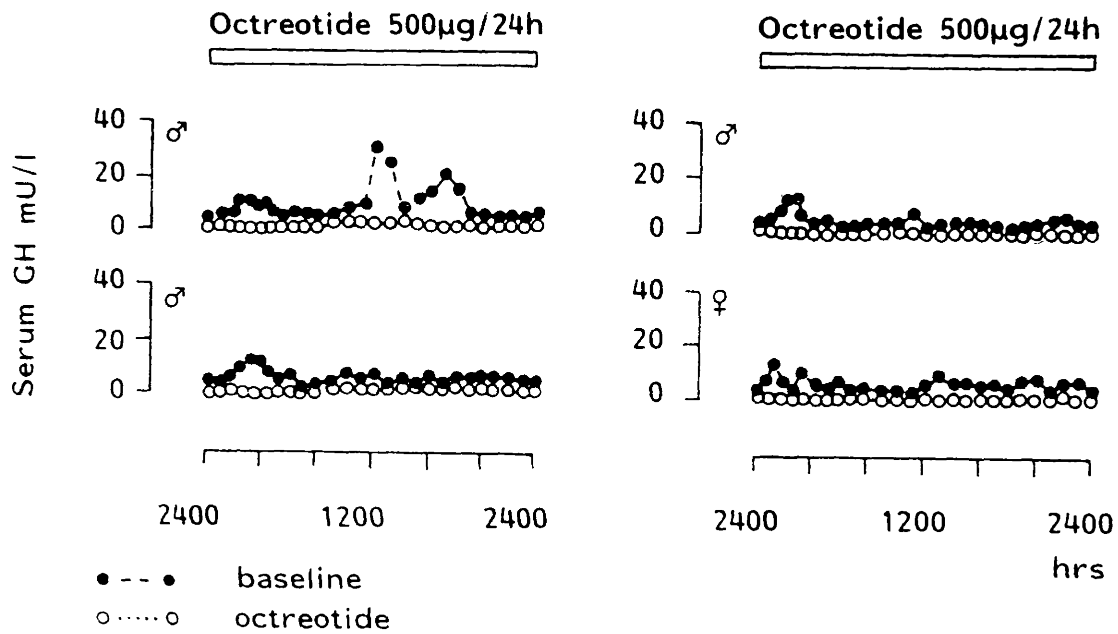


Figure 10.5 Twenty four hour serum GH profiles in four healthy volunteers before and after 3 days continuous octreotide infusion.

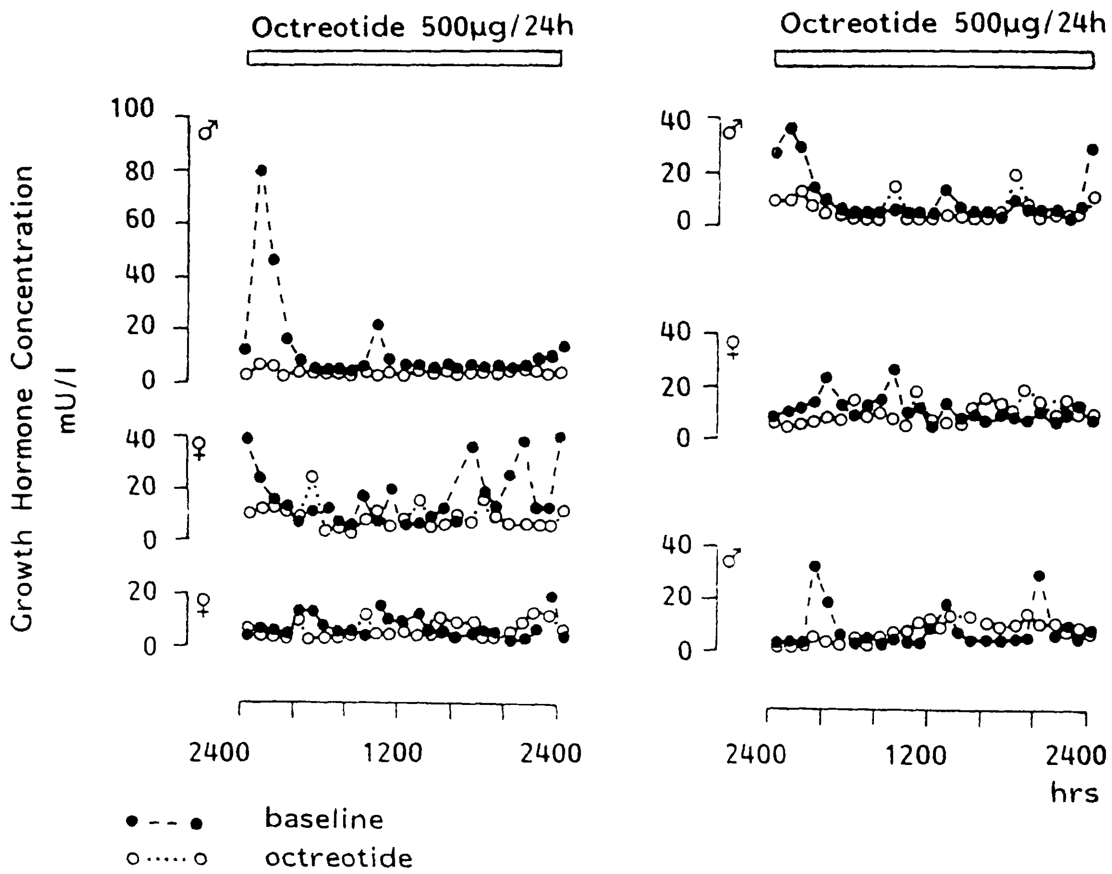


Figure 10.6 Twenty four hour serum GH profiles in diabetic patients before and after 6 weeks (2-16) continuous octreotide infusion.

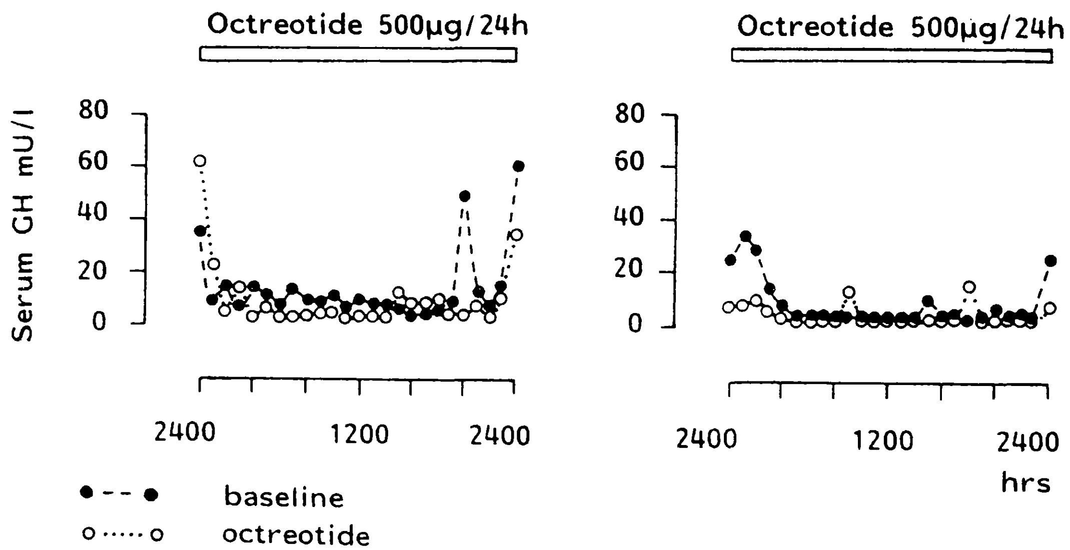


Figure 10.7 Twenty four hour serum GH profile in a patient receiving continuous octreotide pump treatment studied during poor diabetic control (left panel) and again when control had been improved (right panel).

Plasma trough levels of octreotide immunoreactivity greater than 1 mcg/l were sufficient to suppress serum GH in the control subjects. Breakthrough GH secretion in the control subjects occurred on injection treatment when drug levels fell below this level. However, despite high circulating octreotide levels during injection or pump treatment, spontaneous GH peaks continued to occur in the diabetic patients.

(3) Serum IGF-I concentration

Treatment in the patient group by continuous s.c. pump infusion for 3 days resulted in a significant fall in mean IGF-I (203 $\pm$ 62 vs 102 $\pm$ 33 mcg/l; $p < 0.05$). Continued pump treatment for 6 weeks (2-16 weeks) resulted in a much greater reduction in mean IGF-I levels (203 $\pm$ 62 vs 60 $\pm$ 25; $p < 0.05$) than 14 weeks (8-20 weeks) of octreotide by thrice daily injections; 219 $\pm$ 55 (baseline); 195 $\pm$ 73 (200mcg x 3); $p > 0.05$; 180 $\pm$ 75 (500 mcg x 3); $p = 0.05$; Table 10.2. IGF-I values in the patients at the end of pump treatment (median 6 weeks) were in the same range as 14 successfully pituitary ablated diabetic patients; Figure 10.9. Mean IGF-I concentration were reduced by pump (211 $\pm$ 57 vs 128 $\pm$ 45; $p < 0.05$) (n= 4) and injection treatment (211 $\pm$ 57 vs 125 $\pm$ 48) (n= 6) in the controls.

