Bij de voorplaat: De IGF familie zoals die in het bloed aanwezig is.

Vrije IGF-I en IGF-II moleculen kunnen zich aan de IGF-I receptor binden. Deze binding heeft vooral groei-bevorderende effecten tot gevolg. Na binding van IGF-I en IGF-II aan de IGF-I receptor kunnen echter ook metabole effecten optreden.

Insuline bindt zich vooral aan de insuline receptor. Deze binding heeft vooral metabole effecten (zoals bijvoorbeeld een verlaging van de bloedsuiker spiegel) tot gevolg. Na binding van insuline aan de insuline receptor kunnen echter in sommige cellen ook groeibevorderende effecten optreden.

Bovendien kan IGF-I zich aan de insuline receptor en insuline zich aan de IGF-I receptor binden zoals in de tekening te zien is.

De activiteit van het IGF-I (en het IGF-II) wordt sterk beïnvloed door de IGF-bindende eiwitten, die in de tekening met IGFBP no 1 tot 6 zijn aangeduid. De werking van de zes IGFBPs wordt beïnvloed door de zogenaamde IGFBP-proteasen, die specifiek zijn voor een bepaald IGFBP, en die het IGF-I (en het IGF-II) van de IGFBPs kunnen losmaken (zoals in de tekening is aangegeven). Het zo vrijgekomen IGF-I (of IGF-II) kan dan vervolgens een binding met een IGF en/of insuline-receptor aangaan.

(Uit: Lowe WL. Insulin-like growth factors. Science & Medicine 1996; 3: 65; met toestemmining van Science & Medicine).

Insulin-like Growth Factor I and its Binding Proteins in Health, Aging and Disease

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OVER INSULIN-LIKE GROWTH FACTOR I EN HAAR BINDINGSEIWITTEN BIJ GEZONDHEID, BIJ HET OUDER WORDEN EN BIJ ZIEKTEN

PROEFSCHRIFT

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aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. dr P.W.C. Akkermans M.A.
en volgens het besluit van het college voor promoties

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Ter nagedachtenis aan mijn vader en moeder

"A new scientific truth does not triumph by convincing its opponents and making them see the light, but rather because its opponents eventually die, and a new generation grows up that is familiar with it"

Max Planck, in: Scientific Autobiography and Other Papers,
trans. F Gaynor (New York, 1949), pp 33-34.

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List of Abbreviations

IGF-I	:	Insulin-like Growth Factor-I
IGFBP-1	:	Insulin-like Growth Factor Binding Protein-1
IGFBP-3	:	Insulin-like Growth Factor Binding Protein-3
BMD	:	Bone Mineral Density
BMI	:	Body Mass Index
DHEAS	:	DeHydroEpiAndrosteroneSulphate
ERPF	:	Effective Renal Plasma Flow
GFR	:	Glomerular Filtration Rate
GH	:	Growth Hormone
IDDM	:	Insulin-Dependent Diabetes Mellitus
IRMA	:	ImmunoRadioMetric Assay
RIA	:	RadioImmunoAssay
SHBG	:	Sex Hormone-Binding Globulin
WHR	:	Waist Hip Ratio

Conversion factors

-IGF-I ((ng/mL) X 0.1307)= → nmol/L

-IGFBP-1 ((ng/mL) : 25.3)= → nmol/L

-IGFBP-3 ((mg/L) X 1000 : 28.75)= → nmol/L

1

General Introduction and Aim of the Thesis

Introduction

The insulin-like growth factors (IGFs) were discovered in 1956 by William D. Salmon Jr. and William H. Daughaday at Washington University, St Louis, USA (1). Initially IGFs were called sulfation factors because they were able to replace the sulfation factor activity of growth hormone (GH)(1). Moreover, the IGFs were found to be able to stimulate DNA synthesis (2), proteoglycan synthesis (3), glycosaminoglycan synthesis (4), and protein synthesis (5).

In 1963 Froesch et al. from the University of Zürich, Switzerland, described excess insulin-like activity in serum (6). Only a small fraction of the insulin-like activity by normal serum on adipose tissue and muscle could be blocked by specific antibodies against insulin. The mediator of this excess insulin-like activity was not detectable with the radioimmunoassay developed for insulin in 1959, and was termed non-suppressible insulin-like activity (NSILA)(6).

Dulak and Temin demonstrated that serum-free medium conditioned by a rat hepatoma cell line contained mitogenic activity, which they termed multiplication-stimulating activity (MSA) (7).

In 1972 these three apparently distinct peptides (sulfation factor, NSILA, MSA) were unified under the term somatomedin, because it apparently mediated the actions of GH (also called somatotropin) (8).

In 1978 Rinderknecht and Humbel purified somatomedin from serum, and it turned out to be two closely related peptides, which were termed Insulin-like growth factor-I (IGF-I) and Insulin-like growth factor-II (IGF-II) (9, 10).

Evolutionary aspects

The IGF-I molecule is highly conserved throughout vertebrate evolution (11). Amphioxus, a protochordate, expresses a hybrid insulin/IGF-I molecule (12). This strongly suggests that IGF-I diverged from the main line of insulin evolution at a stage antedating the jawless vertebrates.

IGF-I gene stucture and expression

In humans the IGF-I gene is a single gene copy located on chromosome 12 and consists of at least six exons (13). Although the liver was traditionally considered to be the sole producer of IGF-I, recent studies have shown that most tissues express IGF-I (14). Expression of IGF-I mRNA resulting from different transcription initiation sites is tissue-specific, and developmentally and hormonally regulated (11).

Stuctural aspects of IGF-I

IGF-I, IGF-II and insulin are members of a family of polypeptides which are characterized by a high degree of sequence homology (15)(see figure 1). IGF-I is 50-60 % homologous with human proinsulin (16). IGF-I is synthesized as a larger prepro-IGF-I molecule containing a signal peptide as well as a carboxy-terminal extension peptide, the E peptide (17). The signal and E peptide are cleaved off during processing of the mature IGF-I (17). Mature IGF-I is a 70 amino acid peptide, which is different from insulin; at the carboxy terminus it contains an additional sequence the D domain (18).

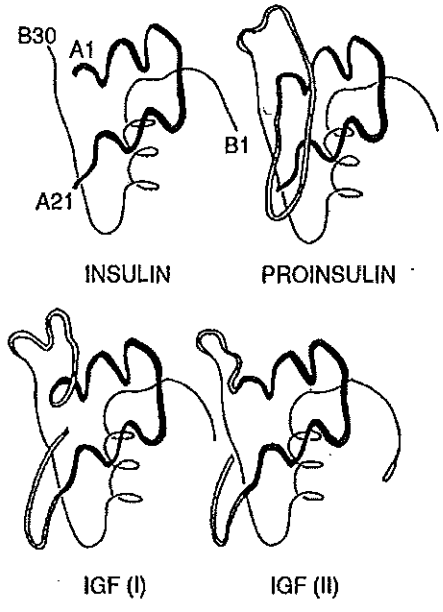

Figure 1

Predicted structure of the insulin-like growth factor family of peptides. The heavy line represents the A chain, double lines represent a D extension to the A chain or the C chain joining the A and B chains (from: LeRoith D. Insulin-like growth factors in health and disease. Ann Intern Med 1992; 116: 855; with permission from the American College of Physicians)

IGF-I purified from brain, bovine colostrum, and uterus lacks three amino-terminal amino acid residues (gly-pro-glu) (19). This truncated form of IGF-I, called des-IGF-I, has a much greater biological potency than mature IGF-I because it has a lower affinity for the insulin-like growth factor binding proteins (IGFBPs) (see infra). It is therefore more available for interacting directly with the IGF-I receptor. The cleavage of the tripeptide presumably occurs as a posttranslational event, although the biomechanisms responsible have not yet been identified.

Functions of IGF-I

IGF-I exerts many biological functions on distant target tissues via the blood stream (endocrine effects), on its own cells of origin (autocrine effects), and on neighboring tissues (paracrine effects)(20). IGF-I has both anabolic and mitogenic effects in vitro and in vivo, which often overlap with the biological effects of insulin (Table 1) (20-23): IGF-I stimulates cell differentiation and it is a mitogen for most cultured cells stimulating [^{3}H] thymidine incorporation into DNA and [^{3}H] uridine incorporation into RNA (21). In vitro glucose uptake, amino acid uptake and ion fluxes are increased by IGF-I (21). In vivo, IGF-I promotes longitudinal bone growth and suppresses GH and insulin secretion (20). IGF-I infusions in normal volunteers increases glucose uptake, reduces free fatty acid and triglyceride levels, and decreases protein breakdown (20). Moreover, insulin stimulates the constitutive secretion of IGF-I from the liver (24). IGF-I in turn, suppresses insulin secretion even in euglycemic conditions (25). In calorically restricted patients administration of IGF-I improves nitrogen balance (26). IGF-I increases glomerular filtration rate and renal plasma flow and enhances creatinine clearance (27).

IGF-I thus influences many cell functions and is considered as growth factor and hormone with important anabolic and metabolic regulatory functions. Another action of IGF-I which is complementary to its stimulatory effect on cell proliferation, is its capacity to inhibit programmed cell death in certain cells, the so called apoptosis (28, 29).

Table I. Biological effects of IGF-I and insulin (modified after references 20-23)

IGF-I	Insulin
Stimulation of glucose uptake	Stimulation of glucose uptake
Stimulation of glucose oxidation	Stimulation of glucose oxidation
Stimulation of glycogen synthesis	Stimulation of glycogen synthesis
	Stimulation of lipogenesis
Antilipolytic effect	Antilipolytic effect
Stimulation of protein synthesis	Stimulation of protein synthesis
Stimulation of DNA synthesis	Stimulation of DNA synthesis
Stimulation of RNA synthesis	Stimulation of RNA synthesis
Stimulation of cell growth and differentiation	Stimulation of cell growth and differentiation
Promotion of longitudinal bone growth	
Stimulation of mitogenesis in culture	
Suppression of GH secretion	
Suppression of insulin and C-peptide secretion	
Stimulation of creatinine clearance	
Stimulation of renal plasma flow	
Stimulation of glomerular filtration rate	
Stimulation of erythropoiesis	
Effect on thymus architecture	
Chemotaxis of T-lymphocytes	
Production of immunoglobulins in plasma cells	

The free insulin-like growth factor-I concentration

Free IGF-I, by analogy with sex and adrenal steroids and thyroid hormones, may be a major biological active hormonal form of IGF-I (30) (Figure 2). Determination of serum free unbound IGF-I was not possible until very recently (30, 31). In 1994 Frystyk et al. and Lee et al. separately described a method to determine free unbound IGF-I in serum (30, 31).

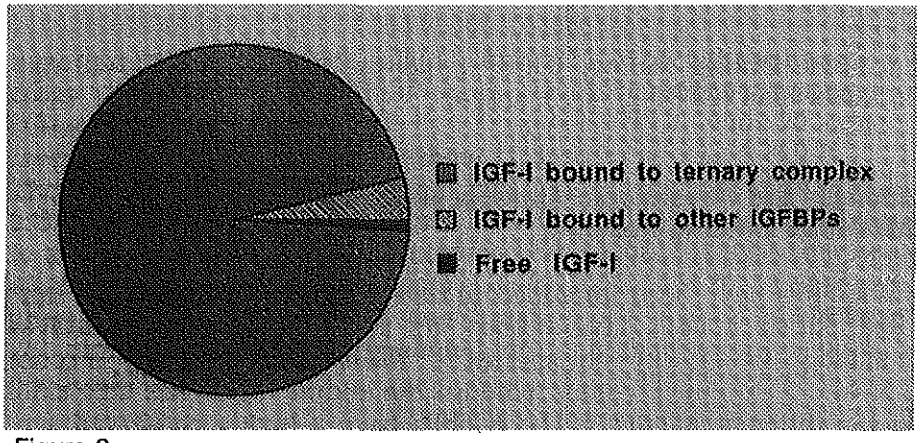

Figure 2

Distribution of IGFs in serum. Ninety-five percent of the IGFs are found as part of the ternary complex of IGF-I and IGF-II bound to IGFBP-3 and acid-labile subunit (ALS). Less than 5% are bound to other IGFBPs, and a very small component circulates as the free form (from: Collett-Solberg PF, Cohen P. The role of insulin-like growth factor binding proteins and the IGFBP proteases in modulation of IGF action. Endocrinol Metab Clin North Am 1996; 25: 593; with permission from WB Saunders)

The method of Frystyk et coworkers used ultrafiltration under in vivo conditions (physiological pH, ionic milieu and temperature) to measure IGF-I, while the free IGF-I assay of Lee and coworkers is an IRMA which initially needs no sample extraction as part of the standard procedure to measure IGF-I. Samples are added directly to tubes containing a dense coating of high-affinity free IGF-I antibody, incubated for 2 hr at room temperature, washed, incubated with a labeled antibody directed to a second epitope, washed and counted. It is likely that the free IGF-I fraction measured with this assay is a combination of the true free and the fraction of IGF-I which can be readily dissociated from IGFBPs under the specific assay conditions (31).

Most circulating IGF-I is bound to the insulin-like growth factor binding proteins (IGFBPs), which titrate the supply of IGF-I to its receptors in the target tissues, while a very small component circulates as the free form (32). The levels of free IGF-I in a system are modulated by the rate of IGF-I production, clearance, and degree of binding to IGFBPs (33). The metabolic clearance of serum free IGF-I is estimated as 2.62 ± 0.94 ml/min with a production rate of 4.75 ± 1.74 mg/day (621 ±227.34 nmol/day) (34). IGF-I bioavailability is probably an important factor in determining the different physiological functions served by IGF-I and insulin (20).

The IGF-I receptor

There are at least two classes of insulin-like growth factor receptors: the insulin-like growth factor-I (IGF-I) receptor and the IGF II/ mannose -6-phosphate (Man-6-P) receptor (35). The IGF-I receptor gene, like the insulin receptor gene, consists of 22 exons (36). It is the product of a single copy gene located at the distal arm of chromosome 15 (37). The insulin-like growth factor receptors are expressed in many cells and in many tissues (38). The overlapping biological effects of IGFs and insulin were initially attributed to the structural homologies between the peptides themselves, but this interpretation turned out to be too simple, after it was shown that the receptors for insulin and IGF-I are also structurally similar (39) (Figure 3).

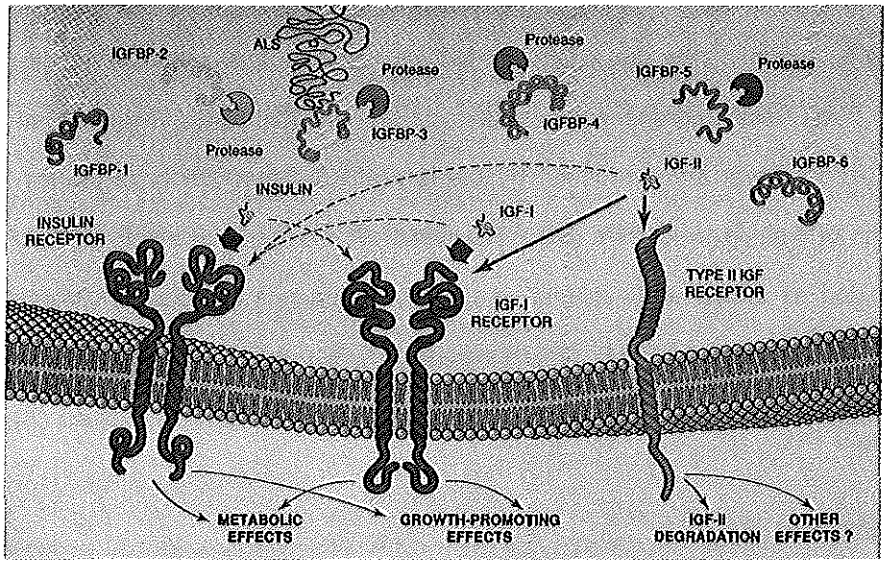

Figure 3

The IGF family of circulating peptides (from: Lowe WL. Insulin-like growth factors. Science & Medicine 1996; 3: 65; with permission from Science & Medicine)

Moreover, IGF-I, IGF-II and insulin can crossreact with each other's receptors (Figure 3) (15). The IGF-I receptor preferentially binds IGF-I, but also binds IGF-II and more weakly insulin. The IGF-II receptor binds IGF-II with higher affinity than IGF-I, but does not interact with even high concentrations of insulin (40). Because IGF-I, IGF-II and insulin can produce the same biological responses, and because many cells possess IGF-I, IGF-II as well as insulin receptors, it has been difficult to determine which receptor is mediating a particular response (40).