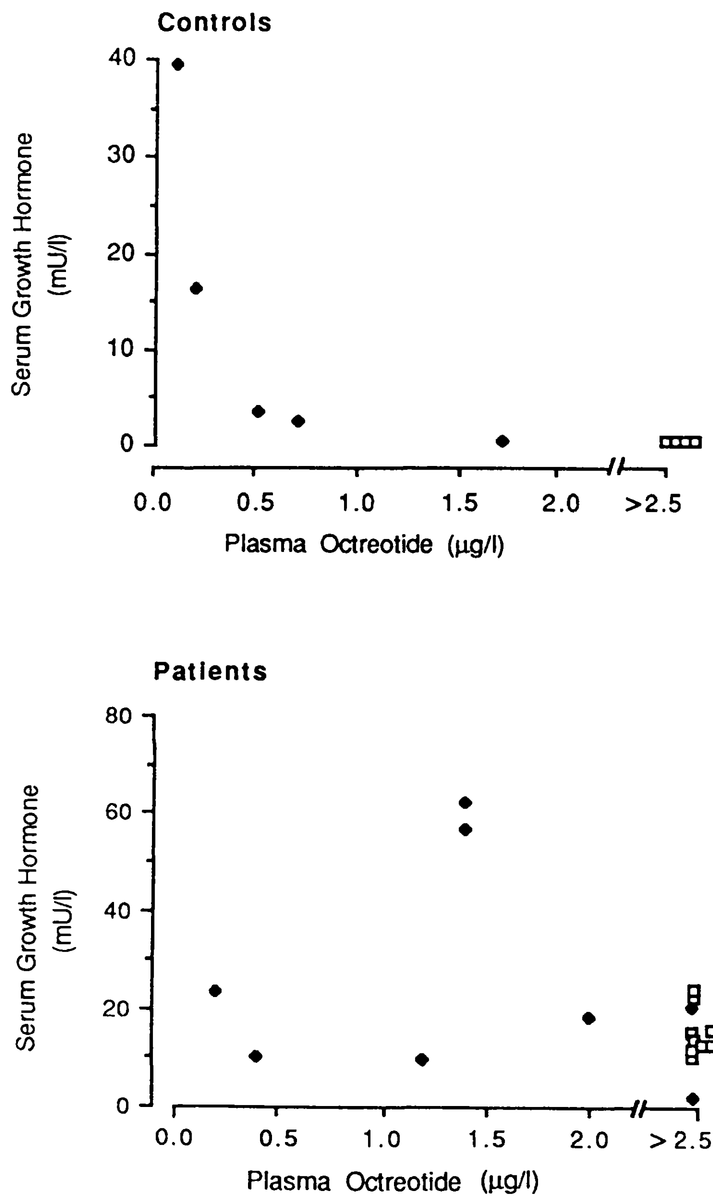


Figure 10.8 Serum GH and trough plasma octreotide values during pump treatment (open squares) and injection treatment (closed diamonds).

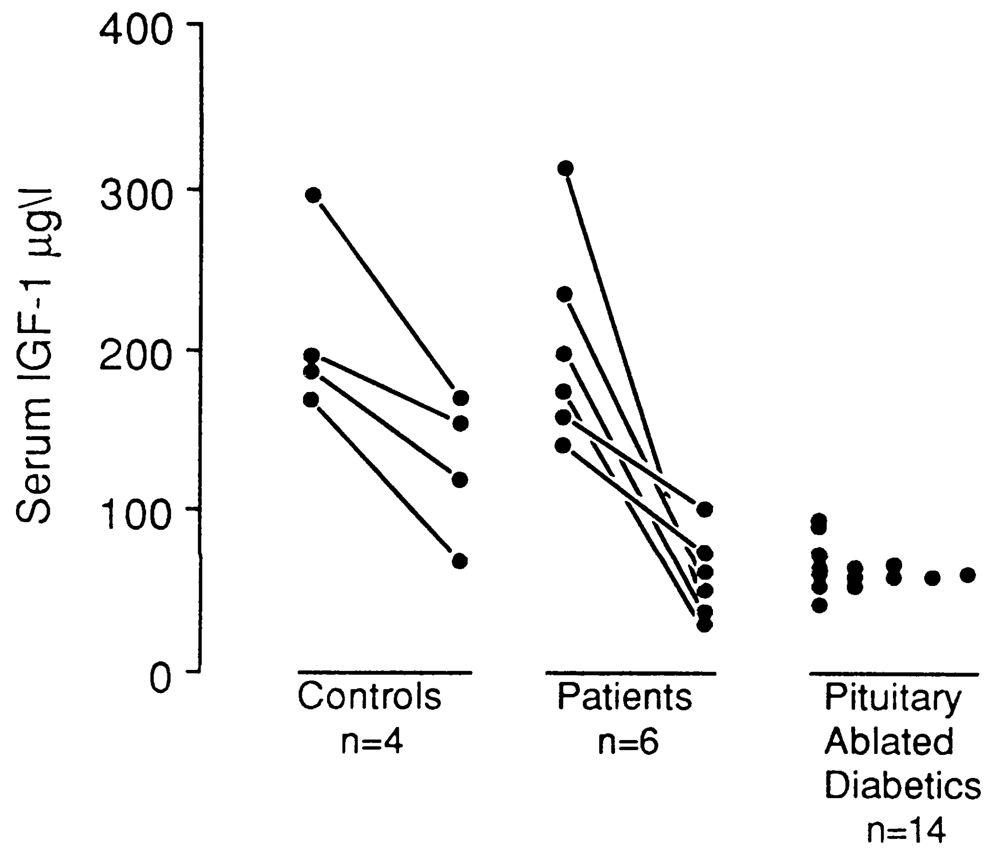


Figure 10.9 Mean serum IGF-I before and after octreotide by continuous pump infusion. IGF-I values in a group of pituitary ablated diabetics are shown for comparison.

(4) Ophthalmic assessment

During injection treatment, visual acuity deterioriated by at least 2 lines in 3 of 4 patients, whereas there was no change (5 patients) or improvement (1 patient) in vision in patients receiving pump treatment. Octreotide treatment did not prevent recurrent haemorrhages and further laser therapy was necessary in the 5 patients with active proliferative retinopathy. One patient who received 2 months of octreotide treatment by injection subsequently required vitrectomy 14 months after cessation of octreotide. There was no progression of preproliferative retinal changes (1 patient) during octreotide treatment.

(5) Adverse effects

All subjects experienced abdominal discomfort and passed pale offensive motions during treatment, particularly at doses in excess of 300mcg daily. The injections were found to be painful although suprisingly, continuous infusion did not produce much local discomfort. There was no tendency for gastrointestinal symptoms to improve with continued therapy. No relation of abdominal symptoms to the presence of autonomic neuropathy was found. One patient lost 7 kg in weight after 3 weeks of pump treatment and another experienced transient amenorrhoea. Daily insulin requirements fell by 50% with no significant change in mean HbA_1 values during the study period (Table 10.1).

There was a small increase in mean blood glucose levels in the normal volunteers during CSI treatment (4.7 $\pm$ 0.2 vs 5.4 $\pm$ 0.5 mmol/l). By contrast, there was an increased incidence of hypoglycaemia in the patient group which tended to be severe and slow to respond to oral glucose. No deteriortiation of renal function as assessed by serum creatinine and degree of proteinuria, occurred during treatment.

10.6 Discussion

In this study, continuous subcutaneous infusion at a dose of 500mcg /24h was superior to three eight hourly injections of up to to 1500mcg /24h in achieving consistently high serum octreotide levels and in suppressing serum GH and IGF-I concentrations. A similar finding was reported in a study comparing octreotide by CSI (100mcg/24h) and injection treatment (100mcg/24h) in patients with acromegaly (Christensen et al., 1987). Twice daily injections were not effective in suppressing GH secretion in a study of five non-insulin dependent diabetics (Davies et al., 1986b). In another study, three injections of 50mcg daily significantly but not completely reduced GH and IGF-I levels in eight insulin-dependent diabetics (Plewe et al., 1987).

Despite adequate drug levels (Kutz et al., 1986), GH levels in the patients in this study were escaping 2-4 hours after injections. Spontaneous GH peaks were also observed in the diabetics (but not in the control subjects) during CSI treatment, again when drug levels were high.

This persistence of GH secretion in the presence of octreotide is in contrast to the marked blunting of GHRH-stimulated GH release by the analogue. The implication from this data is that central regulatory mechanisms of basal GH release in diabetics with retinopathy, probably at the level of the hypothalamus, are relatively resistant to the effects of somatostatin. This would be consistent with the failure of glucose-mediated GH suppression in diabetic patients with retinopathy (Sharp et al., 1984b; Press et al., 1984a).

In addition, an abnormality at pituitary level cannot be excluded as native somatostatin in a dose that fully suppresses GHRH-induced GH release in normal subjects (Davies et al., 1985), failed to completely suppress GH release in 2 patients. However all patients showed rebound GH release on cessation of the infusion. Detailed dose-response studies are needed to confirm relative resistance to somatostatin in these patients.

Interestingly, although continuous infusion of the analogue only partially suppressed GH secretion in the diabetic patients, it reduced IGF-I levels into the same range as pituitary ablated diabetic patients. This could imply that octreotide acts peripherally, possibly on liver receptors, to inhibit IGF-I production. Prolonged treatment could also result in low IGF-I levels as a reflection of the patients' poor nutrition during treatment (Phillips, 1986) although only one patient lost a large amount of weight.

The combination of higher basal 24 hour GH secretion yet similar IGF-I levels in the patient group compared to the control subjects suggests that IGF-I production was already impaired in the diabetic subjects at entry to the study. This is consistent with reports of impaired IGF-I generation in poorly controlled diabetes (Lanes et al., 1985).

IGF-I production may therefore be particularly susceptible to suppression in poorly controlled diabetic patients although the duration of treatment in the normal control subjects may not have been long enough to achieve similar IGF-I suppression. Improvement in diabetic control is likely to increase the effectiveness of somatostatin in suppressing GH secretion as suggested by the results of the patient restudied after control had been tightened. Patients showed no overall change in diabetic control (as assessed by HbA_1 levels) during octreotide treatment. Others have reported diminished post-prandial hyperglycaemia after injections of the analogue before meals (Spinas et al., 1985; Serrono-Rios et al., 1986), probably resulting from glucagon suppression.

Although there was no deterioration in visual acuity during continuous infusion of octreotide, treatment for up to 16 weeks did not lead to regression of persistent new vessels and did not prevent further retinal haemorrhages. It should be noted that all but one patient had long standing proliferative retinopathy only partially responsive to extensive and repeated photocoagulation. At this stage of the eye disease, further progression may not be mediated by GH.

Interestingly, there has been no progression of retinopathy in the one patient with preproliferative changes during eight weeks of treatment. Clearly, more patients with preproliferative retinopathy will need to be studied for longer periods to confirm whether octreotide is of value in halting the progression of the eye disease.

Unfortunately, treatment was associated with unpleasant and persistent gastrointestinal symptoms in all subjects, which were relieved only by discontinuation of the drug. Diarrhoea and abdominal pain probably arise from changes in intestinal motility and reduction in pancreatic enzyme secretion secondary to gut hormone suppression by the somatostatin analogue (Williams et al., 1986). Similar symptoms were reported in normal volunteers (Davies et al., 1986a) and diabetics (Davies et al., 1986b) receiving only 50mcg twice daily. There is, in addition, a theoretical risk of developing malabsorption and gallstones after prolonged treatment (Dieguez et al., 1988b).

Inhibition of the insulin response to meals by octreotide is likely to account for the small increase in glucose levels in the non-diabetic subjects (Davies et al., 1986). By contrast, severe hypoglycaemia was a problem in the patient group despite a 50% reduction in total daily insulin dosage and was poorly responsive to oral glucose. This is reminiscent of the increased frequency of hypoglycaemic episodes and brittleness of control seen after pituitary ablation, and reflects the importance of GH as a counter-regulatory hormone in hypoglycaemia.

Unlike the cholinergic antagonists (chapter 9), octreotide inhibits hypoglycaemia-stimulated GH release (Lightman et al., 1986). In addition, glucagon levels are suppressed for up to 6 hours by the analogue (Davies et al., 1986a). Sudden severe hypoglycaemia has been reported after as little as 50 mcg octreotide s.c. in a patient with insulin-dependent diabetes (Navascues et al., 1988). Insulin-dependent diabetic patients about to be placed on this treatment need to be forewarned of the danger of hypoglycaemia and should perform frequent home glucose measurements. They should also be supplied with glucagon for use in emergencies.

Another potential problem with this somatostatin analogue is attenuation of its GH inhibiting effects with chronic treatment. This has been observed in non-diabetic animals (Lamberts et al., 1987) but does not seem to be a problem in long term studies of acromegalic patients receiving octreotide for up to 15 months (Sandler et al., 1987). The results of this study indicate that IGF-I levels remain suppressed after up to 16 weeks of treatment by CSI.

An oral or intranasal preparation of the analogue may be more acceptable to patients particularly for long term use. Preliminary experience with a cyclic hexapeptide somatostatin analogue (MK-678) suggests that this may be well tolerated in short term studies and may prevent daytime GH peaks after meals (Bolli et al., 1988). Its effectiveness on 24h GH secretion, IGF-I levels and retinopathy as well as long term patient acceptability, remain to be assessed.

10.7 Summary

In this study, the somatostatin analogue octreotide was shown to effectively suppress 24h GH secretion when given to normal volunteers by continuous subcutaneous infusion. Eight hourly injections did not maintain consistent drug levels so that GH release occurred 2-4 hours after injections at times when drug levels were low. The situation in the diabetic patients was different. Although injections were similarly unsuccessful in controlling GH levels, GH peaks were occurring at times when drug levels were elevated. In addition, CSI treatment was only able to partially suppress GH levels in the patient group despite consistently high drug levels whilst IGF-I values were reduced into the hypopituitary range. These results together with incomplete suppression of GHRH-stimulated GH release by native somatostatin suggest relative resistance to the GH suppressing effects of somatostatin. This requires confirmation by dose-response studies.

During treatment, patients experienced an increase in hypoglycaemic episodes which proved slowly responsive to oral glucose. All subjects complained of abdominal discomfort and loose stools. Retinopathy was unaffected by up to 16 weeks of CSI treatment although the single patient with preproliferative retinopathy showed no progression of eye disease during treatment. It remains possible that the analogue will have a beneficial effect on retinopathy if given to patients with less advanced retinal changes. The mechanism of the abnormal GH secretion in these patients will be further explored in the following two chapters.

Chapter 11: Suppression of GHRH-stimulated GH release by pretreatment with hGH in patients with diabetic retinopathy

11.1 Introduction

In this chapter, the control by GH of its own secretion is examined in normal control subjects and diabetics with retinopathy. Previous studies have shown that pretreatment with human GH attenuates the GH response to insulin-induced hypoglycaemia (Abrams et al., 1971), sleep (Mendelson et al., 1983), argine and exercise (Hagen et al., 1972). More recently, hGH pretreatment has been shown to suppress the GH response to GHRH in normal subjects (Rosenthal et al., 1986; Ross et al., 1987). There have been no previous reports of similar studies in diabetic patients in whom failure of GH to inhibit its own release could be important in maintaining excessive GH secretion. In this study, the inhibition of GHRH-stimulated GH release by hGH was investigated during normoglycaemia in diabetic patients with retinopathy and matched healthy volunteers.

11.2 Patients and Methods

Eight diabetic patients and 6 healthy controls, matched for age, sex and weight, were studied after giving their informed consent. Details of the patients are shown in Table 11.1.

Table 11.1 Patient data.

	Male/ Female	Age (years)	Duration diabetes (years)	BMI (kg/m^2)	Retinopathy	HbA_1 (%)	Proteinuria (g/l)
1.	M	35	26	22.0	Prolif	7.8	0
2.	M	32	20	21.1	Bgd	7.2	0
3.	M	33	17	20.4	Prolif	9.1	2
4.	M	36	16	25.2	Bgd	7.4	3
5.	F	34	18	26.1	Prolif	5.2	1
6.	M	29	16	22.1	Prolif	18.5	0
7.	F	45	33	23.1	Prolif	14.1	2
8.	F	30	25	22.1	Bgd	13.5	3

Bgd: Background retinopathy   Prolif:Proliferative retinopathy

Patients were selected from the Diabetic Retinopathy Clinic if they were within 10% of ideal body weight and had normal values for plasma urea and creatinine. The study was approved by the Ethical Committee of the Royal Postgraduate Medical School and Hammersmith Hospital.

All subjects underwent two tests in random order separated by at least 1 week. Studies were performed at 0800h after an overnight fast. Patients were asked to omit their morning insulin. After insertion of an i.v. forearm cannula, subjects received either 2 IU of biosynthetic methionyl hGH (Kabivitrum) or 1 ml of 0.9% saline. Blood was sampled for serum GH, IGF-I and glucose over the following 270 min. Three hours after the injection of saline or hGH, a GHRH test was performed by the administration of 120mcg of GHRH 1-44 (Sanofi, Manchester) i.v. and sampling at 10 minute intervals for 90 minutes. Plasma glucose in the diabetic patients was maintained between 3.5-6 mmol/l throughout each test by a variable continuous insulin infusion.

Two patients agreed to be restudied after a period in which attempts were made to improve diabetic control. Patient 6 (Table 11.1) underwent a repeat study after 6 weeks of intensified insulin treatment; daily insulin dosage was increased from 40 U to 60 U. Patient 7 was restudied after an interval of 4 months during which time no significant change in insulin dosage occurred.

11.3 Analysis

Details of the GH and IGF-I assays are given in section 4.2, and 4.3. All samples from an individual subject were assayed together. Area under the GH curve (AUC) was calculated by the trapezoidal rule and expressed in $mU.l^{-1}.h^{-1}$. HbA_1 concentration was measured by agar gel electrophoresis (reference range 5.5-8.0%). Comparison of IGF-I and GH levels for paired data was made by the Wilcoxon's test and between groups by the Mann-Whitney test; $p < 0.05$ was taken as the level of significance. Results are expressed as mean $\pm$ SEM.

11.4 Results

The mean peak GH concentration following i.v. hGH at 20 minutes was 207.1 $\pm$ 16.9 mU/l. Peak GH following saline injection was < 10 mU/l. By 3h after the injection of hGH, serum GH concentration had fallen to 6.2 $\pm$ 0.4 mU/l (controls) and 8.6 $\pm$ 0.9 mU/l (patients).

Peak GH response to GHRH after saline pretreatment in the 6 controls was 61.6 $\pm$ 12.1 mU/l and area under the GH curve (AUC) 295 $\pm$ 62.2 $mU.l^{-1}.h^{-1}$. The GH response to GHRH was completely abolished by pretreatment with hGH (Figure 11.1).