Attempts to assess the relative contributions of these three receptors to an observed biologic response have included 1] determination of the receptors present on a cell type and the binding affinities for these three ligands, 2] determination of the biological dose response to IGF-I, IGF-II and insulin and 3] selective blockade of IGF-I or insulin receptors with specific antireceptor antibodies without intrinsic biological effects (40). These investigations have yielded the insight that insulin and the IGFs are complementary hormones in their anabolic action: insulin and the insulin receptors are especially involved in the regulation of the short term fluctuations of major metabolites such as glucose, amino acids and free fatty acids. The IGFs and the IGF-I receptor are especially involved in the regulation of the long term processes which are responsible for cell growth and differentiation (41).

In some tissues and cells expressing high numbers of both IGF-I and insulin receptors, hybrids may form (11). These hybrids are tetrameric receptors consisting of an α-β heterodimer of the insulin receptor linked by disulfide bonds to α-β heterodimer of the IGF-I receptor. A possible role for these hybrid receptors is that they might mediate overlapping functions in which insulin, under certain circumstances, may induce cellular proliferation and IGF-I may stimulate metabolic functions (11).

It is assumed that most of the biological effects of IGF-II are mediated through the IGF-I receptor (42). However, IGF-I-mediated intracellular signaling has at present only been demonstrated for the IGF-I receptor (also called the type 1 IGF-I receptor) (20).

The insulin-like growth factor binding proteins (IGFBPs).

The IGFBPs are a family of proteins that non-covalently bind IGF-I and IGF-II with high affinity (dissociation constants in the order of magnitude 10^{-11} mol/L) and specificity (Table 2) (43, 44). To date at least six IGFBPS have been identified (20, 33). They have a high degree of sequence homology (44) and are numbered 1 to 6 in the order in which their primary structure was determined (45)(Figure 3). The IGFBPs have been proposed to have four major functions that are essential to coordinate and regulate the biological activities of the IGF-I: 1] to act as transport proteins in plasma and to control the efflux of IGF-I and IGF-II from the vascular space; 2] to prolong the half-life of IGF-I and IGF-II and thereby regulating its metabolic clearance; 3] to provide a means of tissue- and cell-type specific localization; 4] to directly modulate interaction between IGF-I and the IGF-I receptor, thereby indirectly controlling its biological actions (20). IGFBPs not only regulate the bioavailability of IGF-I but also seem to have their own receptors, which mediate IGF-I- independent actions (33). IGFBPs are produced by a variety of different tissues, each of which contain specific levels of these IGFBPs (33). The cleavage of IGFBPs by IGFBP-protease plays a key role in modulating free IGF-I and IGFBP levels and actions by two mechanisms: 1] IGFBP binding to IGFs decrease the concentration of free IGF-I available to interact with the IGF-I receptor, 2] cleavage of the IGFBPs into fragments with lower affinity to IGF-I allows for increased receptor activation (33). In various biological systems the IGFBPs have been shown to inhibit or potentiate IGF-I action (41).

IGFBP-1 and IGFBP-3 are discussed more extensively, because the role of the four other binding proteins is less well established at present. IGFBP-1 is a 25 Kd protein and found in high concentrations in amniotic fluid (32). IGFBP-1 is secreted by hepatocytes, kidney, decidua and endometrium (33). The half-life in plasma is approximately 90 minutes (46). IGFBP-1 has been proposed as an acute regulator of IGF-I activity (47). Insulin is a major regulator of hepatic IGFBP-1 production (33): plasma IGFBP-1 levels are inversely related to insulin levels in vivo (48). IGFBP-1 exists in different forms (phosphorylated and non-phosphorylated) (49). Non-phosphorylated IGFBP-1 has a 4- to 6-fold lower affinity to IGF-I than phosphorylated IGFBP-1 (49). This would favor sequestration of IGF-I by phosphorylated IGFBP-1 and a more complete release of IGF-I to the IGF-I receptor by the non-phosphorylated form. Most studies have shown inhibition of IGF-I activity by IGFP-1 (20). The IGFBP-1 form which potentiates IGF-I activity is non-phosphorylated, has a lower affinity and is able to associate with the cell surface (50). IGFBP-1 stimulates cell migration by itself (51). The stimulus appears to be the binding of IGFBP-1 to an integrin on the cell surface, initiating an intracellular pathway which involves a series of kinases (45).

Quantitatively the most important IGFBP in the circulation is IGFBP-3 (52). The 150 kD complex consists of IGF-I (or IGF-II) , IGFBP-3, and an acid-labile subunit (ALS) that is glycosylated and does not bind IGF-I (53). The ALS is present in excess in human plasma and is growth hormone dependent (53, 54). IGFBP-3 is secreted by hepatocytes and many other cells. About 75-95 % of IGF-I in the circulation is carried in the 150 kD IGF-I-IGFBP3- complex (20, 33) (see figure 2). It is considered

Table 2. Biochemical properties of human IGFBPs (modified after references 20, 44)

	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6
Amino acids	259	289	264	237	252	216
Cystine residues	18	18	18	18	18	16
Mr(kDa)	28.1	31.3	28.7	26.3	28.6	22.8
Phosphorylation	+	–	+	–	+	Unknown
Glycosylation	–	–	+	+	–	+
IGF-I/IGF-II preference	I>II	I<II	I≤II	I=II	I<II	I<<II
Chromosome	7	2	7	17	5	12

as a reservoir for IGF-I in the circulation: The 150 kD complex cannot cross the endothelial barrier of the blood vessels (52). This is considered critical for avoiding potential hypoglycemic insulin-like effects of the high concentrations of IGF-I which are present in plasma (52). The binding of IGF-I within the the 150 kD complex extends its half life time in the plasma from 10 minutes in the free form to 12 to 15 hours if bound in the complex form (55).

The expression of the gene for IGFBP-3 is increased by p53, the well characterized product of a tumor suppressor gene, which is considered important in the inhibition of tumor formation (56). Recently it has been demonstrated that p53 increases IGFBP-3 production by cells and that the IGFBP-3 produced in response to p53 is able to inhibit IGF-I induced growth of cells (56). IGFBP-3 thus appears one of those proteins which mediate the effects of p53 on cell growth, possibly by apoptosis.

Relations between IGF-I, IGFBPs, growth hormone and insulin.

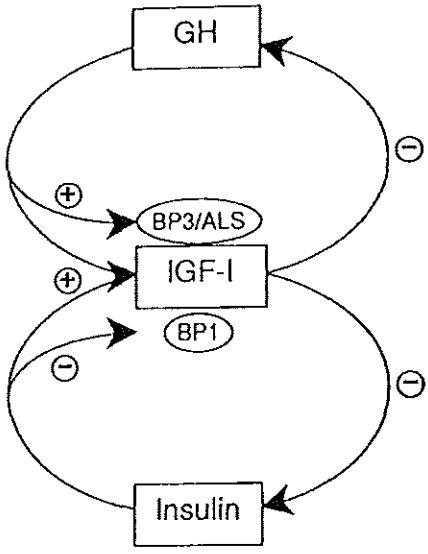

Figure 4

The relations between IGF-I, IGFBPs, growth hormone and insulin(from: Bondy CA. Clinical uses of insulin-like growth factor-I. Ann Intern Med 1994; 120: 594; with permission from the American College of Physicians)

Growth hormone (GH) and insulin both stimulate hepatic IGF-I production, and IGF-I feeds back to suppress GH and insulin release. IGFBP-3 and the associated acid-labile subunit (ALS) are positively regulated by GH, whereas IGFBP-1 levels are negatively regulated by insulin (40) (Figure 4).

IGF-I and age

Age is a major determinant of plasma IGF-I concentrations (57). Serum levels of IGF-I increase significantly from very low levels in cord serum through the first 9 months of life, whereafter there is an almost linear increase from early

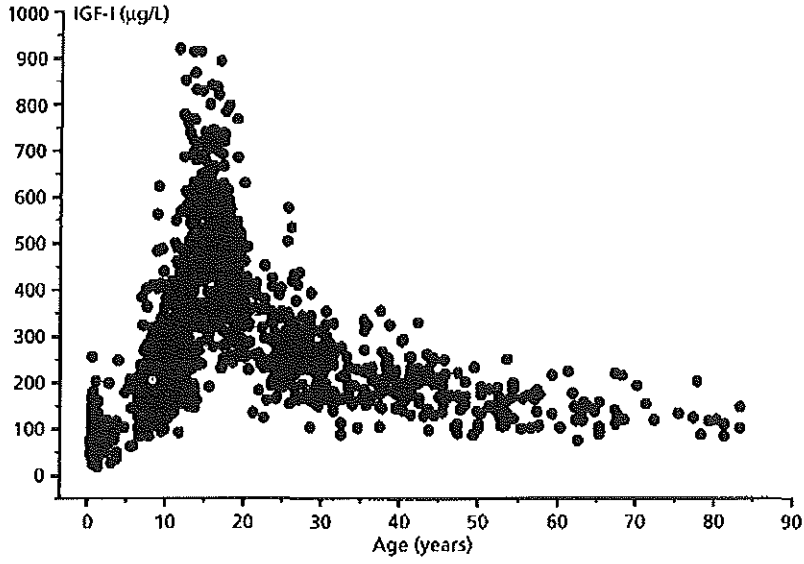

Figure 5

Serum IGF-I levels (vertical axis) according to age (horizontal axis) throughout life in a healthy population (n=1236 subjects). Note the peak at puberty and the wide range of IGF-I levels at each age (from Juul A. Serum levels of IGF-I and IGFBP-3 in healthy children, adolescents and adults. Methodological aspects and definition of reference values with special emphasis on puberty (Ph D Thesis, University of Copenhagen, 1995; with permission of NovoNordisk, Denmark)

childhood into adolescence (Figure 5) (58). After puberty, a 2.5-fold decrease occurs in the mean IGF-I values by the third decade, and a further twofold decrease occurs between the third and eights decade (57). Part of this decrement is believed to be due to the known age-related decrease in GH secretion (57). Very little is known on the effects and clinical consequences of the decrease of the GH-IGF-I system during aging.

IGF-I and diabetes

Many patients with insulin-dependent diabetes mellitus type 1 have some degree of growth hormone resistance, as shown by lowered circulating IGF-I levels, despite increased GH concentrations (59-61). They lack the ability to produce an appropriate increase in IGF-I response to exogenous GH. This functional refractoriness to GH is also seen during starvation and may be due to lowered portal vein insulin concentrations, insufficient to stimulate normal hepatic IGF-I synthesis (40). Treatment with subcutaneous insulin injections may not normalize all liver functions, such as IGF-I synthesis, which mainly depends on the presence of high portal insulin levels (40). In addition, increased growth hormone levels exacerbate hyperglycemia and low IGF-I levels may also contribute to deficient glucose metabolism and hyperglycemia (40).

Hyperglycemia is considered as an important pathogenetic factor for the development of diabetic micro- and macrovascular complications (62). The DCCT-trial has suggested that treatment of type I diabetic patients that does not result in normoglycemia will not prevent the development and progression of diabetic complications. Moreover, it has been reported in an animal model of diabetes that glucose levels play a major role in the regulation of IGF-I receptor mRNA (63). After hyperglycemia an increase in IGF-I receptor mRNA was observed, which was reversible by normoglycemia, even after long-standing hyperglycemia.

Aim of the thesis

The aim of this thesis was to investigate the (patho)physiologic role of the IGF-I/IGFBP system in adult subjects in health and disease under several conditions like aging, GH deficiency, acromegaly, diabetes mellitus and congenital partial lipodystrophy.

The thesis consists out of four parts (I, II, III, IV):

In part I (Chapter 2), we have investigated the relationship between the IGF-I/IGFBP system and blood cells in healthy subjects. Is it possible to measure IGF-I receptors on blood cells in vitro and what is the (clinical) importance of this assay. We studied especially the following questions: do IGF-I receptors on peripheral blood cells reflect what happens with the IGF-I receptors on other IGF-I target cells elsewhere in the body and what are the relationships and effects of (free and total) IGF-I and the IGFBPs on IGF-I receptor characteristics?

In part II (Chapter 3) we have investigated the relationships between the IGF-I/IGFBP system and age, sex steroids, cardiovascular diseases, bone mineral density and quality of health in a healthy elderly population. Other questions studied were: what is the potential significance of measuring serum free IGF-I levels and does the measurement of serum free IGF-I levels give additional and/or other information than the total IGF-I assay?

In part III (Chapter 4) the potential role of the IGF-I/IGFBP system in disorders of GH secretion (GH deficiency, acromegaly) were investigated. We have the studied

the following questions: Whom is to be tested and how to diagnose growth hormone deficiency in adults? Which components of the circulating IGF-I/IGFBP system can be used for this purpose? Do serum IGFBP-3 and/or free IGF-I levels reflect the clinical activity in acromegaly better than serum total IGF-I levels?

In part IV (Chapter 5) we have focused on the relationship between the IGF-I/IGFBP system, age and the presence of diabetic microvascular complications in insulin-dependent diabetes mellitus (IDDM) type I.

In this Chapter we have also studied the relationships between the IGF-I/IGFBP system and the IGF-I receptors on peripheral blood cells in patients with congenital partial lipodystrophy in order to study the role of the IGF-I/IGFBP system in the development of this syndrome which is characterized among other things by severe insulin resistance, hyperinsulinemia and acromegaloid features.

Finally, conclusions and the general discussion are presented in Chapter 6.

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2

The IGF-I/IGFBP system and blood cells

§ 2.1 Introduction: The role of IGF-I and IGFBPs in blood cells under physiologic and experimental circumstances.