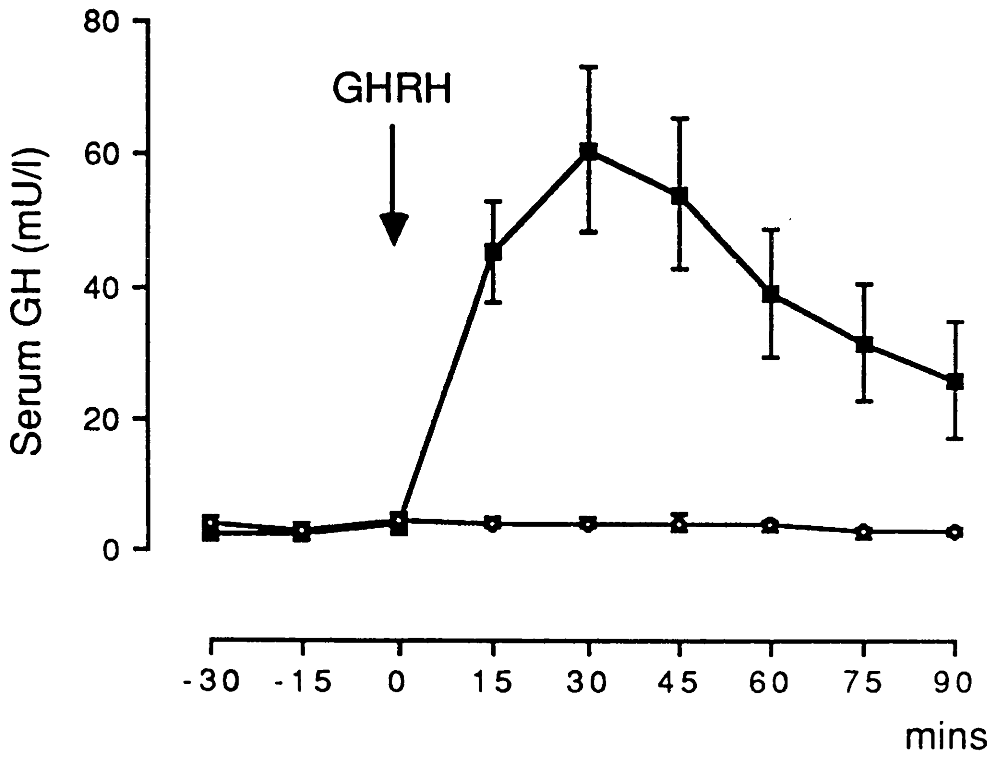


Figure 11.1 GH response to 120 mcg GHRH in 6 normal controls, 3 hours after pretreatment with saline (closed squares) or 2 IU hGH (open squares) i.v.

The response to GHRH after pretreatment with saline was more variable in the diabetic patients (peak GH: 76.4 ± 14.8 mU/l; AUC: 535 ± 129 $mU.l^{-1}.h^{-1}$) although not significantly different from the control subjects ($p > 0.05$). Five patients (patients 1-5, Table 11.1) showed significant suppression of the GHRH response after pretreatment with hGH (AUC: 555 ± 199 vs 167 ± 43; $p < 0.01$; peak GH: 77 ± 22 vs 18.2 ± 8.3; $p < 0.05$) (Figure 11.2). Previous glycaemic control in these patients was good as reflected by a mean HbA_1 concentration of 7.36 ± 0.64%.

By contrast, there was no significant GH suppression in the 3 diabetic patients with the poorest glycaemic control (mean HbA_1:15.36 ± 1.58%); AUC: 497 ± 150 vs 588 ± 267; $p > 0.05$ (Figure 11.3). GHRH responses in these patients after pretreatment with saline were no different from the 5 patients with good glycaemic control (AUC: 555 ± 199 vs 497 ± 150; $p > 0.05$; peak GH: 76.7 ± 22 vs 75.6 ± 21.1; $p > 0.05$). Patient 6 was restudied after 6 weeks of intensified insulin treatment (HbA_1 reduced from 16.5% to 12.2%; daily insulin dosage (40 U) increased to 60 U). GHRH-induced GH release was now suppressed by hGH pretreatment (AUC 471 vs 174; $p < 0.01$) (Figure 11.4). GH release remained unsuppressed in patient 7 on retesting (AUC: 257 vs 197). In this patient, HbA_1 concentration had not improved significantly between the two studies (14.1 vs 13.4%).

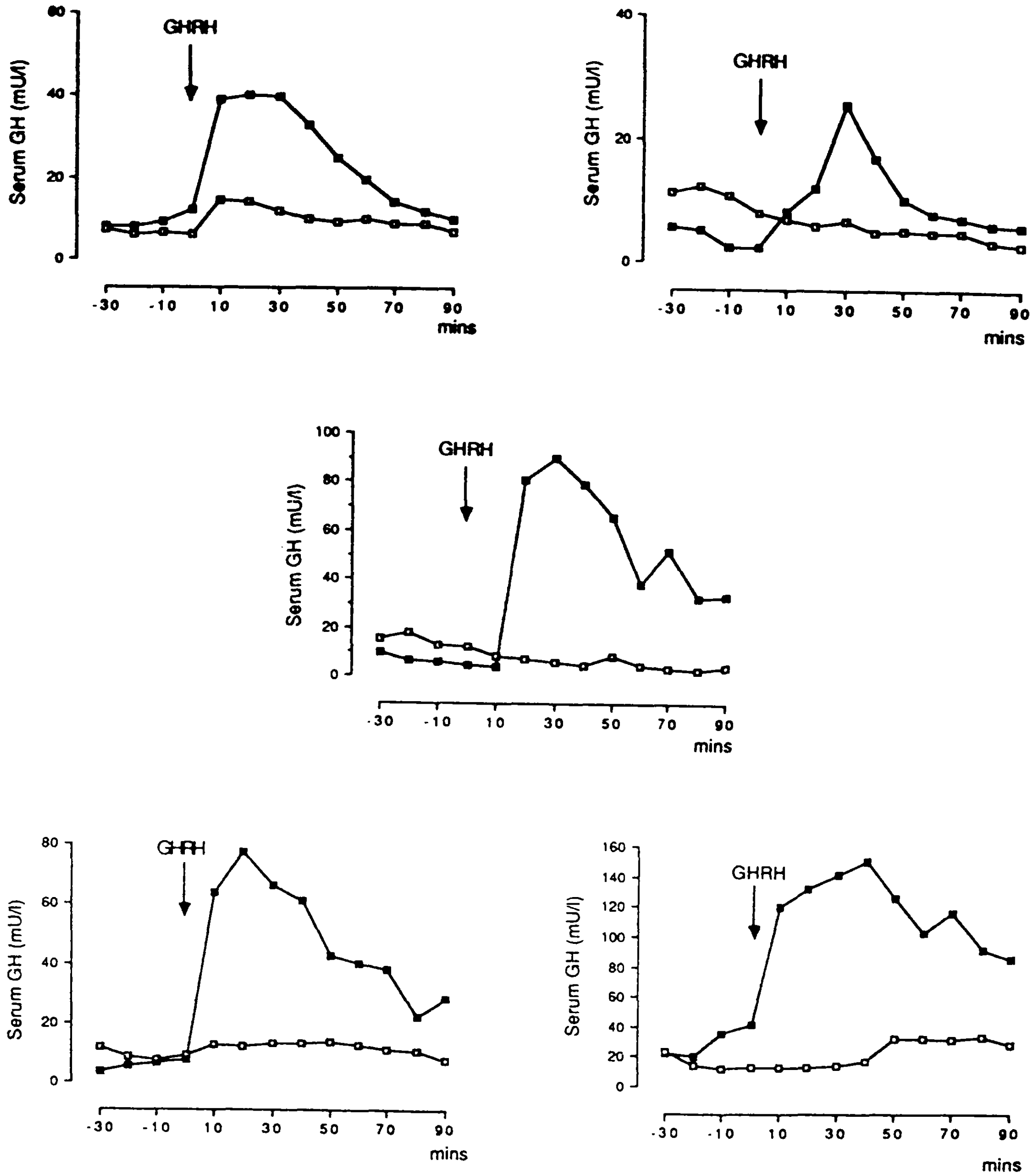


Figure 11.2 GH responses to 120 mcg GHRH in 5 diabetic patients, 3 hours after pretreatment with saline (closed squares) or 2 IU hGH (open squares) i.v.

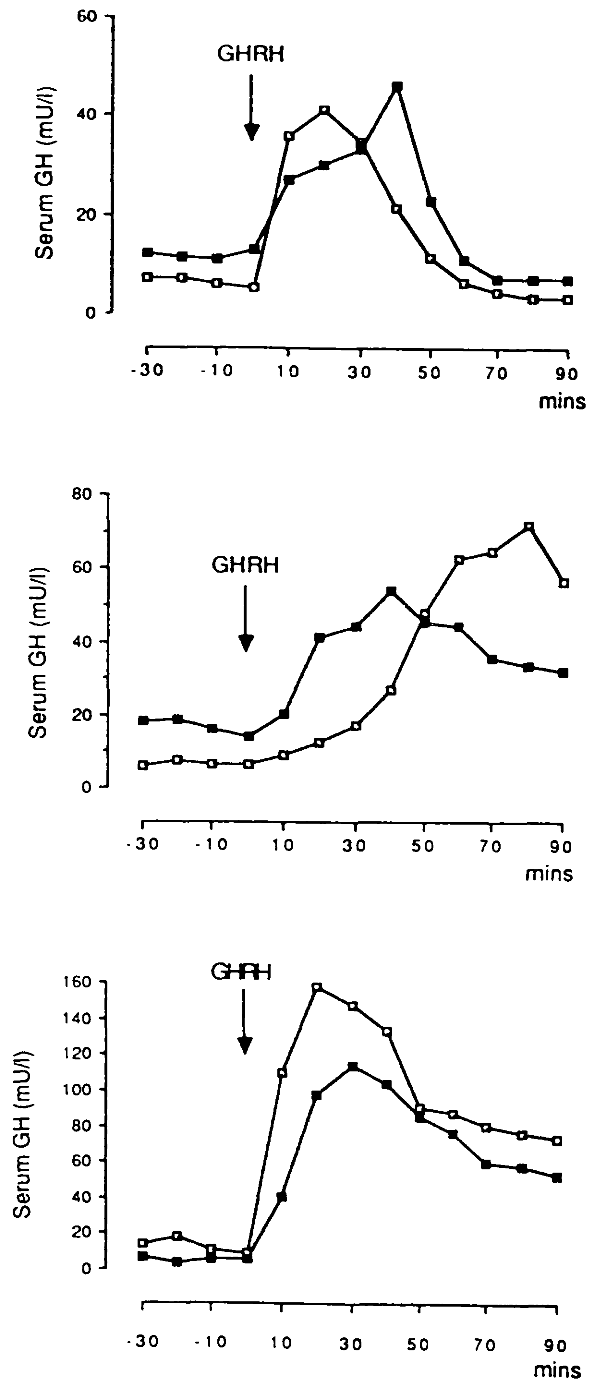
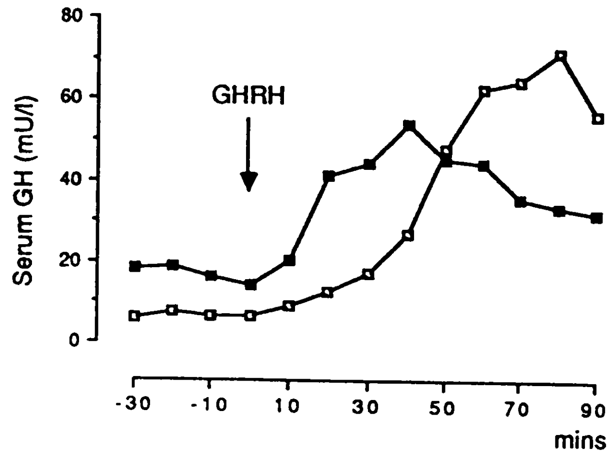


Figure 11.3 GH responses to 120 mcg GHRH in 3 diabetic patients with poor diabetic control, 3 hours after pretreatment with saline (closed squares) or 2 IU hGH (open squares) i.v.