IGF-I has been implicated as a hematopoietic regulatory cytokine as it has growth-promoting effects on a range of hematopoietic precursor cells. IGF-I enhances the growth of erythroid and granulocytic cells in vitro, but does not substitute the requirement for erythropoietin or granulocyte colony-stimulating factor (1-7). IGF-I increases thymocyte cell content and the size of the thymus size (8). Geffner et al. showed that locally generated IGF-I stimulates the growth of T-lymphoblasts after previous growth hormone stimulation, an effect which is mediated via the IGF-I receptor (9). These in vitro results were heralded by the observation that the stimulating effect of serum on the proliferation of PBMCs is increased in acromegalics and decreased in pituitary dwarfs (10). IGF-I may be involved in lymphocyte transformation and appears to be important for the process of lymphocyte proliferation. Mitogen-activated lymphocytes bear cell surface IGF-I receptors, which are only minimally expressed on quiescent lymphocytes (11-14). Nanomolar concentrations of IGF-I have been found to enhance anabolic processes or DNA synthesis in activated lymphocytes (10, 15, 16). IGF-I can partially substitute for serum in mitogen-induced T-cell proliferation (10). Moreover, IGF-I may stimulate chemotaxis of T-lymphocytes (17) and cytotoxic T cell function in vitro can be potentiated by IGF-I (18). Immunosuppressive effects of IGF-I have also been demonstrated in vitro (19). These divergent findings can at least be partly explained by the used culture methods in which the inclusion of serum with its high content of IGF-I and IGFBP- binding proteins was a confounding factor (20). In the absence of serum and phytohaemagglutin (PHA), IGF-I has also been demonstrated to stimulate [^{3}H] thymidine incorporation into mononuclear cells (21) and to enhance immunoglobulin production and proliferation of plasma cells (22).

However, the in vitro actions of IGF-I and the IGFBPs on blood cells are very complex: IGF-I has been found to be a potent signal for priming human polymorphonuclear neutrophils to secrete superoxide anion (23). IGF-I acting via

the IGF-I receptor is able to potentiate the release of histamine from basophils in response to immunoglobulin E (24).

Growth factors are believed to play an important role in the regulation of blood cell apoptosis, i.e. programmed cell death (25). In human erythroid progenitor cells, DNA breakdown from apoptosis due to serum deprivation is prevented by IGF-I (26). Apoptosis in an IL-3-dependent cell line of murine pre-B lymphocytes is inhibited by IGF-I (27). Overexpression of recombinant IGF-I receptors relieves hematopoietic cells of their dependency on IL-3 for their growth and survival (28). All these observations support the hypothesis that the inhibition of apoptosis in hematopoietic cells is mediated by the IGF-I receptor (28). IGF-I can thus influence both cell viability as well as the proliferation of hematopoietic cells. Inhibition of apoptosis in hematopoietic cells by IGF-I may help to expand a cell population which normally is destined to die (29).

Finally, human peripheral lymphocytes are not only potential target cells for IGF-I action, but are also a potential source of IGF-I and the IGFBPs in human serum (30). Stimulation of lymphocytes can affect the expression of IGF-I receptors, the production of IGF-I as well as of IGFBPs (30). Furthermore, human macrophages and rat leukocytes have also been reported to produce IGF-I (31, 32).

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§ 2.2. The IGF-I receptor on human blood cells: theoretical background.

Studies of adipocytes, muscle, and liver cells in vitro are unsuitable for the assessment of IGF-I receptors in a large number of subjects or for a repeated rapid evaluation of the characteristics of IGF-I receptors within the same individual. Blood cells offer a feasible alternative for this dilemma but this choice has also some disadvantages and shortcomings. Which type(s) of blood cell is (are) the most appropriate one(s) to use in an IGF-I receptor study? Do blood cells represent pathophysiological IGF-I receptor states in other target tissues? The study of IGF-I receptors resembles in this respect in many aspects the study of insulin receptors (1).

Circulating mononuclear cells can be divided into monocytes and lymphocytes. Most mononuclear cells make only a brief appearance in the blood stream on their way from the bone marrow to the tissues. Under normal conditions blood monocytes leave the blood stream with a half time of 4.5 to 10 hours (2, 3). In some disease states alterations in monocyte kinetics have been observed. For B-lymphocytes the transit time in the blood is less than 30 minutes (4).

T-Lymphocytes have the property of migrating continuously between the circulation and the lymphatic system. For the IGF-I receptor, differences in the number and Kd of T-cells, B-Cells, natural killer cells and monocytes have been described (5-8).

In contrast, different age groups of red cells from a single individual demonstrate distinctly different degrees of IGF-I binding capacity. It has been found that mature erythrocytes possess only 15% of the number of IGF-I binding sites of reticulocytes (6). Erythrocytes lack the capacity to synthesize new receptors and are thus unable to renew IGF-I receptors lost during their aging process.
Consequently, the majority of detectable IGF-I receptors per cell type is probably present on reticulocytes and other young red blood cells (9).
Hematological disorders that alter the normal erythrocyte half life of 60 days may in theory significantly affect IGF-I binding on this cell population (10). Many factors, including previous phlebotomy, as well as various hemolytic and other diseases

may affect the reticulocyte percentage and the mean erythrocyte age (6). Such confounding factors complicate the interpretation of IGF-I binding studies involving erythrocytes. However, some authors find this phenomenon unlikely, since they did not observe a correlation between IGF-I binding to erythrocytes and the number of reticulocytes (11).

IGF-I receptors are subject to down-regulation in the same manner as many other peptide hormone receptors, and expression of the IGF-I receptor gene inversely relates to serum levels of IGF-I (12, 13). No results of studies have been published sofar, in which in various clinical conditions the number and Kd of the IGF-I receptor on blood cells were compared to those on other important target organs for IGF-I. However, it has been found that defects of insulin binding on erythrocytes, which are not metabolically important targets for insulin action, correspond to defects of insulin binding on liver receptors in vivo (14). Since the insulin and IGF-I receptor are structurally similar, it suggests that the same relationship may exist for the IGF-I receptor.

The characteristics of insulin receptors in both mononuclear and erythrocytes have been repeatedly investigated in various clinical conditions (6). Significant correlations were observed between specific insulin binding to both cell types for a large number of subjects under a variety of physiological and pathological conditions, while the total number of binding sites and the affinity of the insulin receptor did not correlate between both cell types. In general, insulin binding to red blood cells changed more slowly after a change in the metabolic status than in monocytes (6).

Although there are no data available concerning the IGF-I receptor status on mononuclear cells and erythrocytes under different clinical situations, it seems very likely that similar to the findings for the insulin receptor (6), IGF-I receptors on red cells primarily reflect the chronic IGF-I receptor status, wheras IGF-I receptors on mononuclear cells reflect adaptation to the most recent metabolic changes.

Beck-Nielsen and Pedersen have found a diurnal variation for insulin binding to peripheral monocytes in healthy subjects, with a nadir at 1400 h and a peak at 2200 h (15). As fasting abolished these diurnal variations, this pattern seems likely

to be influenced by the diet. At present there are no data available whether IGF-I binding to peripheral blood cells also shows a diurnal rhythm.

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§ 2.3. **IGF-I receptors on blood cells: their relationship to circulating total and free IGF-I, IGFBP-1, IGFBP-3, and insulin levels in healthy subjects.**

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Summary

We have studied the relationships between the IGF-I/IGFBP system and the IGF-I receptor characteristics on erythrocytes and PBMCs in healthy subjects in the fasting state to establish whether this would be a valid way of examining IGF-I receptors in vivo. The Kd of the IGF-I receptor on erythrocytes was positively related to circulating free IGF-I levels. For the IGF-I receptor on PBMCs no relation was observed with free IGF-I levels.

IGFBP-3 levels were inversely related to the number of IGF-I binding sites on erythrocytes and to the Kd of the IGF-I receptor on PBMCs. Total IGF-I, insulin and IGFBP-1 levels showed no relation to the IGF-I receptor on erythrocytes and PBMCs in the fasting state. Our report suggests that studies of IGF-I receptor characteristics on erythrocytes and PBMCs in the fasting state are cell specific and cannot cannot be extrapolated to other cell types, which may be more relevant target tissues for IGF-I action in vivo.

Introduction

Insulin-like growth factor I (IGF-I) binds to high-affinity receptors which are expressed in a variety of cell types and tissues. In addition, IGF-I is capable of binding to the insulin receptor (1).The overlapping biological effects of IGF-I and insulin were initially attributed to the structural homologies between the peptides themselves, but subsequently it was shown that the receptors for insulin and IGF-I also have structurally similarities (2). Insulin and IGF-I receptors are both plasma membrane glycoproteins composed of two alpha and two beta subunits. The extracellular alpha subunit contains the ligand-binding domain, whereas the beta subunit contains the transmembrane anchoring domain as well as the putative tyrosine kinase activity (3).

In previous investigations significant correlations were found between insulin binding to insulin receptors on both peripheral blood mononuclear cells (PBMCs) and on erythrocytes in large numbers of subjects under a variety of physiological and pathological conditions (4).

Although there is considerable homology between IGF-I and insulin, and between the IGF-I and insulin receptor, insulin-like growth factor binding proteins (IGFBPs) form an important difference between these systems (5). IGFBPs are exclusively able to bind IGF-I but not insulin. Therefore they are considered as important modulators of IGF-I action. Previous studies demonstrated the presence of IGF-I receptors on both erythrocytes and PBMCs (6, 7).

We report here the results of simultaneously performed studies of IGF-I receptor characteristics on erythrocytes and PBMCs in healthy volunteers under fasting conditions. Their relation with the circulating IGF-I/IGFBP system and insulin was investigated in parallel to establish whether the study of the IGF-I receptor on these cells can be used to get an impression of IGF-I actions on more important target tissues for IGF-I in vivo.

Methods

Subjects

The study was performed according to the rules of the hospital medical ethics committee. Healthy human volunteers entered the study after informed consent. Twenty-one subjects (aged 23-47 yr) were investigated after an overnight fast. Blood was drawn from these subjects between 8.00-9.00 am.

Cells

Human erythrocytes were isolated from freshly obtained heparinized blood. Cell separation was performed by density centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) at 400 G for 30 min at room temperature. The interphase containing the mononuclear cells and granulocytes was collected and prepared as described below. The pellet containing the erythrocytes was resuspended in 0.9 % saline and centrifuged again on Ficoll-Paque under the same conditions. Erythrocytes were recovered from the pellet after the second centrifugation, and washed with two volumes ice-cold assay buffer (For composition see Peptides and Medium). Next the erythrocytes were centrifuged at 400 G for 10 min at 4 °C. After this centrifugation step the erythrocytes were resuspended in ice-cold assay buffer (two volumes cells, one volume assay buffer). This resulted in all experiments in a cell concentration above 5 x 10^9 cells/mL. The remaining part of the IGF-I receptor assay was performed at 4 °C. The number of cells was determined with the aid of an electronic cell counter model AD-260 (Sysmex, Kobe, Japan).

Human PBMCs were isolated from the same freshly taken heparinized blood sample. Isolation was performed by density centrifugation on Ficoll-Paque, as described above. Centrifugation on Ficoll-Paque yields an interface of PBMCs in which 95 +/- 5 % are mononuclear lymphocytes. PBMCs were recovered from the Ficoll medium interface, washed twice with 0.9% saline and after each washing, centrifuged during 5 minutes at 670 G at room temperature. The PBMCs were resuspended in 1,75 mL assay, which resulted in cell concentrations above 15 x 10^6 /mL. The number of cells per ml was counted with the aid of a

hematocytometer. After resuspension in assay buffer cells were kept at 4 °C.

Peptides and Medium

3-[^{125}I]iodotyrosyl-IGF-I was purchased from Amersham International Inc. (Amersham, Buckinghamshire, United Kingdom) and used as radioligand in the competitive IGF-I receptor assay. The radiolabeled IGF-I had a specific activity of 2,000 Ci/mmol. Competitive binding studies were performed with 0.05 nM ^{125}I-labeled hIGF-I and increasing concentrations of unlabeled purified human recombinant IGF-I (hIGF-I), IGF-II (hIGF-II) and insulin. hIGF-I and hIGF-II were purchased from Saxon Biochemicals GmBH (Hannover, Germany). Human recombinant insulin was purchased from Novo Nordisk (Bagsvaerd, Denmark).
The monoclonal anti-IGF-I receptor antibody (IgG), clone α-IR3 was purchased from Oncogene Science (Uniondale, NY, USA).
The assay buffer in the IGF-I receptor assay consisted of 50 mM Hepes, 50 mM Tris,10 mM dextrose, 50 mM $MgSO_4$, 2 mM EDTA, 10 mM $CaCl_2$, 45 mM NaCl, 4.5 mM KCl, and 0.1% BSA (6). The pH of the assay buffer was adjusted to 8.0 at room temperature. The experiments were performed at a pH of 8.0 since it has been found in previous experiments that there is optimal binding of insulin to erythrocytes at this pH (8). Moreover, Hizuka et al. have demonstrated that binding of [^{125}-I] IGF-I to cells did not change in the pH range between 7.0 and 8.0 (9). In order to reduce of the non-specific binding bacitracin was added in a concentration of 2 mg/mL to the incubation medium (10).

Competitive IGF-I receptor assay

Competitive binding studies were carried out in Eppendorf tubes. For the IGF-I receptor assay the methods described by Hizuka et al. (9) and Izumi et al. (6) were used with three modifications: The final incubation volume was decreased to 250 μL for measurement of IGF-I binding to erythrocytes, and to 100 μL for measurement of IGF-I binding to PBMCs. Secondly, bacitracin was added to the assay buffer. Thirdly, after 20 h incubation bound and unbound ligand were

separated by three washing steps with saline instead of separation with dibutyl phtalate as described in the original method.

Erythrocytes: In a final volume of 250 μL erythrocytes (> 1.0 x 10^9 / mL final concentration) or 100 μL PBMCs (> 15 x 10^6/mL), were incubated with ^{125}I-IGF-I (10-30.000 cpm) in assay buffer and in the absence or presence of increasing concentrations of unlabeled hIGF-I, hIGF-II, and insulin at 4 °C for 20 h. Each experiment was performed in duplicate. For the competition studies, added unlabeled concentrations of hIGF-I and hIGF-II ranged from 10^{-11} to 10^{-7} M, while insulin was added in concentrations ranging from 10^{-11} to 10^{-4} M.

After 20 h incubation, bound and unbound radioligand were separated by three washing steps with saline at 4 °C. In each washing step, the cells were centrifuged at 10.000 G during 2 minutes and the supernatant was removed. After the third centrifugation step the cell-bound radioactivity was measured in a gamma counter. Non-specific binding was defined as the amount of radioligand bound in the presence of excess of unlabeled IGF-I (10^{-7} M). Specific binding of ^{125}I-IGF-I was determined by subtracting the amount of radioactivity bound in the presence of excess of unlabeled IGF-I (10^{-7}M).

In the experiments with the anti-IGF-I receptor antibody αIR-3 competitive binding studies were carried out after preincubation of the cell suspensions during 3 h with αIR-3 (final concentration 1μg IgG_1/100 μl). Gelatine was added to the control tubes, because the solution provided by the manufacturer contained 0.2 % (w/v) gelatine. After 3 h preincubation the cell suspensions were incubated with $^{[125I]}$I- IGF-I and unlabeled IGF-I and the rest of the IGF-I receptor assay was performed as described above.

The binding data were analyzed according to the method of Scatchard (11). The number of binding sites is expressed as sites per cell. The dissociation constant (Kd) is given in nmol/L.

Hormone assays

Blood samples were taken by venipuncture simultaneously with the IGF-I receptor study, and was allowed to coagulate for 60 minutes. All subjects were fasting . Subsequently, serum was separated by centrifugation and quickly frozen to -20 ^{o}C. Free IGF-I was measured with a commercially available two-site immunoradiometric assay (Diagnostic System Laboratories Inc., Webster, Texas, U.S.A.; intra-assay and inter-assay C.V.: 10.3% and 10.7%, respectively) (12). Estimation of free IGF-I typically involves size-exclusion extraction followed by a displacement immunoassay. Sample matrix alterations with consequent disturbance of the free/bound equilibrium is an inevitable consequence. The used free IGF-I assay needs initially no sample extraction as part of the standard procedure to measure IGF-I. Samples are added directly to tubes containing a dense coating of high-affinity free IGF-I antibody, incubated at 2 hr at room temperature, washed, incubated with ^{125}I labeled antibody directed to a second epitope, washed and counted. Assay standards are rhIGF-I: 0.04-2.6 nmol/L, the minimal detection limit is 3.9 x 10^{-3} nmol/tube. There is no cross-reactivity with IGF-II and no residual IGFBP-1 or IGFBP-3 is detectable after the first wash. It is likely that the free IGF-I fraction measured with the free IGF-I assay represents a combination of the true free and the fraction of IGF-I which can be readily dissociated from IGFBPs under the specific assay conditions (12). Total IGF-I was determined by a commercially available radioimmunoassay (Medgenix Diagnostics, Brussels, Belgium, intraassay and interassay coefficients of variation (C.V.): 6.1%; 9.9%) after the acid/ethanol extraction method, originally described by Daughaday et al.(13).The purpose of this step is to convert the different forms of IGF-I present into free IGF-I. Commercially available immunoradiometric assays were also used for measurement of IGFBP-1, and IGFBP-3 (Diagnostic System Laboratories Inc., Webster, Texas, U.S.A.; intraassay and interassay C.V. for IGFBP-1: 4.0% and 6.0%, respectively; and for IGFBP-3: 1.8 % and 1.9%, respectively). Insulin was determined by a commercially available radioimmunoassay (Medgenix Diagnostics, Brussels, Belgium, intraassay and interassay C.V.: 8.0%; 13.7%).

Statistical Analysis

Results are expressed as the mean +/- SEM. Pearson 's correlation coefficients were calculated to assess the association between variables. A two-sided p value of < 0.05 was considered significant.

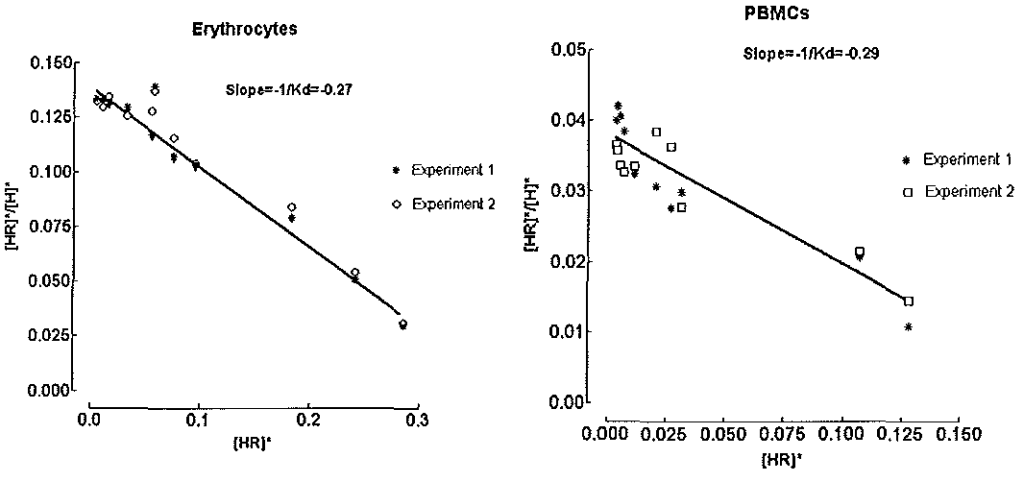

Figure 1

Scatchard analyses of ^{125}I-IGF-I binding to human erythrocytes (left) and PBMCs (right) show both a linear plot.
[H] = concentration of Free IGF-I
[HR] = concentration of Bound IGF-I receptor complex
[HR]/[H] = Bound/Free ratio
The showed linear regression analysis is the mean of one experiment, done in duplicate

Results

^{125}I-IGF-I specifically bound to both human erythrocytes and PBMCs and the binding linearly increased for both cell types as a function of the number of cells (data not shown). Scatchard analyses for IGF-I binding to erythrocytes and PBMCs, respectively, showed a linear plot (Figure 1).

Specificity of the IGF-I receptor assay was established as follows: 1] unlabeled IGF-I, unlabeled IGF-II, and unlabeled insulin were able to displace ^{125}I-IGF-I to erythrocytes and PBMCs, although with striking differences of more than 100-fold between IGF-I and insulin in affinity (Figure 2). Binding of ^{125}I-IGF-I to erythrocytes and PBMCs was inhibited by the unlabeled peptides in the following order of potency: IGF-I>IGF-II>>>insulin (Figure 2). 2]After preincubation with αIR-3 (a specific anti-IGF-I receptor antibody) the specific binding of ^{125}I-IGF-I in the IGF-I receptor assay was totally lost (Figure 3).

Table 1 shows the results of the characteristics of the IGF-I receptor on erythrocytes and PBMCs, respectively, as well as the circulating hormone levels. The number of IGF-I binding sites was closely positively related to the Kd of the

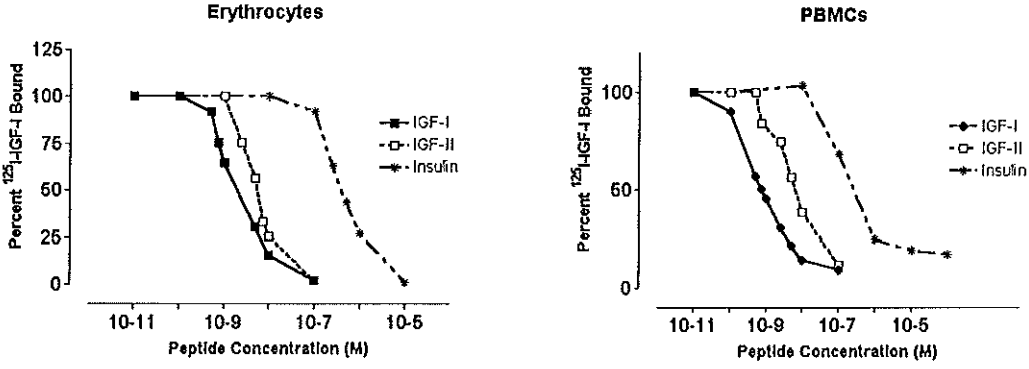

Figure 2

Competition of IGF-I tracer binding by unlabeled IGF-I, IGF-II and insulin.
Left: Erythrocytes (± 5 x 10^9 per mL) were incubated with tracer plus the indicated hormone concentrations at 4 °C for 20 hours. The percentage ^{125}I-IGF-I specific bound is plotted as function of the unlabeled concentration. Data are the means of one experiment, done in duplicate.
Right: PBMCs (± 15 x 10^6 per mL) were incubated with tracer plus the indicated hormone concentrations at 4 °C for 20 hours. The percentage ^{125}I-IGF-I specific bound is plotted as function of the unlabeled concentration. Data are the means of one experiment, done in duplicate.

IGF-I receptor on both erythrocytes and PBMCs in the fasting state (in all cases p ≤ 0.001)(Table 2). The Kds of the IGF-I receptor on erythrocytes and PBMCs, and the numbers of the IGF-I receptor on erythrocytes and PBMCs, respectively, were closely correlated (Kds: p=0.009 , numbers: p=0.04)(Table 2).

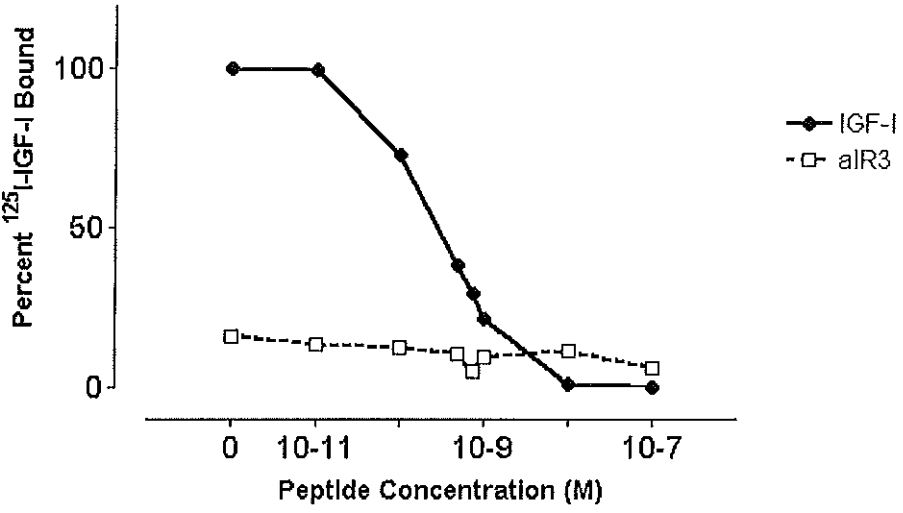

Figure 3

Effect of preincubation with α-IR-3 (final concentration 1μg IgG_1 /100 μl) on competitive IGF-I binding assay of human PBMCs. The percentage ^{125}I-IGF-I specific bound to PBMCs is plotted as function of the unlabeled IGF-I concentration. Solid line: competitive IGF-I binding assay without preincubation with α-IR-3. Broken line: competitive IGF-I binding assay with preincubation with α-IR-3.Data are the means of one experiment, done in duplicate.

IGF-I receptor on both erythrocytes and PBMCs in the fasting state (in all cases p ≤ 0.001)(Table 2). The Kds of the IGF-I receptor on erythrocytes and PBMCs, and the numbers of the IGF-I receptor on erythrocytes and PBMCs, respectively, were closely correlated (Kds: p=0.009 , numbers: p=0.04)(Table 2).

Free IGF-I was positively related to the Kd of the IGF-I receptor on erythrocytes in the fasting state (p=0.03), while no significant correlation was found with the

Table 1. Characteristics of IGF-I binding to Erythrocytes and PBMCs, circulating IGF-I and IGFBPs, and insulin in healthy volunteers in the fasting state.

		mean	(SEM)	Range
Age subjects (years)		33.4	(1.8)	23-47
Erythrocytes				
No of specific IGF-I binding sites/cell		33.7	(3.5)	11-57
Kd (nM)		2.33	(0.26)	0.42-4.75
PBMCs				
No of specific IGF-I binding sites/cell		3547	(476)	430-8774
Kd (nM)		2.05	(0.24)	0.22-3.73
Total IGF-I	(nmol/L)	28.0	(1.6)	13.3-46.4
Free IGF-I	(nmol/L)	0.145	(0.015)	0.034-0.257
IGFBP-1	(nmol/L)	1.23	(0.28)	0.17-5.46
IGFBP-3	(nmol/L)	100.0	(2.4)	80.0-121.7
Insulin	(mU/L)	18.4	(3.4)	5.2-69.4

* For used abbreviations, see text

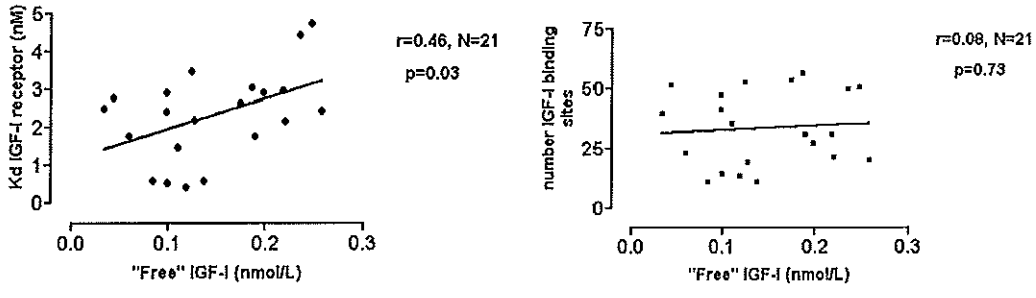

Figure 4

Relation between the Kd of the IGF-I receptor on erythrocytes (vertical axis) and serum free IGF-I levels (horizontal axis)(left) and relation between IGF-I binding sites per erythrocyte (vertical axis) and serum free IGF-I levels (nmol/L) (horizontal axis)(right), both in 21 healthy volunteers and in the fasting state.

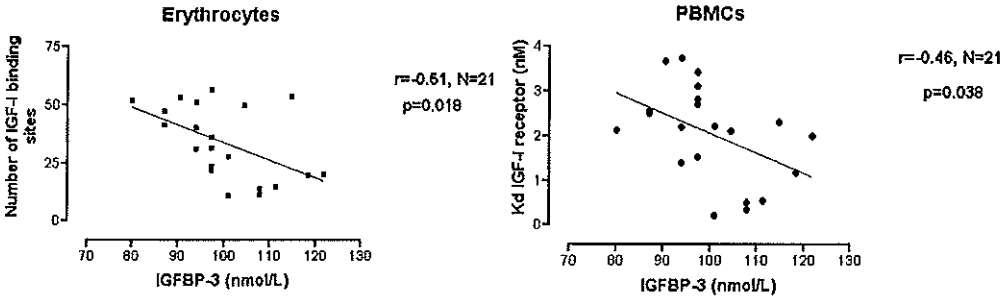

Figure 5

Relation between serum IGFBP-3 levels and number of IGF-I binding sites on erythrocytes (left) and relation between serum IGFBP-3 levels and the Kd of the IGF-I receptor on PBMCs (right).

number of receptors (Table 2, and Figure 4). IGFBP-3 was inversely related to the number of IGF-I binding sites on erythrocytes ($p=0.02$) and to the Kd of the IGF-I receptor on PBMCs ($p=0.04$) (Table 2 and Figure 5). The relation between free IGF-I levels and the Kd of the IGF-I receptor on erythrocytes in the fasting state, remained significant after adjustment for IGFBP-3 levels using multivariate analyses, and this relation became even stronger. No relations were observed between total IGF-I, IGFBP-1, and insulin, respectively, and the number or the Kd of the IGF-I receptor on erythrocytes and PBMCs (Table 2)

Table 2. Pearson 's correlation coefficients between the circulating IGF-I/IGFBP system, insulin, and the IGF-I receptor on erythrocytes and PBMCs in the fasting state (21 subjects)

	Total IGF-I	Free IGF-I	IGFBP-1	IGFBP-3	Erythrocytes IGF-I Receptor Binding Sites	Erythrocytes IGF-I Receptor Kd	PBMCs IGF-I Receptor Binding Sites	PBMCs IGF-I Receptor Kd
Free IGF-I	0.56 §							
IGFBP-1	-0.54 *	-0.31						
IGFBP-3	0.67 §	0.33	-0.36					
Erythrocytes								
Binding sites	-0.02	0.08	0.12	-0.51 *				
Kd	0.21	0.46 *	0.05	-0.32	0.78 #			
PBMCs								
Binding sites	-0.03	-0.15	-0.02	-0.35	0.38 *	0.23		
Kd	-0.03	0.12	-0.13	-0.46 *	0.60 §	0.55 §	0.74 #	
Insulin	-0.17	0.06	-0.44 *	-0.26	0.04	0.11	0.07	0.24

* $p<0.05$, § $p<0.01$, # $p<0.001$

Discussion

We have studied whether the characteristics of the IGF-I receptor on erythrocytes and PBMCs can be used to investigate the relation between the circulating IGF-I and IGFBP levels and the IGF-I receptor characteristics in the fasting state and so to get an impression of IGF-I actions on more important target tissues for IGF-I in vivo. The methods to measure IGF-I characteristics on blood cells described by by Izumi et al. (6) and by Hizuka et al. (9) were used for this purpose with a few modifications: the volume of the incubation medium was reduced in order to reduce the amount of blood and materials needed for each assay, and bacitracin at a concentration of 2 mg/mL was added to reduce non-specific binding without altering receptor affinity. Thirdly, after 20 h incubation bound and unbound ligand were separated by three washing steps with saline in stead of separation with dibutylphthalate as described in the original method.