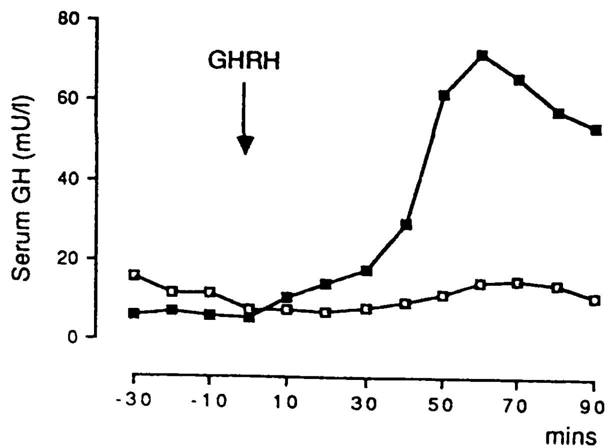


Figure 11.4 GH responses to 120 mcg GHRH in patient 6 studied when diabetic control was very poor (HbA_1:18.5%) (upper panel) and 6 weeks later after intensified insulin treatment (HbA_1: 12.4%) (lower panel). Symbols as in previous figures.

Mean serum IGF-I (mcg/l) was similar in diabetics and control subjects (161.5 ± 12.4 vs 202.6 ± 19.6; $p > 0.05$). There was no significant change in serum IGF-I during the 270 min following hGH or saline injection. IGF-I values at the start of the GHRH test were similar following saline or hGH pretreatment; 194 ± 11.3 vs 210 ± 13.8 [controls]; 159.5 ± 19.3 vs 162.1 ± 16.5 mcg/l; [patients]; $p > 0.05$. IGF-I values in the 3 most poorly controlled patients were not significantly different from other patients; 159 ± 44.6 : 163 ± 41.7 ; $p > 0.05$. In patient 6, mean serum IGF-I concentration rose from 91 ± 2.4 during poor control to 114 ± 6.9 following improvement although this did not achieve statistical significance ($p > 0.05$).

11.5 Discussion

Consistent with previous studies in normal subjects (Rosenthal et al., 1986; Nakamoto et al., 1986; Ross et al., 1987), all six normal volunteers and five of the eight diabetic patients showed blunting of the GH response to GHRH after hGH pretreatment. In contrast to these studies, the three poorest controlled diabetic patients showed no suppression despite peak serum GH levels following hGH injection of more than 200 mU/l. It is possible that an even larger dose of hGH would have been more effective in these patients.

Interestingly, this relative resistance to GH appeared reversible in the patient whose diabetic control had improved by the time he was restudied.

Studies were not performed on patients without retinopathy but since the severity of retinopathy in the patients who demonstrated GH suppression was similar to those who did not suppress, and since the abnormality in GH control was reversed by changes in metabolic control only, it is reasonable to assume that this alteration in GH feedback inhibition relates to the metabolic state rather than the presence of retinopathy.

The GH rise after GHRH in the absense of previous hGH injection appeared more variable in the diabetic group but this alone could not explain the failure of suppression by hGH. The variability in GH response did not correlate with glycaemic control, as reflected by HbA_1 concentration, or serum IGF-I concentration. It may reflect variability in central somatostatin tone in diabetes (Delitala et al., 1988). It is also possible that the greater variability in GHRH responses in diabetic patients accounts for the conflicting reports of normal (Press et al., 1984a; Richards et al., 1984; Giampietro et al., 1986, Kopelman et al., 1988) or increased (Kaneko et al., 1985; Pietschmann et al., 1987; Krassowski et al., 1988) GH responses to GHRH in non-obese diabetic patients compared to normal controls (section 1.5).

No correlation was found between changes in serum IGF-I and blunting of the GHRH response, consistent with a previous report (Ross et al., 1987). This implies that circulating IGF-I does not mediate the inhibition by GH of GHRH-stimulated GH release.

The feedback could, however, operate through locally produced IGF-I acting at pituitary level to inhibit GH release by a paracrine mechanism before increases in serum IGF-I become apparent. Tissue IGF-I levels after GH administration, peak much earlier than serum levels and indeed may rise before any change in circulating levels becomes apparent (Underwood et al., 1986). Secretion of IGF-I by pituitary cells has also been previously reported (Fagin et al., 1987). If pituitary IGF-I release is important in mediating GH autoregulation, the lack of GHRH suppression in this study could have resulted from impaired IGF-I generation in response to GH. This would be analagous to the impaired IGF-I response in response to exogenous GH in poorly controlled diabetic children (Lanes et al., 1985).

Alternatively, circulating IGF-I bioavailability in the poorest controlled patients, may have been reduced by increased concentration of low molecular weight binding protein despite normal immunoassayable IGF-I values (Suikkari et al., 1988). Intensified insulin treatment would be expected to reduce levels of this binding protein and thus restore IGF-I activity.

There is also evidence from animal studies that both GH and IGF-I may induce release of hypothalamic somatostatin and this could provide a mechanism of GH feedback inhibition (Sheppard et al., 1978; Berelowitz et al., 1981; Tannenbaum et al., 1983). Relative resistance to the GH-suppressing effects of somatostatin could then account for the inability of GH to suppress the GHRH response in poorly controlled diabetic patients (chapter 10).

Against this possibility is the lack of a reduction in TSH levels in normal subjects after treatment with hGH (Ross et al., 1987) which would have been expected if somatostatin release is augmented (Weeke et al., 1975).

Whatever the mechanism(s) involved, the failure of GH to suppress its further secretion in poorly controlled diabetics and the resulting GH hypersecretion, will in turn aggravate the metabolic problem leading to even worse control. Administration of GH to well controlled diabetics leads to marked hyperglycaemia and hyperketonaemia (Press et al., 1984b; Campbell et al., 1985) attributable to stimulated hepatic glucose production, increased peripheral insulin resistance and stimulation of lipolysis (Rizza et al., 1982; Press et al., 1986). The enhanced GH secretion in these patients may therefore make their diabetes more difficult to control as well as contributing to long term complications.

11.6 Summary

Pretreatment with hGH effectively suppressed GHRH-stimulated GH release in well controlled diabetic patients and normal volunteers but failed to inhibit the GHRH response in diabetics with poor glycaemic control. In one patient with poor control, the GHRH response subsequently became suppressible after intensification of insulin treatment. The mechanism of the short negative feedback loop whereby GH controls its own secretion is not known. The effects of hGH appeared independent of changes in serum IGF-I but may have been mediated by local changes in IGF-I concentration within the pituitary.

Inability of hGH to suppress the GHRH-induced GH release would then imply relative pituitary resistance to GH resulting in an impaired tissue IGF-I response. Alternatively, an increase in the level of low molecular weight IGF-I binding protein associated with poor diabetic control could have reduced functional IGF-I levels despite normal serum concentrations. A further possibility is that somatostatin release is stimulated by hGH and that the inability of hGH to suppress GHRH-stimulated GH release results from relative somatostatin resistance. Failure of GH feedback control could be important in maintaining excessive GH secretion in poorly controlled diabetic patients. The increased GH levels will in turn aggravate the already poor diabetic control.

Chapter 12: The GH response to galanin in diabetics with retinopathy

12.1 Introduction

In the last chapter an abnormality in GH feedback inhibition was demonstrated in poorly controlled diabetic subjects. In this chapter, the hypothalamic-pituitary axis is examined further by investigating the pituitary response in diabetic patients to the neuropeptide galanin. As described in section 2.3, this peptide appears to act at hypothalamic level to release GH, although the exact mechanism of GH release is not known. In studies of healthy male volunteers, an infusion of galanin stimulated GH release (Bauer et al., 1986a) whilst a concomitant infusion of somatostatin with galanin completely inhibited this GH rise (Davis et al., 1987).

There have been no previous reports of the GH response to galanin in diabetic patients. It is conceivable that exaggerated GH release by galanin contributes to the hypersecretion of GH in diabetes. The aim of this study was therefore to investigate the GH response to galanin in a group of diabetic patients with retinopathy and GH hypersecretion, as assessed by 24h GH levels. The GH rise induced by galanin and the suppressibility of the GH response to galanin by somatostatin were studied. The results in the diabetics were compared with those obtained in a group of healthy volunteers matched for age and weight, and investigated in the same way.