For both PBMCs as well as erythrocytes, 10% to 90% of the competition of cold IGF-I against ^{125}I-IGF-I occurred over a concentration range of competitor spanning two orders of magnitude (see Figure 2). This is the typical result for a simple competitive interaction of an unlabeled and labeled ligand for a single class of sites. Moreover, for both PBMCs and erythrocytes linear Scatchard plots were found in the concentration range of 10^{-11} to 10^{-9} M IGF-I, which can be considered as the physiological range of (free) circulating IGF-I levels (see below).

We observed a comparable number of IGF-I receptors on erythrocytes in normal individuals as has been reported before (6, 9, 10, 14, 15). These previous studies reported numbers of high affinity IGF-I receptors on erythrocytes varying between 2 and 58 sites per cell in the fasting state. The Kd measured for erythrocytes in the fasting state was also similar to the values reported by Izumi et al and Hizuka et al. (6, 9). In previous studies, also using competitive binding assays to measure IGF-I receptors, the number of high affinity IGF-I receptors on PBMCs varied between 45 and 4,500 sites per cell (16, 17, 18). The number of IGF-I binding sites and the Kd per PBMC, observed in our experiments, were in the same range.

Measuring free IGF-I levels in serum, may have physiological relevance because it theoretically represents the biologically active fraction (19). We found in this group

of healthy individuals, mean levels of 0.145 (range 0.034-0.257) nmol/L in the fasting state. In the past a significant inverse correlation has been demonstrated in non-healthy subjects between plasma total IGF-I levels and the number of IGF-I receptors on erythrocytes by some (20-21), but not all investigators (22). In agreement with the latter study, we did not find such a relation in the fasting state in erythrocytes and PBMCs. Most circulating IGF-I is bound to the insulin-like growth factor binding proteins (IGFBPs), which titrate the supply of IGF-I to its receptors, while a very small component circulates as the free form (23-24). Free IGF-I was positively related to the Kd of the IGF-I receptor on erythrocytes in the fasting state, but not to the number of IGF-I receptors on erythrocytes. Moreover, free IGF-I was not related to the number and the Kd of IGF-I receptors on PBMCs in the fasting state. We do not have an explanation for this cell-specific difference, but this suggests that findings from erythrocytes and PBMCs could not be extrapolated to other cell types, which may be more relevant target tissues for IGF-I action.

It has been suggested that the study of insulin receptors on red cells reflects the chronic and long-term insulin receptor status better, whereas the insulin receptors on mononuclear cells primarily might reflect the most recent (acute) IGF-I receptor changes (25). Our study does not give information whether the study of IGF-I receptors on blood cells in this respect is similar to the study of insulin receptors on blood cells.

IGFBP-3 -quantitatively the most important IGFBP- was inversely related to the number of IGF-I receptors on the erythrocytes. IGFBP-3 was also inversely related to the Kd of the IGF-I receptor on PBMCs. These observations suggest that IGFBP-3 may modulate the interaction of IGF-I with the IGF-I receptors on blood cells. Our study also suggests that this modulation is celltype-specific. It may thereby indirectly control biological actions of IGF-I (21).

No statistical relationship was observed between circulating insulin levels and the characteristics of the IGF-I receptor (number, Kd) or circulating total IGF-I, free IGF-I and IGFBP-3 in the fasting state in our study. Only the well known inverse relationship between insulin and IGFBP-1 was confirmed. It has been suggested

that IGF-I receptor regulation by insulin is a complex and dynamic phenomenon (26, 27). In addition, insulin can downregulate IGF-I receptors in some tissues and upregulate IGF-I receptors in some other tissues (26, 27).

In conclusion: We have studied the relationships between the IGF-I/IGFBP system and the IGF-I receptor characteristics on erythrocytes and PBMCs in healthy subjects in the fasting state. The Kd of the IGF-I receptor on erythrocytes in the fasting state was positively related to circulating free IGF-I levels. For the IGF-I receptor on PBMCs no relation was observed with free IGF-I levels. IGFBP-3 levels were inversely related to the number of IGF-I binding sites on erythrocytes and to the Kd of the IGF-I receptor on PBMCs. Total IGF-I, insulin and IGFBP-1 levels showed no relation to the IGF-I receptor characteristics on erythrocytes and PBMCs in the fasting state. Our data suggest that IGF-I and IGFBPs may modulate the IGF-I receptor status on blood cells celltype-specific. Therefore, studies of IGF-I receptor characteristics on erythrocytes and PBMCs in the fasting state cannot be extrapolated to other cell types, which may be more relevant target tissues for IGF-I action in vivo.

Acknowledgement

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§ 2.4 The relationships between circulating total and free IGF-I, IGFBP-1, IGFBP-3, and insulin levels after an oral glucose load.

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Summary

We studied the relationships between the circulating insulin-like growth factor/insulin-like growth factor binding proteins (IGF-I/IGFBP) system, insulin and glucose levels in the fasting state and during a 75 g oral glucose tolerance test (OGTT) in six healthy volunteers. In three of these volunteers IGF-I receptors on peripheral mononuclear blood cells (PBMCs) were also studied at the start and at the end of the OGTT.

Serum insulin increased, while free IGF-I, and IGFBP-3 levels showed no significant changes during the first 120 minutes after an oral glucose load. The free IGF-I/IGFBP-1 ratio, a measurement of IGF-I bioactivity, started to increase 60 minutes after the glucose load. The number and affinity of IGF-I binding sites on PBMCs did not change 120 minutes after an oral glucose load.

This study suggests that circulating insulin and IGFBP-1 play a role in the regulation of IGF-I bioactivity in response to an acute glucose load.

Introduction

The hypoglycemic insulin-like effects of IGF-I suggest that the IGF-I/IGFBP system is involved in glucose homeostasis (1). There is considerable homology between IGF-I and insulin, and between the IGF-I and insulin receptor (2), while insulin-like growth factor binding proteins (IGFBPs) form an important difference between these systems (3). The binding proteins are important modulators of IGF-I action, and exclusively bind IGF-I but not insulin.

Nevertheless, insulin also influences IGFBPs: IGFBP-1 levels are negatively regulated by insulin (4) and it has been suggested that insulin plays an important role in the regulation of IGFBP-3 protease activity (5). Moreover, insulin stimulates hepatic IGF-I production, while IGF-I feeds back to suppress insulin release (4).

We report here the results of a study in six healthy volunteers into the dynamic relation between the circulating IGF-I/IGFBP system and insulin levels in response to an oral glucose load, while in parallel in three of these volunteers IGF-I receptor characteristics on PBMCs were studied under fasting conditions and after 120 minutes.

Methods

Subject and Methods

Four healthy non-obese human male volunteers (31, 33, 34 and 39 years) and two female volunteers (21 years and 46 years) participated in the study and were investigated after an overnight fast and after an oral 75 gram glucose load during two hours in the morning. The first sample was drawn in the fasting state in the morning between 8.30 am and 9.30 am immediately before the glucose load (=0 minutes). Blood samples were taken by an indwelling catheter (venflon), and were allowed to coagulate for 60 minutes.

Subsequently, serum was separated by centrifugation and quickly frozen to -20 ^{o}C. After the glucose load, blood was drawn after 30, 60 and 120 minutes. The subject, who developed a reactive hypoglycemia during the OGTT was excluded from the

statistical analysis, since a hypoglycemia can induce a paradoxical rise of IGFBP-1 levels by suppression of endogeneous insulin levels (6). The results of this subject are presented separately.

In three subjects (2 men an 1 woman) IGF-I receptors were performed during the OGTT. For the IGF-I receptor study a total of 120 mL of blood was drawn. The fasting sample and the sample drawn after 120 minutes were used for the IGF-I receptor studies (see infra). The study was performed according to the rules of the hospital medical ethics committee. All subjects entered the study after informed consent.

Hormone assays

Free IGF-I was measured with a commercially available two-site immunoradiometric assay (Diagnostic System Laboratories Inc., Webster, Texas, U.S.A.;
intraassay and interassay C.V.: 10.3% and 10.7%, respectively)(7). Total IGF-I was determined by a radioimmunoassay (Medgenix Diagnostics, Brussels, Belgium; intraassay and interassay coefficients of variation (C.V.): 6.1%; 9.9%).

Immunoradiometric assays were also used for measurement of IGFBP-1, and IGFBP-3 (Diagnostic System Laboratories Inc., Webster, Texas, U.S.A.; intraassay and interassay C.V. for IGFBP-1: 4.0 % and 6.7 %, respectively; and for IGFBP-3: 1.8 % and 1.9%, respectively). Insulin was determined by a radioimmunoassay (Medgenix Diagnostics, Brussels, Belgium; intraassay and interassay C.V. 13.7% and 8.0%). Blood glucose concentrations were measured in venous whole blood by an automatic hexokinase method (Boehringer Mannheim Gmbh, Germany).

IGF-I receptor study

PBMCs were isolated from freshly obtained heparinized blood and cell separation was performed by density centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) at 400 G for 30 min at room temperature.

For the IGF-I receptor assay the methods described by Hizuka et al. (8) and Izumi et al. (9) were used with three modifications: The final incubation volume was decreased to 100 μL for measurement of IGF-I binding to PBMCs. Secondly,

bacitracin was added to the assay buffer (10). Thirdly, after 20 h incubation bound and unbound ligand were separated by three washing steps with saline in stead of separation with dibutylphthalate as described in the original method.

In a final volume 100 μL 60 μL PBMCs (> 15 x 10^6/mL), were incubated with ^{125}I-IGF-I(10-30.000 cpm) in assay buffer and in the absence or presence of increasing conentrations of unlabeled hIGF-I at 4 °C for 20 h. Each experiment was performed in duplicate. For the competition studies, added unlabeled concentrations of hIGF-I ranged from 10^{-11} to 10^{-7} M.

After 20 h incubation, bound and unbound radioligand were separated by three washing steps with saline at 4 °C. In each washing step, the cells were centrifuged at 10.000 G during 2 minutes and the supernatant was removed. After the third centrifugation step the cell-bound radioactivity was measured in a gamma counter. Non-specific binding was defined as the amount of radioligand bound in the presence of excess of unlabeled IGF-I (10^{-7} M). Specific binding of ^{125}I-IGF-I was determined by subtracting the amount of radioactivity bound in the presence of excess of unlabeled IGF-I (10^{-7}M).

The binding data were analyzed according to the method of Scatchard (11). The number of binding sites is expressed as sites per cell. The dissociation constant (Kd) is given in nmol/L.

Statistical analysis

Results are given as the mean with ± SE. Changes in variables during the OGTT were analyzed by Wilcoxon signed-rank sum test. A $p<0.05$ was considerd statistically significant.

Results

Mean serum free IGF-I, IGFBP-3 and GH levels did not significantly change during the first 120 minutes after an OGTT, while IGFBP-1 decreased and as a consequence the IGF-I/IGFBP-1 ratios increased the last 60 minutes during the OGTT (Figure 1, Table 1).

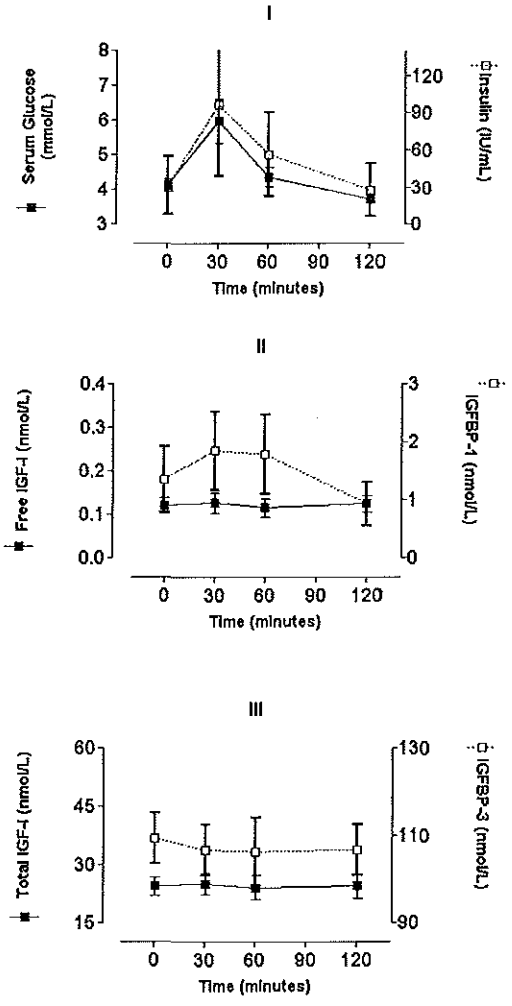

Figure 1. The mean results (with SE) from an oral glucose tolerance test during a 75 g OGTT in five healthy individuals. Measurements were performed after 0 (fasting), 30, 60 and 120 min during the OGTT. The changes in mean glucose (I, left axis), Insulin (I, right axis), Free IGF-I (II, left axis), IGFBP-1(II, right axis), Total IGF-I (III, left axis), and IGFBP-3 (III, right axis) are shown for these five subjects. Individual data used were the means of one experiment, done in duplicate.

Table 1. Changes in mean values for free IGF-I, total IGF-I, IGFBP-1, IGFBP-3 and insulin levels during an oral glucose tolerance in 5 healthy subjects.

	0 min	30 min	60 min	120 min
Glucose (mmol/L)	4.2 (0.2)	6.0 (0.6) ■	4.4 (0.3)	3.7 (0.1) †
Insulin (mU/L)	32.0 (23.6)	96.6 (57.2) ■	56.3 (33.9) ■ †	27.5 (21.5) ■ † ‡
Free IGF-I (nmol/L)	0.123 (0.019)	0.126 (0.023)	0.116 (0.021)	0.124 (0.019)
IGFBP-1 (nmol/L)	1.37 (0.56)	1.85 (0.69)	1.79 (0.70)	0.93 (0.37) † ‡
Free IGF-I/IGFBP-1 ratio	0.14 (0.05)	0.12 (0.05)	0.11 (0.05)	0.25 (0.10) † ‡
Total IGF-I (nmol/L)	24.7 (2.4)	25.1 (2.8)	24.1 (3.1)	24.5 (3.1) ‡
IGFBP-3 (nmol/L)	109.5 (5.8)	106.7 (5.7)	106.3 (7.8)	106.9 (5.8)
GH (μg/L)	2.3 (2.05)	4.8 (5.1)	1.6 (1.7)	1.1 (1.0)

Data showed are the means with SE values between brackets
Changes in variables during the OGTT were analyzed by Wilcoxon signed-rank sum test
■ $p < 0.05$ when compared with baseline value (t=0 min)
† $p < 0.05$ when compared with t=30 min
‡ $p < 0.05$ when compared with t=60 min

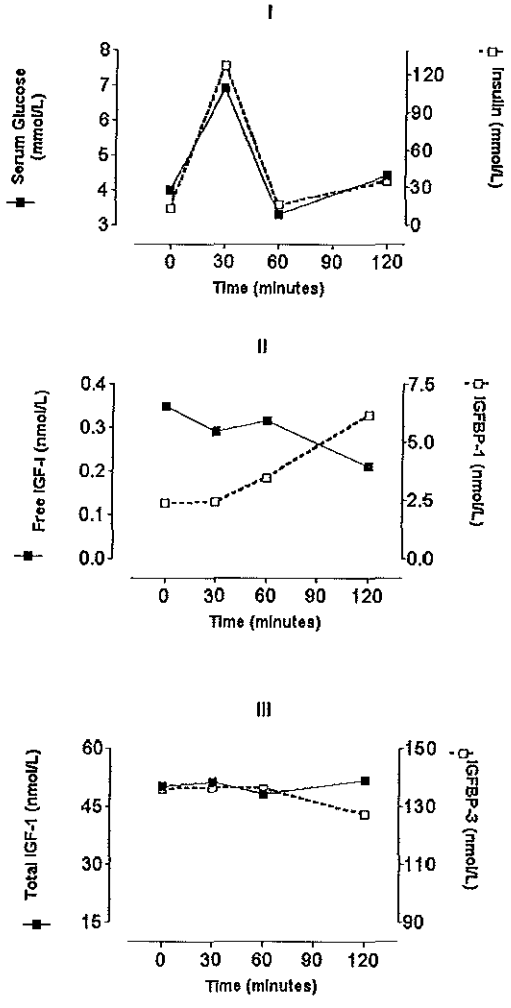

Figure 2. The results from an oral glucose tolerance test during a 75 g OGTT in one healthy individual. Measurements were performed after 0 (fasting), 30, 60 and 120 min during the OGTT. The changes in glucose (I, left axis), Insulin (I, right axis), Free IGF-I (II, left axis), IGFBP-1(II, right axis), Total IGF-I(III, left axis), and IGFBP-3 (III, right axis) are shown for this Data are the means of one experiment, done in duplicate. Note the reactive hypoglycemia in this subject.