12.2 Patients and Methods

Six insulin-dependent diabetic patients with retinopathy and six healthy controls (4 males, 2 females), matched for age, weight and sex were studied after informed written consent. Clinical details of the patients are given in Table 12.1. The study was approved by the Ethical Committee of the Royal Postgraduate Medical School. Patients were selected if they had normal serum creatinine concentration (<120 micromol/l) and if 24h GH secretion was elevated on 2 control days (Area under the GH curve: 206-428 $mU.l^{-1}.h^{-1}$; normal range: 72-149). Two patients were currently taking twice daily conventional insulin, two were receiving insulin by continuous subcutaneous infusion (CSII) and two were using three injections of short acting insulin by the NovoPen. Patients omitted their morning insulin on each study day.

Each subject underwent three tests in random order on separate occasions at 0800h after overnight fasting:

(1) an infusion of 150 mmol/l saline 1ml/min from 0 to 60 minutes
(2) an infusion of galanin 40 pmol/kg/min from 0 to 40 minutes
(3) an infusion of somatostatin (Sanofi, Manchester) 50 pmol/kg/min from -10 to 40 minutes together with an infusion of galanin 40 pmol/kg/min from 0 to 40 minutes.

Table 12.1 Clinical details of study patients

Patient	Sex M/F	Age (years)	Duration diabetes (years)	BMI (kg/m^2)	HbA_1 (%)	Proteinuria (g/l)	Retinopathy
1.	M	32	13	22.1	8.7	0	Background
2.	M	37	19	22.9	4.3	0	Proliferative
3.	M	27	19	20.9	8.1	2	Proliferative
4.	F	34	19	24.5	5.2	1	Proliferative
5.	F	46	35	23.3	14.1	0	Proliferative
6.	M	36	16	25.2	7.4	3	Background

Plasma glucose in the patient group was maintained between 3.5-6 mmol/l throughout each infusion by a variable continuous subcutaneous infusion of insulin. For each test, indwelling cannulae were inserted into antecubital veins in both arms; one for sampling, the other for infusion.

Sterile endotoxin-free synthetic porcine galanin (Institut Armand-Frappier, Laval, Canada) was reconstituted in 1.5 ml of the patient's own plasma containing 5000 U aprotonin and 20 U heparin. Reconstituted galanin was then diluted in 150 mmol/l saline and delivered as a constant infusion via a syringe pump. Blood samples were taken for serum GH and plasma glucose at 10 minute intervals from -20 to 70 minutes.

12.3 Assays

Serum GH was measured by radioimmunoassay (RIA) (section 4.3). Plasma galanin was evaluated by a RIA specific for galanin (Bauer et al., 1986b). Plasma glucose was estimated by the glucose oxidase method (Beckman Glucose Analyser) and HbA_1 by cellulose acetate electrophoresis. The reference range for HbA_1 was 3.5-5.0%.

12.4 Analysis

The data was analysed by repeat measures analysis of variance. Serum GH results are expressed as the median value and range or mean $\pm$ SD. Area under the GH curve (AUC) was calculated by the trapezoidal rule.

12.5 Results

Serum GH values during saline infusion ranged from 3-10 mU/l (median 7.5 mU/l); there was no significant change in GH levels between any time points during the infusion in either patients or controls ($p > 0.2$).

Galanin was well tolerated producing only a transient metallic taste in the mouth. Plasma galanin levels (median 1.17 nmol/l; range 0.44-1.37) were similar regardless of whether galanin was given alone or with somatostatin.

Intravenous galanin induced a significant rise in serum GH (mU/l) in the normal subjects starting at 30 minutes (median 5.4 [4.7-13.2]) and reaching a peak at 50 minutes (median 15.4 [6.9-17.1] ; $p < 0.01$). The GH rise induced by galanin is shown in Figure 12.1. In the presence of somatostatin, the galanin infusion failed to induce a significant change in GH levels ($p > 0.05$).

The GH response to galanin in the patient group is shown in Figure 12.2. A significant rise in serum GH (mU/l) occurred starting at 40 minutes (median 5.8 [2.3-25.4]) and reaching a peak at 50 minutes (median 13.0 [6.3-26.8]). Mean area under the GH curve in response to galanin ($mU.l^{-1}.h^{-1}$) was not significantly different in the patient group compared to the normal controls (86.3 ± 42.39 vs 60.0 ± 13.13; $p > 0.05$).

In one male patient (patient 2), blood glucose levels were transiently elevated up to 9.6mmol/l for 10 minutes during the galanin infusion. The GH rise during the infusion in this patient was similar to the other patients and not significantly different when the infusion was repeated at normoglycaemia.

In all patients, galanin failed to induce a significant rise in serum GH during 40 minutes of infusion in the presence of a concomitant somatostatin infusion (Figure 12.3). Rebound GH secretion occurred following cessation of the somatostatin with GH levels starting to rise at 50 minutes (median 6.12 [4.1-43.7]) and reaching a peak at 70 minutes (median 28.5 [7.3-58.4]) (Figure 12.3). Complete suppression of galanin-induced GH release by somatostatin was also observed in one patient (patient 3), who was studied during hyperglycaemia (blood glucose during the infusion 8-12 mmol/l).

Mean IGF-I levels (mcg/l) were similar in the patient and control groups (158 $\pm$ 54.6 vs 162 $\pm$ 46.6; NS). There was no significant change in serum IGF-I levels during the infusions of galanin or somatostatin. Serum IGF-I levels at the start of the galanin infusion showed no relation to the subsequent rise in serum GH induced by galanin.

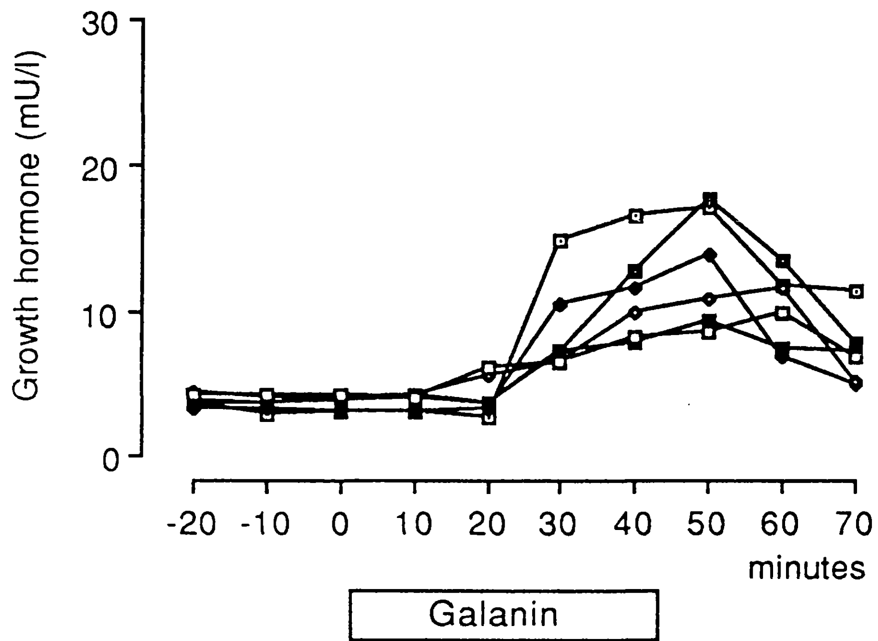


Figure 12.1 Serum GH response to intravenous galanin 40 pmol/kg/min in six healthy volunteers.

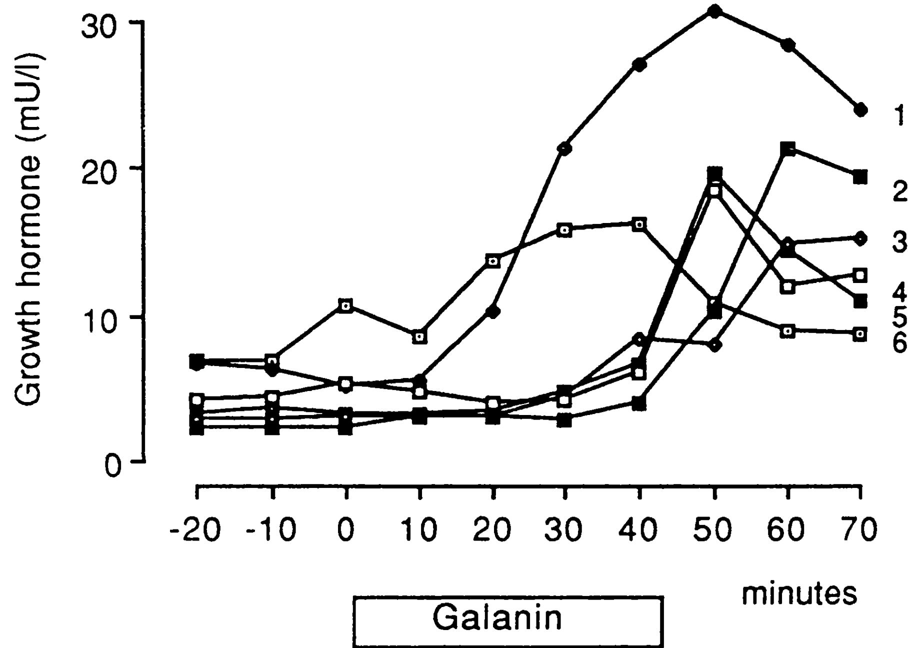


Figure 12.2 Serum GH response to intravenous galanin 40 pmol/kg/min in 6 diabetic patients with retinopathy.

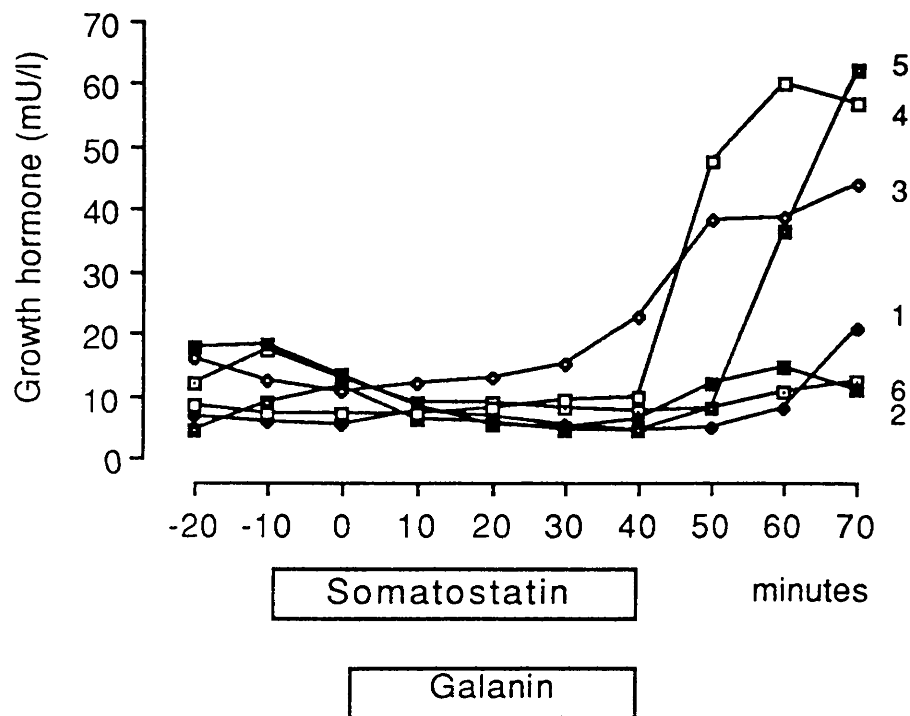


Figure 12.3 Serum GH response to intravenous galanin in the presence of a concomitant infusion of somatostatin 50 pmol/kg/min.

12.6 Discussion

The GH rise induced by galanin in the control group in the present study is very similar to that seen in a previous study performed under identical conditions in normal male volunteers (Davis et al., 1987). Although peak GH levels achieved and mean area under the GH curve were not significantly different in the diabetic group, the GH rise appeared delayed in 4 of the 6 patients (patients 2-5). In these patients, GH levels did not begin to rise until the end of the galanin infusion (i.e. after 40 minutes) in contrast to the control subjects in whom significant GH rises occurred at 30 minutes.

Interestingly, the highest and earliest responses to galanin occurred in the two female patients. This might imply that oestrogen augments galanin-stimulated GH release. Indeed oestrogen stimulates galanin mRNA and galanin synthesis in the anterior pituitary of female rats (Ottlecz et al., 1988). However, no increased response occurred in the 2 female control subjects. By comparison, sex differences in response to GHRH have been reported with greater GH rises in normal premenopausal women compared to age-matched men (Lang et al., 1987). Female diabetic patients, however, appear to respond similarly to GHRH as diabetic men (Krassowski et al., 1988).

Increased basal GH secretion and excessive GH responses to various stimuli in diabetics with retinopathy (section 1.5) especially at times of poor glycaemic control, could result from reduced endogenous SS tone.

It is conceivable that excessive galanin production or release could contribute to lowered SS tone and GH hypersecretion (section 2.3 (3). The results of this study, however, provide no evidence of an exaggerated GH response to galanin in a group of diabetic patients with GH hypersecretion.

24h GH concentration in the patients showed no relation to peak or total GH release induced by galanin. Additionally, exogenous somatostatin, at a dose that was effective in normal subjects, was equally effective in suppressing galanin-stimulated GH release in the diabetic patients. These findings argue against a role for galanin in producing the excessive GH secretion. Nevertheless, the variability and lack of uniformity of the GH response observed in the patient group may reflect an underlying defect in the regulation of GH release in these patients. Furthermore, an abnormality in GH response to higher or lower concentrations of galanin cannot be excluded without more extensive dose-response studies in patients and normal controls.

Abnormal GH release by galanin may only become apparent during periods of high blood glucose concentration. In a previous report in normal subjects (Bauer et al., 1986a), the peak GH concentration achieved with a comparable dose of galanin after an i.v. 25g glucose bolus (40.4 $\pm$ 14.1 mU/l) was considerably greater than the peak achieved either in this study (13 [4.4-14.6] mU/l) or in that of Davis et al. (17.3 [12.5-19.8] mU/l) (Davis et al., 1987).

This may suggest that glucose augments the pituitary response to galanin. However, there was considerable individual variability in the GH response induced by galanin so that repeated measurements in the same individuals before and after glucose are required to investigate this possibility.

In one patient observed during mild hyperglycaemia, the GH response induced by galanin was similar to the response at euglycaemia. Galanin-induced GH release was also effectively inhibited by somatostatin in another patient despite hyperglycaemia. Galanin reduces glucose clearance (Bauer et al., 1986a) and may therefore be expected to worsen diabetic control but no increase in insulin requirements needed to maintain euglycaemia was observed at the end of the galanin infusion.

12.7 Summmary

In this study, an infusion of galanin at a dose of 40 pmol/kg/min induced a small but significant rise in GH concentration in a group of normal volunteers which was suppressed by somatostatin. Galanin induced similar peak and total GH release in a group of diabetic patients with increased 24h GH secretion and retinopathy. Patients showed more variable responses and in four of the six patients, the GH rise was delayed until the end of the galanin infusion. Galinin-stimulated GH release was not related to 24h GH concentration or IGF-I levels.

The variability of GH responses to galanin in the patients could reflect defective control of GH release but no evidence was found of excessive GH release in response to galanin as a cause of GH hypersecretion. It is possible that diabetic patients do show enhanced GH release in response to other doses of galanin. Since glucose may augment galanin-stimulated GH release, galanin could contribute to increased GH levels at times of poor glycaemic control. Further work including dose-response studies and investigations performed at different ambient glucose concentrations are required to help determine the potential importance of galanin in the GH abnormalities of diabetes.

Chapter 13: Concluding Remarks

13.1 Introduction

In attempting to shed some light on the hormonal mechanisms involved in the developement of diabetic retinopathy, this work has focused on two issues; (1) the role of GH/IGF-I and bFGF in the pathogenesis of diabetic retinopathy, particularly proliferative retinopathy and (2) the factors responsible for the excessive GH secretion in diabetic patients. Definitive answers to these problems cannot be given. Part of the difficulty in addressing the first issue is the lack of an animal model of diabetic proliferative retinopathy. Clinical studies, as described in this work, can only hope to provide indirect evidence of cause and effect. Other approaches, such as cell culture studies, provide useful information on the response of cells in a variety of experimental conditions, but the situation in vivo may be quite different. Conversely, extrapolation from animal models of diabetes to human diabetes in an attempt to unravel the GH abnormalities of diabetes is also problematic because of inter-species differences in GH dynamics. For example, unlike in clinical diabetes, experimental diabetes in the rat is accompanied by low levels of GH and IGF-I (Tannenbaum, 1981). Again, in contrast to the situation in man, stress and hypoglycaemia are both accompanied by suppression of GH levels in the rat (Painson & Tannenbaum, 1985).

With these thoughts in mind, inferences can be made from the results of data presented above, in the light of current understanding of diabetic retinopathy and GH regulation.

13.2 The mechanism of excessive GH secretion in diabetes

Despite increased 24h GH secretion in patients with diabetic retinopathy and relatively poor diabetic control, IGF-I levels were found to be in the normal range (chapter 9 and chapter 10) suggesting that the increased GH levels in these patients fail to stimulate IGF-I production effectively. A relation of these GH and IGF-I abnormalities with diabetic control was suggested by the patient restudied after improved metabolic control, in whom tighter control resulted in a reduction in 24h GH levels and increase in IGF-I values. In a separate study, intensified insulin treatment by means of continuous subcutaneous insulin treatment, was found to be associated with a significant rise in IGF-I levels (chapter 6). A weak but significant inverse relationship between IGF-I level and HbA_1 was also demonstrated in chapter 4. A more striking negative correlation may have been demonstrated with more severe diabetic decompensation (Rieu et al., 1985). The above results could provide a possible mechanism for excessive GH secretion during poor diabetic control and could explain why GH levels return towards the normal range as control is improved.

As discussed in chapter 2 (section 2.2 (2)), IGF-I can inhibit GH release either by acting at hypothalamic level to stimulate somatostatin release (Berelowitz et al., 1981) or by suppressing GHRH (Brazeau et al., 1982) or by a direct effect on the pituitary (Ceda et al., 1985). Impaired IGF-I production could therefore result in reduced feedback inhibition and hence increased GH output. As control is improved, the rise in IGF-I concentration would be expected to inhibit GH release, and hence restore GH levels to normal (Tamborlane et al., 1981; Amiel et al., 1984).

This then raises the further question of why IGF-I production should be impaired at times of poor glycaemic control. Evidence from studies of normal and streptozotocin-induced diabetic rats, indicates that the hepatic GH receptor appears to be regulated by insulin (Baxter et al., 1980b), and that in diabetes the expression of GH receptors is suppressed. Insulin therapy both reverses this defect and restores IGF-I levels to normal (Baxter et al., 1980a). Insulin may also affect GH gene expression either stimulating or inhibiting expression, depending on the metabolic state of the cells (Isaacs et al., 1987). There is also evidence of a permissive effect of insulin on post GH-receptor events (Maes et al., 1986). As diabetic control worsens, intermediate metabolites and ketone bodies may also interfere with IGF-I production (Phillips & Unterman, 1984). In addition to decreased IGF-I generation, increased levels of low molecular weight IGF-I binding protein level during poor metabolic control may reduce IGF-I bioavailabilty despite normal serum IGF-I levels (Suikkari et al., 1988).