Table 2. Characteristics of IGF-I binding to PBMCs, circulating IGF-I and IGFBPs, and insulin in three healthy volunteers in the fasting state and after an oral glucose load at t=120 minutes

		Fasting		After 120 min OGTT		
		Mean	SE	Mean	SE	p-value
PBMCs						
No of specific IGF-I binding sites/cell		2389	(868)	2995	(2018)	ns
Kd (nM)		1.86	(0.23)	1.85	(0.14)	ns
Total IGF-I	(nmol/L)	24.7	(4.6)	24.5	(5.7)	ns
Free IGF-I	(nmol/L)	0.127	(0.037)	0.134	(0.033)	ns
IGFBP-1	(nmol/L)	1.52	(1.08)	0.76	(0.44)	ns
Free IGF-I/IGFBP-1 ratio		0.165	(0.093)	0.295	(0.177)	ns
IGFBP-3	(nmol/L)	108.4	(1.2)	106.7	(10.9)	ns
Insulin	(mU/L)	45.1	(43.5)	39.9	(39.9)	ns
Serum glucose	(mmol/L)	4.3	(0.3)	3.8	(0.2)	ns

For used abbreviations, see text
Data are the means from one experiment done in duplicate, standard deviation between brackets
Changes in variables during the OGTT were analyzed by Wilcoxon signed-rank sum test

Serum insulin levels transiently increased after 30 minutes and had returned to fasting values after 120 minutes (Table 1, Figure 1). Individual analysis of serum free IGF-I levels showed no changes during the OGTT in five subjects, but in the subject, who had a reactive hypoglycemia during the OGTT, serum free IGF-I levels decreased significantly while serum IGFBP-1 levels simultaneously increased after 120 minutes (Figure 2). As a consequence, GH level in this latter subject was increased after 120 minutes (GH at t=0 min: 0.7 μg/L; GH at 30 min: 0.4 μg/L;GH at t=60 min: 0.7 μg/L; and GH at t=120 min: 7.4 μg/L) while the free IGF-I/IGFBP-1 ratio decreased from 0.15 at t=0 min to 0.05 at t=120 min.
The number and affinity of the IGF-I receptors on PBMCs did not change when the values in the fasting state and 120 minutes after start of the OGTT were compared (see Table 2)

Discussion

Mean serum free IGF-I levels did not change significantly in five healthy subjects during the first 120 minutes of an OGTT in our study. Mean IGFBP-1 levels decreased, while the free IGF-I/IGFBP-1 levels ratios increased after 60 min.
Also no changes in free IGF-I levels were observed until 4 hours after a morning meal in a recent study using the same assay (12). Insulin and IGF-I intercommunicate at many levels and act in a dual effector model regulating basal and postabsorptive metabolism (13). Under normal conditions acute variations in IGFBP-1 levels are inversely related to insulin levels (14), while IGFBP-1 has been proposed as an regulator of IGF-I bioactivity (15). It has been demonstrated that low IGFBP-1 levels result in high IGF-I activity, and vice versa (14). The increase in the mean free IGF-I/IGFBP-1 ratio during the last 60 minutes of the OGTT (Table 1) suggests thus an increased IGF-I bioactivity and supports the idea that IGF-I is a main modulator of postabsorptive metabolism in the postprandial period (16).
Previously it has also been suggested that a lowered total IGF-I/IGFBP ratio may reflect a lower bioavailability of IGF-I (17). Thus our observation in the subject who tended to develop a reactive hypoglycemia after the glucose load, suggests that

the increase of IGFBP-1 levels, indeed lowered IGF-I bioactivity acutely in this subject in order to counteract the development of hypoglycemia.

It took about one hour after the glucose load before a significant decrease in IGFBP-1 levels was observed in the other five subjects. The transient increase in serum insulin levels after a glucose load (peak at 30 min) as observed in our study eventually induces this decrease in serum IGFBP-1 levels. Insulin exerts an inhibitory effect on m-RNA IGFBP-1 production in the liver (18). The observed changes in serum IGFBP-1 levels are in accordance with the estimated half life of IGFBP-1 in plasma (30-90 minutes) (19).

Because of the considerable amount of blood needed, it was investigated only in three subjects whether the characteristics of the IGF-I receptor on PBMCs are acutely affected by changes in the circulating levels of insulin, GH, IGF-I and its IGFBPs. Our study suggests that IGF-I receptors on PBMCs are unaffected after a glucose load. IGF-I receptors are subject to up- and down-regulation in the same manner as many other peptide hormone receptors, and it has been suggested that expression of the IGF-I receptor gene varies inversely with serum levels of IGF-I (20, 21). Since the free IGF-I/IGFBP-1 ratios increased at the end of the OGTT, however, our observations suggest that no acute changes in the IGF-I receptor characteristics occur during 120 minutes after a glucose load. In contrast to these findings, a marked increase in the affinity of insulin receptors of PBMCs has previously been demonstrated after an oral glucose load, suggesting that changes in the insulin receptor characteristics acutely occur as part of the physiological and dynamic regulation of target cell sensitivity (22).

In conclusion: We have studied the reaction of the circulating IGF-I/IGFBP system to an oral glucose load. Free IGF-I levels and IGFBP-3 levels did not significantly change during the first 120 minutes after the glucose load, despite significant changes in insulin, glucose, and IGFBP-1 levels. The free IGF-I/IGFBP ratio, a measurement for IGF-I bioactivity, increased 60 minutes after a glucose load.The characteristics of the IGF-I binding sites on PBMCs (Kd and number) did not change 120 minutes after a glucose load. This preliminary study suggests that circulating insulin and IGFBP-1 play a role in the dynamic regulation of IGF-I

bioactivity in response an acute glucose load.

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3

The IGF-I/IGFBP system and aging

§ 3.1 Serum free IGF-I, total IGF-I, IGFBP-1 and IGFBP-3 levels in an elderly population: relation to age and sex steroid levels

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Summary

Background: Most previous studies concerning the relationship between IGF-I and age used assays measuring total IGF-I. Although free IGF-I is considered of greater biological relevance, little is known about its relationship with sex steroids levels in elderly healthy subjects.

Measurements: In a cross-sectional study of 218 healthy persons (103 men, 115 women) aged 55 to 80 years we measured serum total and free IGF-I, IGFBP-1 and IGFBP3 levels and sex steroids. Free androgen index and free oestradiol index were used as an indicator for free oestradiol and free testosterone levels, respectively.

Results: Free IGF-I levels did not decline with age in the whole study population. Free IGF-I levels even increased in individuals above 70 yrs of age in comparison to those aged between 55 and 70 yrs (0.086(SE 0.004) nmol/L vs 0.106 (SE 0.007) nmol/L , p=0.009). Total IGF-I and IGFBP-3 decreased with age (r= -0.20, p=0.005 and r=-0.24, p=0.001, respectively). Total IGF-I levels were positively related with free oestrogen index in both sexes. Free IGF-I did not relate to free oestrogen and androgen index. In women only, free IGF-I was positively related with DHEAS while IGFBP-1 was inversely correlated with DHEAS.

Conclusions: Free IGF-I levels do not decrease with age and are even higher in individuals above 70 yrs. There was no relationship between free IGF-I and free androgen and oestrogen indices in both sexes. We hypothesize that higher free IGF-I levels in older persons may be the consequence of a selective survival in the cohort: subjects with high free IGF-I levels may live longer. The absence of a relationship between free IGF-I levels and free androgen and oestrogen indices suggests that there is no direct interaction between the biological activity of circulating IGF-I levels and sex hormone production in a healthy aging population.

Introduction

The changes in total circulating insulin-like growth factor-I (IGF-I) with age parallel those of growth hormone (GH) (1). After a peak during early adulthood, there appears to be a gradual decrease in the serum concentrations of total IGF-I and insulin-like growth factor binding protein-3 (IGFBP-3), which is progressive with increasing age (2-4). However, IGF-I measured in serum is the total extractable IGF-I, which offers only a crude estimate of biologically active IGF-I, due to the wide inter-individual variation in circulating IGFBP (5). Free IGF-I likely has greater physiological and clinical relevance than total IGF-I in analogy with sex and adrenal steroids and thyroid hormones, and accounts for approximately 1% of total IGF-I (6). The amount of total IGF-I is dependent on the free IGF-I level and on the concentrations of the specific insulin-like growth factor binding proteins (IGFBPs). IGFBP-3 is quantitatively by far the major binding protein and it is thought to function as an intravascular reservoir and buffer for IGF-I (7). IGFBP-1 has been proposed as an acute regulator of IGF-I bioactivity and might simultaneously both inhibit or potentiate IGF-I action at different sites (8).
Consequently, it seems desirable to distinguish bound and unbound components of IGF-I when studying the IGF/IGFBP system (5). Recently a method has been developed and validated to measure free IGF-I levels (9,10). The increase in serum total IGF-I and IGFBP-3 levels during puberty suggest the influence of estrogens and /or androgens on circulating IGF-I and IGFBP-3 levels (11, 12). This effect might be at least partially mediated through stimulated growth hormone (GH) secretion (11).

Little is known about the free IGF-I levels and the relationships of the circulating IGF-I/IGFBP system to the circulating sex steroids at older age. We investigated the free IGF-I, total IGF-I, IGFBP-1 and IGFBP-3 and plasma sex steroid levels in a healthy older population.

Subjects and methods

Study population For the present study, a sample of participants from the Rotterdam Study was invited for an additional examination. The Rotterdam Study is a population-based cohort study of the determinants of chronic disabling diseases in the elderly. All approximately 10,000 inhabitants of a suburb of Rotterdam, aged 55 years and over were invited to participate as described elsewhere (13). Overall 7983 participants were examined in the Rotterdam Study (response rate 78 %).

The population for the present study included 218 persons aged 55 to 80 years, who had completed the baseline visit of the Rotterdam Study not more than six months earlier. Subjects with acute, psychiatric or endocrine diseases including diabetes mellitus treated with medications, were not invited. Compared to the other participants of the Rotterdam Study of the same age without known diabetes mellitus, there were no differences in age and gender distribution. Only 4 women in this study population were on hormone replacement therapy.These were (n=4) excluded from analysis.The study population was stratified by age in three groups of about equal size: group 1 (55-64 yrs old, 73 subjects), group 2 (65-69 yrs old, 72 subjects) and group 3 (>70 yrs old, 69 subjects). From all subjects informed consent was obtained and the study was approved by the medical ethics committee of Erasmus University Medical School.

Measurements Participants were examined in the morning after an overnight fast. Fasting blood samples were taken by venipuncture between 8.00 and 9.00 a.m. and allowed to coagulate for 30 minutes. Subsequently serum was separated by centrifugation and quickly frozen in liquid nitrogen. Free IGF-I was measured with a commercially available two-site immunoradiometric assay (Diagnostic System Laboratories Inc., Webster, Texas, U.S.A.; intraassay and interassay C.V.:10.3% and 10.7%, respectively)(9, 10). Estimation of free IGF-I in other assay systems usually involves size-exclusion extraction followed by a displacement immunoassay. Sample matrix alterations with consequent disturbance of the

free/bound equilibrium is an inevitable consequence. The free IGF-I assay used in this study needs initially no sample extraction as part of the standard procedure to measure IGF-I. Samples are added directly to tubes coated with IGF-I antibodies, washed, incubated with ^{125}I labeled antibody directed to a second epitope on IGF-I, washed and counted. Assay standards are rhIGF-I: 0.04-2.6 nmol/L, the minimal detection limit is 4 pmol/L. There is no cross-reactivity with IGF-II and no residual IGFBP-1 or IGFBP-3 is detectable after the first wash. The free IGF-I fraction measured in this assay, is a combination of the true free and the fraction of IGF-I which can be readily dissociated from IGFBPs under the specific assay conditions (9). Total IGF-I was determined by a commercially available radioimmunoassay (Medgenix Diagnostics, Brussels, Belgium, intraassay and interassay coefficients of variation (C.V.): 6.1%; 9.9%). Commercially available immunoradiometric assays were also used for the measurement of IGFBP-1 and IGFBP-3 (Diagnostic System Laboratories Inc., ; intraassay and interassay C.V. for IGFBP-1: 4.0% and 6.0 %, respectively; and for IGFBP-3: 1.8 % and 1.9%, respectively). Oestradiol and oestrone were assayed with a radioimmunoassay (Diagnostic Products Corporation, Los Angeles, U.S.A.; oestradiol: intraassay and interassay C.V. : 7.0% and 8.1%; oestrone: intraassay and interassay C.V. : 9.4% and 11.1%, respectively). SHBG was assayed with an commercially available immunoradiometric assay (Diagnostic Products Corporation; intraassay and interassay C.V. : 3.6% and 6.9%, respectively). The ratio of oestradiol to SHBG was used as an index of free oestradiol (14). Testosterone was measured with a non-commercial radioimmunoassay (intraassay and interassay C.V: 5.6%; 9.0%) (15). The ratio of total testosterone to SHBG was used as an index of free testosterone (16, 17). DHEAS and androstenedione were assayed by radioimmunoassay (Diagnostic Products Corporation; DHEAS: intraassay and inter-assay C.V. : 5.3% and 7.0%; androstenedione intraassay and interassay C.V. : 8.3% and 9.2%). Insulin was determined by a commercially available radioimmunoassay (Medgenix Diagnostics, intra-assay and inter-assay C.V.: 8.0%; 13.7%). Serum glucose levels were determined, using a standard glucose hexokinase method. Height and weight of the subjects were measured while wearing indoor clothes and without shoes.

Body mass index was defined as weight divided by the square of height (kg/m^2), body fat distribution was estimated using the ratio of waist and hip circumferences.

Statistical Analysis The clinical characteristics of the study population stratified by gender are expressed as the mean and standard error (SE). Mean total IGF-I, free IGF-I, IGFBP-1 and IGFBP-3 levels with SE were also calculated after stratification for age. Linear regression analyses were used to calculate differences in the baseline characteristics between men and women with adjustment for age. Pearson 's correlation coefficients were calculated to assess associations between the variables. One way ANOVA was used to test for difference in the free IGF-I levels across age groups and multiple linear regression analysis was performed to adjust for confounding variables. A two-sided p-value of <0.05 was considered significant. Analyses in which the values were logarithmically transformed yielded results similar to those with untransformed data: the non-transformed results are presented. All statistical analyses were performed with the Stata statistical package (Computing Resource Center, Santa Monica, Ca, U.S.A).

Results

In Table 1 the general characteristics of the study population are presented. Men were slightly older than women. BMI, and mean (free or total) IGF-I and IGFBP-1 levels did (age-adjusted) not differ between the sexes. Mean free over total IGF-I ratio and IGFBP-3 levels were significantly lower in men than in women. Mean insulin and glucose levels did not differ between both sexes. As expected, serum SHBG levels were significantly lower in men. Although the differences in mean serum oestradiol levels did not reach statistical significance, mean free oestradiol index and oestrone levels were significantly higher in men than in women as were total and free testosterone. Mean DHEAS and androstenedione levels were also significantly higher in men.

Table 1. General characteristics of the study population

	Men n=103 mean	(SE)	Women n=111 mean	(SE)	p value*
Age (years)	67.6	(0.56)	65.9	(0.59)	0.05
BMI (kg/m^2)	26.4	(0.29)	26.6	(0.41)	0.70
Total IGF-I (nmol/L)	19.3	(0.83)	17.9	(0.70)	0.11
Free IGF-I (nmol/L)	0.088	(0.005)	0.095	(0.005)	0.22
Percentage					
Free IGF-I/Total IGF-I	0.51	(0.03)	0.59	(0.04)	0.05
IGFBP-1 (nmol/L)	0.71	(0.07)	0.84	(0.10)	0.21
IGFBP-3 (nmol/L)	103.5	(2.5)	116.6	(2.5)	0.002
Insulin (IU/mL)	14.2	(0.8)	13.0	(0.8)	0.47
Glucose (nmol/L)	6.0	(0.1)	5.8	(0.1)	0.24
SHBG (nmol/L)	50.9	(2.0)	60.2	(2.7)	0.006
Oestradiol (pmol/L)	106.4	(2.0)	82.1	(4.8)	0.10
Free Oestradiol Index (pmol/nmol)	2.26	(0.25)	1.65	(0.13)	0.009
Oestrone (pmol/L)	194.2	(7.1)	126.6	(5.4))	< 0.001
Testosterone (nmol/L)	20.4	(0.5)	1.4	(0.06)	< 0.001
Free Androgen Index (nmol/nmol)	0.45	(0.02)	0.03	(0.002)	< 0.001
DHEAS (μmol/L)	4.1	(0.2)	2.6	(0.2)	< 0.001
Androstenedione (nmol/L)	6.6	(0.3)	4.6	(0.2)	< 0.001

Values are means with standard error (SE) between parentheses

* Difference between men and women, adjusted for age

BMI, body mass index; IGF-I: insulin-like growth factor I; IGFBP: Insulin-like growth factor binding protein; SHBG: sex-hormone binding globulin; DHEAS: dehydroepiandrosterone sulphate

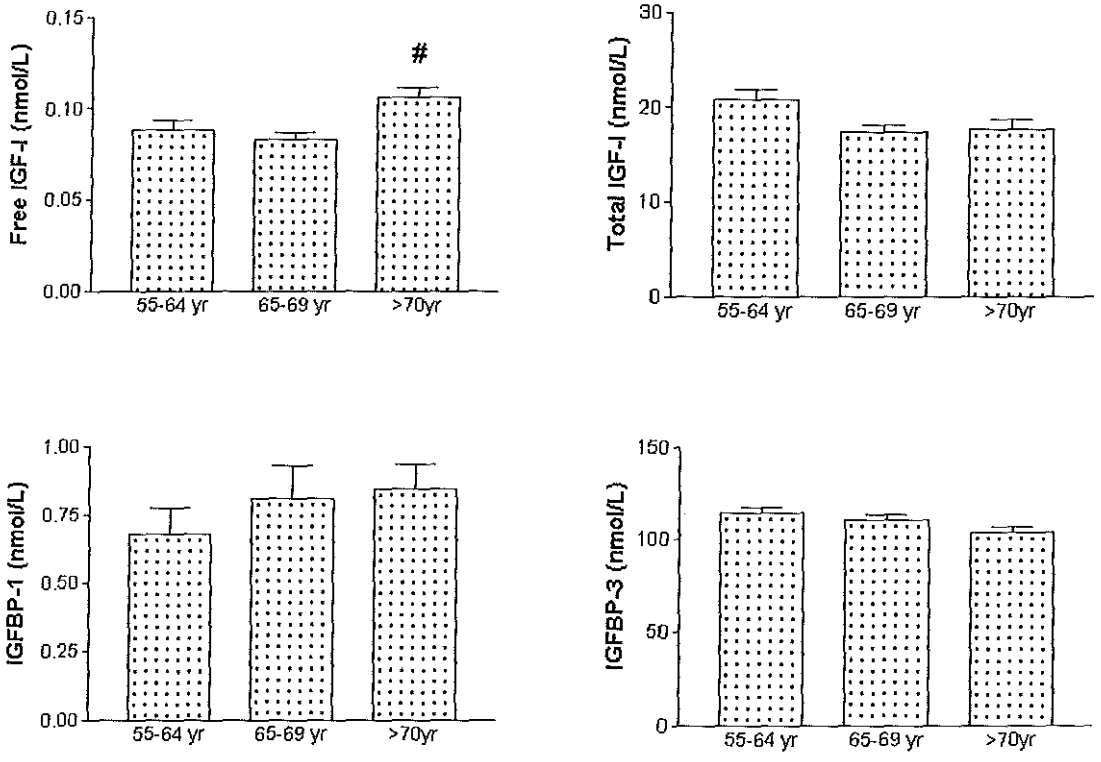

Figure 1. Free IGF-I (Upper left), total IGF-I (Upper right), IGFBP-1 (Lower left) and IGFBP-3 (Lower right) levels according to three groups of increasing age (see text). Note the different scales on the vertical axes, the increase in free IGF-I levels in individuals after 70 yrs in comparison to those between 55 and 70 yrs (# : ANOVA, $p=0.009$), and the comparable decreases in total IGF-I and IGFBP-3 levels with increasing age (see text).

Total IGF-I levels decreased significantly with age in the whole study population (r=-0.20, p= 0.005) (Figure 1). Free IGF-I levels did not significantly decline with age (r=0.12, p= 0.08), but showed a significant increase in individuals above 70 yrs when compared with those between 55 and 70 yrs (p=0.009) (Figure 1). This difference remained in multivariate analysis after adjustments for BMI and insulin. IGFBP-1 levels showed no relation with age and IGFBP-3 levels decreased with age (r=-0.24, p= 0.001) (Figure 1).

Serum total IGF-I was positively related with free IGF-I, IGFBP-3, and inversely related with IGFBP-1 (Table 2). Serum free IGF-I levels were positively correlated with IGFBP-3, and inversely with IGFBP-1 (Table 2). IGFBP-1 was negatively related to IGFBP-3, and insulin (Table 2). After adjustment for age and gender these relations remained unchanged.

Table 2. Pearson's correlation coefficients between (total and free) IGF-I, IGFBP-1 IGFBP-3, insulin and glucose levels for the whole study population

	Total IGF-I	Free IGF-I	IGFBP-1	IGFBP-3	Insulin
Free IGF-I	0.35 #				
IGFBP-1	-0.30 #	-0.17 *			
IGFBP-3	0.30 #	0.19 §	-0.17 *		
Insulin	-0.03	0.05	-0.19 §	0.07	
Glucose	0.11	0.09	-0.12	0.03	0.46 #

* $p<0.05$, § $p<0.01$, # $p<0.001$

Table 3. Pearson's (age-adjusted) correlation coefficients between (total and free) IGF-I, IGFBP-1, IGFBP-3, sex steroids and SHBG in men (A) and women (B).

	Total IGF-I	Free IGF-I	IGFBP-1	IGFBP-3
A. Men				
Oestradiol	0.15	0.10	-0.08	-0.13
Free Oestradiol Index	0.24 *	0.13	-0.15	-0.07
Testosterone	-0.03	0.08	-0.08	-0.14
Free Androgen Index	0.20	0.12	-0.35 #	0.28 *
SHBG	-0.20 *	-0.05	0.38 #	-0.24 *
DHEAS	0.13	0.01	-0.05	-0.03
Androstenedione	0.08	0.01	0.07	0.01
Oestrone	0.12	-0.009	-0.03	-0.07
B. Women				
Oestradiol	-0.003	0.08	0.01	0.07
Free Oestradiol Index	0.26 §	0.16	-0.15	0.38 #
Testosterone	0.07	-0.009	-0.05	-0.07
Free Androgen Index	0.29 §	0.12	-0.21 *	0.31 §
SHBG	-0.30 §	-0.18	0.31 §	-0.43 #
DHEAS	0.06	0.24 *	-0.22 *	-0.002
Androstenedione	-0.002	0.07	-0.002	-0.02
Oestrone	-0.01	0.15	-0.12	-0.03

* $p<0.05$, § $p<0.01$, # $p<0.001$

Free IGF-I levels were inversely related to BMI in men (r=-0.23, p=0.03), but not in women (r=0.004, p=0.97). No relations were observed between total IGF-I and BMI (men: r=-0.03, p=0.77; women: r=0.09, p=0.35).
IGFBP-1 levels were inversely related with BMI in women(r=-0.20, p=0.004) but not in men (r=-0.16, p=0.12), while no relations were observed between IGFBP-3 levels and BMI in both sexes (men: r=0.12, p=0.24; women: r=0.09, p=0.38).
Both in men and women serum total IGF-I was positively related to the free oestradiol index, and inversely to SHBG (Table 3A and 3B). In women serum total IGF-I was also positively related to the free androgen index (Table 3B), while in women also serum free IGF-I was positively related to DHEAS (Table 3B). Serum IGFBP-1 was positively related to SHBG and inversely to the free androgen index both in men and women (Table 3). Only in women IGFBP-1 was also inversely related to DHEAS (Table 3). IGFBP-3 was positively related to the free androgen index and negatively to SHBG in both sexes, and positively related to the free oestradiol index in women only (Table 3). Free oestradiol index was (age-adjusted) inversely related to SHBG in women (r=-0.53, p<0.001) while free androgen index was inversely related to SHBG in both sexes (M: r= -0.68, p<0.001; F: r=-0.52, p<0.001).

Discussion

In this population based study in the elderly total IGF-I and IGFBP-3 levels decreased with age, while free IGF-I levels did not. They even tended to be higher in subjects over seventy years of age (Figure 1). A close relationship was found between total IGF-I and IGFBP-3 levels with free androgen and free oestrogen levels both in elderly men and women. No relation was found between free IGF-I levels and free sex steroid indices in both sexes.

Before these findings can be accepted some issues need to be addressed. An important, at present insufficiently answered question in this regard remains whether the free IGF-I assay used in our study represents a better, more biologically active representation of the IGF-I/IGFBP system, than the currently used total

IGF-I, IGFBP-1 and IGFBP-3 assays. The free IGF-I assay used in our study measures in the unextracted serum the unbound and readily dissociable IGF-I (the so called easily dissociable free fraction) (18). It has become clear that during the transport of hormones into cells (e,g. steroid and thyroid hormones) the unbound hormone fraction is not the only component of the hormone in the circulation which enters the cells (19). This might also be true for IGF-I. Dissociation of IGFBP-bound IGF-I might also occur within the capillary bed, suggesting that the active fraction is larger than the unbound fraction measured in vitro. Although not yet proven, measurement of the easily dissociable free IGF-I fraction probably reflects the "true" bioavailable IGF-I (the amount of IGF-I available for entry in most tissues) better than the currently used assays. In a previous study three fractions of IGF-I have been found to be present in the circulation: the total amount, the ultrafiltrated free fraction (measured by an ultrafiltrated centrifuge method) and the easily dissociable free fraction (measured by the same free IGF-I assay as used in our study) (10). Still, these authors concluded that it remains to be proven to what extent the two latter fractions reflect bioavailable IGF-I.

It should be realized that cross-sectional studies do not reflect the rate of change of free IGF-I in an individual. As our study was cross-sectional, the higher free IGF-I levels in subjects above 70 years might be the consequence of a selective survival in the cohort: subjects with high free IGF-I levels may live longer. Alternatively, subjects with lower free IGF-I levels might have been excluded from the study because their physical condition (illness, frailty, or other causes) prevented a visit to the research centre.

Apart from these limitations in the study itself, an explanation for our observation of higher free IGF-I levels in subjects above 70 years might be that free IGF-I levels indeed increase with aging. An age-related increase has also been described for IGF-II levels (6). It has become clear that IGF-I levels are not only GH-dependent and that there are a number of other factors which influence IGF-I production (11). The increase in free IGF-I levels might be due to other age-related phenomena, such as a decreased metabolism and clearance of IGF-I, increased IGFBP-3 protease activity, increased abdominal adiposity, hyperinsulinemia, and

hyperglycaemia, which might modify IGF-I levels independently, despite the decrease in GH, total IGF-I and IGFBP-3 levels during aging (3, 20, 21).
Alternatively, an increase in free IGF-I levels may also represent an age-related IGF-I receptor or post-receptor defect (22).

According to previous findings the decline in serum GH concentration with age in men and women correlates with changes in gonadal steroid levels (23-25). Indeed we confirmed a close relationship between total IGF-I levels and free oestrogen levels both in elderly men and women. Total oestradiol levels in our study were slightly higher in elderly men than in women, which is in accordance to the study reported by Pfeilshifter et al. (25). Mean total IGF-I levels were comparable in both sexes in both studies. In neither study a correlation was observed between total IGF-I and total oestradiol levels.
The absence of a relationship between free IGF-I and free androgen and oestrogen levels suggests that there is during aging no direct relationship between IGF-I bioactivity in peripheral blood and the free sex steroid levels. Such a conclusion might have important consequences with regard to therapeutic interventions in the elderly, which aim to increase serum IGF-I levels with combinations of IGF-I modifying drugs (e.g. GH, growth hormone releasing hormone (GHRH), growth hormone binding protein (GHBP 's)) and sex steroids (e.g. androgens and/or oestrogens).

In our study serum IGFBP-1 levels were positively related to SHBG in men and women. The direction of the associations between IGFBP-1 and SHBG were the mirror image of the observed associations between IGFBP-1 and the free androgen index and free oestradiol index. These results suggest a common regulatory mechanism for the hepatic production of IGFBP-1 and SHBG. Previous studies have also reported a positive correlation between SHBG and IGFBP-1 levels (26-28). In cell cultures of human hepatoma cells both IGF-I and insulin inhibit the production of SHBG and IGFBP-1 (29, 30). In agreement with this both total and free IGF-I levels were negatively related to SHBG levels.

It has been previously found that DHEA administration increases IGF-I concentrations in middle-aged and elderly individuals (31). The relationships

between free IGF-I and DHEAS, and between IGFBP-1 and DHEAS in women as observed in our study, support the view that DHEAS influences free IGF-I levels in women via an unknown mechanism. DHEAS might thus exert a direct effect on the hepatic production of IGF-I in women. Free IGF-I may also influence DHEA production in the adrenals by its previously demonstrated positive effects on the number of corticotrophin (ACTH) receptors (32). The absence of a relation between serum IGF-I and DHEAS in men might be the consequence of a considerable contribution of testicular DHEA to serum DHEAS levels (33), which may have masked the relation between free IGF-I and adrenal DHEAS.