An alternative mechanism of GH hypersecretion was suggested by the study outlined in chapter 11. In patients with relatively poor diabetic control, pretreatment with GH failed to suppress the subsequent response to GHRH, independent of serum IGF-I levels. This feedback loop whereby GH regulates its own secretion might operate through stimulation of hypothalamic somatostatin release (Sheppard et al., 1978; Ross et al., 1987). Failure to inhibit GH release could imply GH resistance at the hypothalamus in an analagous way to GH resistance and impaired IGF-I production at the level of the liver. Other possibilities include inadequate IGF-I generation within the pituitary and loss of paracrine control of GH release (Underwood et al., 1986), or resistance to endogenous somatostatin released by GH.

In chapter 10, patients with diabetic retinopathy were found to have relative resistance to the GH suppressing effects of the long acting somatostatin analogue, octreotide, and by implication, relative resistance to somatostatin. Unlike in healthy volunteers in whom GH secretion was totally suppressed, continuous infusion of the analogue only partially suppressed GH levels in the patients. Circulating IGF-I levels, however, were markedly suppressed by treatment possibly because production of this peptide was already impaired. The suppressed IGF-I levels could have contributed to the continued GH release in these patients although the reduced IGF-I concentration in the control subjects did not induce GH secretion in the presence of adequate circulating levels of the analogue.

It is also possible that the effectiveness of somatostatin was antagonised by increased circulating levels of various metabolites such as amino-acids or free fatty acids associated with poor metabolic control (Fulks et al., 1975; Boden et al., 1980). These factors, acting as GH secretogogues, could contribute to the enhanced GH secretion and exaggerated GH responses to various stimuli during poor diabetic control.

Alternatively, chronic hyperglycaemia may induce changes at the level of the somatostatin receptor, as a result of chronic stimulation of somatostatin release by glucose. If this were so, and assuming that the analogue acts at the same receptors, chronic treatment with high doses of octreotide would be expected to induce similar receptor changes and somatostatin resistance, but this doesnt seem to occur, at least in acromegalic patients (Ch'ng et al., 1985; Sandler et al., 1987; Dieguez et al., 1988a).

Finally in chapter 12, an abnormality in the pituitary responsiveness to galanin was sought as an indication of a disturbance in neuropeptide control of GH release in diabetes. The peak GH response after an infusion of galanin was similar to that seen in healthy volunteers and was suppressible with somatostatin. The GH rise in 4 of the 6 patients was delayed until the end of the galanin infusion, possibly indicating an abnormality in the control of GH release. Nevertheless, there was no evidence of increased pituitary sensitivity to galanin as a mechanism of increased GH secretion.

The presence of diabetes, particularly when poorly controlled, would seem to impose a need for the body to conserve energy in an analagous way to the situation in the fasted individual. Metabolic fuels in diabetes may be abundant but cannot be utilised because of insulin deficiency and/or insulin resistance. Excessive GH secretion and reduced IGF-I generation may be considered as an adaptation whereby metabolic fuels are shunted away from growth (reduced IGF-I), protein is spared and alternative metabolic fuels are mobilised (GH actions on muscle and fat) (Phillips, 1986). However, this adaptation is not without problems, as GH impairs tissue responses to insulin and worsens hyperglycaemia and hyperketonaemia (Press et al., 1984b; Campbell et al., 1985).

To summarise, GH hypersecretion in diabetes is likely to be a product of several abnormalities. These include reduced IGF-I production, relative resistance to somatostatin and possibly also resistance to GH at hypothalamic or pituitary level. Other factors such as an increase in somatomedin inhibitors and changes in binding protein levels (Baxter and Martin, 1986; Suikkari et al., 1988) may also be important.

13.3 GH/IGF-I and bFGF in the pathogenesis of diabetic retinopathy

The role of GH and tissue growth factors in the pathogenesis of diabetic retinopathy is more difficult to address. No relation between the appearance of cotton wool spots and haemorrhages and serum IGF-I was noted in the study described in chapter 6. This would suggest that IGF-I (and presumably GH) are not essential for the early changes in diabetic retinopathy. A similar conclusion can be drawn from the patient with congenital GH deficiency who developed background diabetic retinopathy following post-pancreatectomy diabetes (Rabin et al., 1984).

The importance of GH in more advanced retinopathy remains unclear as attempts to selectively suppress its release by cholinergic antagonists or octreotide proved unsuccessful. Future studies must await more acceptable and more specific therapy. Although initial studies with pirenzepine looked promising, the effects of this drug also appear transient and are accompanied by rebound GH secretion (Hindmarsh et al., 1987).

In favour of a role for IGF-I in the development of proliferative retinopathy, the results from the prospective study of conventionally treated diabetic patients in chapter 5 indicated a significant increase in IGF-I levels around the time when new vessels were just starting to appear. Levels were subsequently restored to previous values by laser photocoagulation.

In addition, the patient in chapter 6 who developed proliferative retinopathy during CSII treatment, showed a marked increase in serum IGF-I corresponding to the time of new vessel formation. The finding of a transient rise in circulating IGF-I levels may explain why differences in mean IGF-I values in different groups of diabetic patients could not be detected in the cross-sectional study described in chapter 4.

However, a reduction of serum IGF-I concentration to hypopituitary levels after up to 12 weeks of octreotide treatment in chapter 10, had no detectable effect on retinopathy appearance. This may simply reflect the advanced stage of retinopathy in these patients. Treatment at an earlier phase in the process of new vessel formation may have proved more successful as suggested by the patient with preproliferative retinopathy who showed no progression of retinopathy during octreotide treatment.

In view of the paracrine functions of IGF-I, tissue concentrations may be particularly important and are not always reflected by circulating levels (Orlowski & Chernausek, 1988). In chapter 8, bovine retinal endothelial cells were found to release IGF-I into the cell medium. In proliferative retinopathy, the retina could be an important source of IGF-I locally and may also contribute to circulating levels especially when hepatic production is impaired and retinal production is enhanced. After panretinal photocoagulation, previously raised serum levels declined, although it is not known if this would still have occurred if patients were left untreated.

Other serum factors are likely to be important in promoting endothelial cell proliferation and new vessel formation, probably acting at different phases in the process. The proliferative activity of diabetic serum as demonstrated by Petty et al. (Petty et al., 1987) showed no relation to either GH or IGF-I concentration (chapter 7).

Other tissue growth factors are also likely to be important, and in particular, the role of the heparin binding growth factors (homologous to acidic and basic FGF) and their relation with GH/IGF-I, remains to be elucidated. The apparent paradox that the normal non-proliferating retina contains significant amounts of potent angiogenic growth factors (acidic and basic FGF) is explained by the fact that the FGFs do not appear to be secreted in vivo but remain sequestered in the cell or its associated basement membrane. FGF is thus rendered inaccessible to stimulate endothelial cell proliferation. Injury to the retina, however, results in FGF release (chapter 8). Contact between the endothelial cell and pericytes reinforces the normal non-proliferative state although this contact inhibition can be overcome by FGF (D'Amore & Orlidge, 1988).

In diabetic retinopathy, pericyte loss and hence loss of contact inhibition occurs early (Cogan et al., 1961; Speiser et al., 1968). If in addition, FGF becomes available perhaps because of ischaemic damage in the preproliferative retina, the normally quiescent surviving endothelial cells will be stimulated to enter the cell cycle.

GH/IGF-I and other local and systemic factors acting as progression factors, would then allow the cell to complete the cycle and proliferate, leading ultimately to new vessel formation.

Knowledge regarding the nature and action of these growth factors is expanding very rapidly. The situation appears to be far more complex than previously appreciated. Not only is the importance of growth inhibitors such as TGF-B in diabetic retinopathy coming to be increasingly recognised (Glaser, 1988) but it is also now appreciated that under different circumstances the same growth factor can be either stimulatory or inhibitory (Sporn & Roberts, 1988). Whilst the complex inter-relationships remain to be elucidated, an interaction of serum factors including GH/IGF-I and tissue factors -likely to include IGF-I and bFGF - provide the most plausible explanation for the progression to proliferative diabetic retinopathy.

13.4 Future studies

Each of the factors invoked in the mechanism of GH hypersecretion (13.2) require further examination. Thus GH resistance could be further explored by a study of the GH response to repeated GHRH boluses or a continuous infusion of GHRH in patients with varying degrees of diabetic control. Possible antagonism of the GH suppressive effects of somatostatin (or octreotide) could be examined by administation of free fatty acids or branch chain amino-acids to healthy subjects in whom GH secretion has been suppressed by a concomitant infusion of somatostatin (or octreotide).

Formal assessment of somatostatin resistance and galanin sensitivity require detailed dose-response studies. The GH response to galanin needs to be examined during hyperglycaemia in both control subjects and patients. With the availability of recombinant human IGF-I (rhIGF-I), the negative feedback loop can be investigated in diabetics by observing the effects on 24h GH secretion, of a continuous infusion of rhIGF-I, at a dose calculated to avoid acute hypoglycaemia.

Future studies investigating the hormonal mechanisms of diabetic retinopathy and particularly the role of GH and IGF-I , will continue to concentrate on cells in culture until an animal model of proliferative diabetic retinopathy is developed. Now that purified 34kDa protein (PP12) is available, it will be of great interest to assess whether it enhances or inhibits the growth promoting effects of IGF-I on retinal endothelial cells. The interaction of GH and IGF-I with bFGF and also TGF-B, obviously requires further investigation. The effects of laser photocoagulation on the release of these factors is also of great interest.

Finally, the potential benefit of other drugs on the course of diabetic retinopathy, should be explored. When and if an oral somatostatin analogue becomes available, this should be assessed in patients with preproliferative diabetic retinopathy to determine if it will be able to delay progression of the retinopathy.

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