In conclusion, free IGF-I levels did not decrease with age and were even higher in individuals above 70 yrs. There was no relation between free IGF-I and free androgen and oestrogen indices. We hypothesize that high free IGF-I levels in older persons may be the consequence of a selective survival in the cohort: subjects with high free IGF-I levels may live longer.The absence of a relationship between free IGF-I levels and free androgen and oestrogen indices suggests that there is no direct interaction between the biological activity of circulating IGF-I levels and sex hormone production in a healthy aging population.

Acknowledgement

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§ 3.2 Serum total IGF-I, free IGF-I and IGFBP-1 levels in an elderly population: relation to cardiovascular risk factors and disease

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Summary

Background: Recently a method to measure free insulin-like growth factor (IGF-I) levels has been developed. Free IGF-I levels may have greater physiological and clinical relevance than total (bound and free) IGF-I. The associations between the circulating IGF-I/ IGF binding protein (IGFBP) system and cardiovascular disorders was studied.

Methods: In a cross-sectional study of 218 healthy persons (103 men, 115 women) aged 55 to 80 years, fasting serum (total and free) IGF-I, and IGFBP-1 levels, lipid profile, insulin, and glucose were measured. In addition blood pressure, body mass index (BMI) and waist hip ratio (WHR) were measured. Ultrasonography of both carotid arteries was performed to investigate the presence of atherosclerotic lesions. A history of angina pectoris, the presence of a possible or definite myocardial infarction on the electrocardiogram, and plaques in the carotid arteries were used as indicator of presence of cardiovascular signs and symptoms.

Results: Free IGF-I was inversely related to serum triglycerides ($p=0.04$, adjusted for age and sex). Mean free IGF-I levels in subjects without signs or symptoms of cardiovascular diseases were significantly higher than in those with at least one cardiovascular symptom or sign ($p=0.002$, adjusted for age and sex). Free IGF-I levels were also higher in subjects who had no atherosclerotic plaques in the carotid arterties ($p=0.02$, adjusted for age and sex) and who had never smoked ($p=0.02$, adjusted for age and sex).

IGFBP-1 showed an inverse relation with insulin, BMI and WHR and a positive relation with HDL-cholesterol. The associations between IGFBP-1 levels and HDL cholesterol, WHR, and BMI remained significant after adjustment for fasting insulin levels.

Conclusions: High fasting serum free IGF-I levels are associated with a decreased presence of atherosclerotic plaques, coronary artery disease and lower serum triglycerides while high fasting IGFBP-1 levels are associated with a more favourable cardiovascular risk profile.The findings suggest that the IGF-I/IGFBP system is related to cardiovascular risk factors and atherosclerosis.

Introduction

A possible involvement of circulating Insulin-like Growth Factor-I (IGF-I) and Insulin-like Growth Factor Binding Proteins (IGFBPs) in the pathogenesis of cardiovascular disorders has recently been suggested (1). Total IGF-I levels are lower in patients with an atherogenic lipid profile and may contribute to the development of atherosclerosis (2). Many in vitro studies have shown proliferation of vascular smooth muscle cells after stimulation with IGF-I (3, 4). Moreover, IGF-I has also been shown to be an important regulator of vascular function by stimulating nitric oxide (NO) release from cultured vascular endothelium (5).

The commonly measured total extractable IGF-I in serum provides only a crude estimate of biologically active IGF-I, due to the wide inter-individual variation in IGFBPs (6). Free IGF-I, by analogy with sex and adrenal steroids and thyroid hormones, may have greater physiological and clinical relevance and accounts for only 1% of total IGF-I (7). Recently a method has been developed to measure free IGF-I levels (8).

The six IGFBPs comprise a family of structurally homologous proteins that prolong the half-life of IGF-I in the circulation and modulate IGF-I action at its target cells (9, 10). IGFBP-1, one of these IGFBPs, is not restricted to the circulation and is considered to function as a transport protein that shuttles IGF-I from the intravascular space through the endothelial walls of the capillaries (11, 12). IGFBP-1 has been proposed as an acute regulator of IGF-I bioactivity and might simultaneously both inhibit or potentiate IGF-I action at different sites (13). Recent evidence even suggests that IGFBP-1 has intrinsic mitogenic and metabolic activity (14). Consequently, it seems desirable to distinguish bound and unbound components of IGF-I when studying the IGF-I/IGFBP system.

To broaden our understanding of a possible role of the IGF-I/IGFBP system in the development of cardiovasular disorders, we studied the relationships of fasting serum circulating levels of (free and total) IGF-I and IGFBP-1 with the presence of cardiovascular risk factors and diseases in an elderly population.

Methods

Subjects

For the present study, a sample of participants from the Rotterdam Study was invited for an additional examination. The Rotterdam Study is a population-based cohort study of the determinants of chronic disabling diseases in the elderly. All approximately 10,000 inhabitants of a suburb of Rotterdam, aged 55 years and over were invited to participate as described elsewhere (15). Overall 7983 participants were examined (response rate 78 %).

The population for the present study included subjects aged 55 to 80 years, who had completed the baseline visit of the Rotterdam Study not more than six months earlier. Subjects with psychiatric or endocrine diseases, including diabetes mellitus treated with medication, were not invited. From these subjects a random sample of 218 persons was examined. Compared to the other participants of the Rotterdam Study of the same age without known diabetes mellitus, there were no differences in age and gender distribution, mean blood pressure, use of antihypertensive medication, echographic evidence of atherosclerotic plaques in the carotid arteries, and electrocardiographic abnormalities. From all subjects informed consent was obtained and the study was approved by the medical ethics committee of Erasmus University Medical School.

Cardiovascular assessment Resting standard 12-lead electrocardiogram was made with an ACTA Gnosis IV (EsaoteBiomedica) and an automated diagnostic classification system of the Modular Electrocardiogram Analysis System (MEANS) was used (16, 17). The presence of a possible or definite myocardial infarction on the electrocardiogram was used as indicator of presence of coronary artery disease (18).

Information on medical history of myocardial infarction, medical history, drug use and smoking was obtained by a trained research assistant using a computerized questionnaire, which includes a Dutch version of the Rose questionnaire to determine the presence of angina pectoris and intermittent claudication (19).

To measure carotid artery intima-media thickness ultrasonography of the left and

right common carotid artery, carotid bifurcation and the internal carotid artery was performed with a 7.5 Mhz linear array transducer (ATL Ultramark IV, Advanced Technology Laboratories). Following the Rotterdam Study ultrasound protocol, a careful search was performed for all interfaces of the near end and far wall of the distal common carotid artery (20-22). The actual measurements of the intima-media thickness were performed off_line. This procedure has been described previously (23).

The common carotid artery, carotid bifurcation, and internal carotid artery were also evaluated for the presence (yes/no) of atherosclerotic lesions on both the near and the far wall of the carotid arteries. Plaques were defined as a focal widening relative to adjacent segments, with protrusion into the lumen composed of only calcified deposits or a combination of calcification and non-calcified material (24). The size or extent of the lesions was not quantified. The presence of plaques was used an indicator of presence of atherosclerosis.

Subjects were categorized into groups of current smokers, former smokers and those who had never smoked. BMI was defined as weight divided by the square of height (kg/m^2), and body fat distribution was estimated using the ratio of waist and hip circumferences (WHR) in cm. Sitting blood pressure was measured at the right upper arm with a random-zero sphygmomanometer. The average of two measurements obtained at one occasion was used in the analyses. Hypertension was defined as a systolic blood pressure of 160 mm Hg or a diastolic pressure of 95 mm Hg or the use of antihypertensive drugs (25).

Biochemical measurements Fasting blood samples were taken by venipuncture between 8 and 9 A.M. and allowed to coagulate for 30 minutes.

Subsequently, serum was separated by centrifugation and quickly frozen in liquid nitrogen. Dissociable free IGF-I was measured with a commercially available non-competitive two-site immunoradiometric assay (Diagnostic System Laboratories Inc.; intraassay and interassay C.V. : 10.3% and 10.7%, respectively) (8). The free IGF-I assay used needs no initial sample extraction as part of the standard

procedure to measure IGF-I. Samples are added directly to tubes containing a dense coating of high-affinity free IGF-I antibody, incubated for 2 hr at room temperature, washed, incubated with ^{125}I labeled antibody directed to a second epitope, washed and counted. Assay standards are rhIGF-I: 0.04-2.6 nmol/L, the minimal detection limit is 0.004 nmol/L. There is no cross-reactivity with IGF-II and no residual IGFBP-1 or IGFBP-3 is detectable after the first wash. It is likely that the free IGF-I fraction measured with the free IGF-I assay, is a combination of the true free IGF-I and the fraction that can be readily dissociated from IGFBPs under the specific assay conditions (8). Addition of pure IGFBP-1 and -3 to an IGF-I containing buffer caused a dose-related decrease in measurable free IGF-I (8). Total IGF-I was determined by a commercially available radioimmunoassay (Medgenix Diagnostics; intraassay and interassay C.V.: 6.1%; and 9.9%) after an acidification/neutralization step. The purpose of this step is to convert the different forms of IGF-I present into free IGF-I. A commercially available immunoradiometric assay was also used for measurement of IGFBP-1(MW 25.3 kD) (Diagnostic System Laboratories Inc.; intraassay and interassay C.V. : 4.0% and 6.0%). Insulin was determined by a commercially available radioimmunoassay (Medgenix Diagnostics, intraassay and interassay C.V.: 8.0% and 13.7%). Serum glucose, creatinine, cholesterol, HDL-cholesterol and triglyceride levels were determined with standard laboratory methods.

Statistical Analysis The clinical characteristics are presented as means (SE). Differences between subgroups with or without cardiovascular symptoms and signs were analyzed by linear regression after adjustment for age and sex. Multiple linear regression analyses were used to further assess the associations of free IGF-I, and IGFBP-1 with other parameters. A two-sided propability of $p<0.05$ was considered statistically significant. Analyses in which the values were logarithmically transformed yielded similar results to those with untransformed data. Because the interpretation of logarithmic data is difficult, the non-transformed data are presented. Age-adjusted OR (with 95 % CI) were calculated as an approximation of the relative risk of free IGF-I for the presence of atherosclerotic

plaques and coronary artery disease. All statistical analyses were performed with Stata statistical package (Computing Resource Center).

Results

In Table 1, the general characteristics of the study population are presented. The percentage free over total serum IGF-I varied between 0.14 and 2.77 %. Serum free IGF-I increased 0.003 nmol/L per nmol/L total IGF-I (SE 0.0004, P<0.001, adjusted for age and sex) and decreased -0.013 nmol/L per nmol/L IGFBP-1 (SE 0.004, P=0.003, adjusted for age and sex). Serum total IGF-I levels decreased 2.766 nmol/L per nmol/L serum IGFBP-1 (SE 0.596), P<0.001(adjusted for age and sex). Age- and sex adjusted fasting free IGF-I levels were inversely related to serum triglycerides, but showed no relation with most cardiovascular risk factors (Table 2). After further adjustment for insulin, the relation between free IGF-I and serum triglycerides became even stronger (regression coefficient -3,1 (mmol/L) per nmol/L free IGF-I, p=0.01).

Total IGF-I was positively related to serum glucose (regression coefficient: 0.022 (SE0.008) mmol/L per nmol/L serum IGF-I, p=0.008) but not to any of the other investigated cardiovascular risk factors mentioned in Table 2 (data not shown). IGFBP-1 showed an inverse relation with insulin, glucose, BMI and WHR and a positive relation with HDL-cholesterol (Table 2). The associations between IGFBP-1 and, HDL cholesterol, WHR, and BMI remained significant after adjustment for fasting insulin levels (regression coefficients per nmol/L IGFBP-1 were respectively: 0.06 (SE 0.03) (mmol/L, p=0.03); -0.02 (SE 0.01), p=0.009, and -0.63 kg/m^2 (SE 0.27, p=0.02), while the association between IGFBP-1 and serum glucose lost statistical significance after this adjustment.

Free IGF-I and presence of cardiovascular diseases.

Age- and sex adjusted mean free IGF-I levels were lower in subjects with at least one plaque in the carotid arteries compared to those without plaques (difference 0.017 nmol/L (SE 0.008), p=0.02). Free IGF-I levels were also lower in subjects with coronary artery disease than in those without (difference 0.018 nmol/L

Table 1. General characteristics of 218 subjects (103 men, 115 women)

	Mean	SE	Range
Age (years)	66.7	(0.4)	55.0-82.4
BMI (kg/m^2)	26.4	(0.2)	16.4-43.1
WHR (cm/cm)	0.92	(0.006)	0.66-1.12
Total IGF-I* (nmol/L)	18.7	(0.5)	3.1-41.3
Free IGF-I* (nmol/L)	0.092	(0.004)	0.02-0.28
Percentage			
Free IGF-I/ Total IGF-I*	0.55	(0.02)	0.14-2.77
IGFBP-1 (nmol/L)*	0.77	(0.06)	0.04-6.07
Insulin *(IU/mL)	13.5	(0.5)	2.4-44.2
Glucose*(mmol/L)	5.9	(0.06)	4.7-11.9
Cholesterol* (mmol/L)	6.8	(0.08)	2.8-12.5
HDL-cholesterol* (mmol/L)	1.36	(0.03)	0.70-2.80
Triglycerides* (mmol/L)	1.96	(0.07)	0.47-7.42
Systolic BP (mm Hg)	139.2	(1.31)	96-185
Diastolic BP (mm Hg)	74.8	(0.67)	44-97
Hypertension † (%)	30.1		
Anamnestically Angina Pectoris (%)	7.6		
Anamnestically Myocardial Infarction (%)	20.3		
Myocardial Infarction on ECG (%)	22.1		
Atherosclerotic plaques in carotid arteries (%)	62.8		
Carotid intima media thickness (mm)	0.76	(0.13)	0.49-1.49
Smoking (%)			
Current	24.3		
Former	50.5		
Never	25.2		

Values are means with standard error (SE) between parentheses
BMI, body mass index; WHR, waist-to-hip ratio; IGF-I: insulin-like growth factor I; IGFBP: Insulin-like growth fac binding protein; BP=blood pressure

* Fasting blood samples

† Hypertension was defined as a systolic BP of 160 mm Hg or over and/or diastolic pressure of 95 mm Hg or o and/or use of antihypertensive drugs

Table 2. Age- and gender adjusted associations between fasting free IGF-I and IGFBP-1, respectively, and several cardiovascular risk factors in an elderly population (aged 55-80 years).

	Regression Coefficient	(SE)	p value	Regression Coefficient	(SE)	p value
	A. Free IGF-I (nmol/L)			B. IGFBP-1 (nmol/L)		
Systolic BP (mm Hg)	-6.3	(24.8)	0.80	-1.9	(1.5)	0.21
Diastolic BP (mm Hg)	4.5	(13.0)	0.73	-1.0	(0.8)	0.22
Cholesterol* (mmol/L)	-2.1	(1.6)	0.18	-0.01	(0.09)	0.89
HDL Cholesterol* (mmol/L)	0.16	(0.46)	0.73	0.08	(0.03)	0.004
Triglycerides* (mmol/L)	-2.8	(1.4)	0.04	-0.02	(0.08)	0.85
Insulin*(IU/mL)	6.0	(10.2)	0.56	-2.0	(0.6)	0.002
Glucose* (mmol/L)	1.8	(1.2)	0.14	-0.18	(0.07)	0.02
WHR	-0.11	(0.10)	0.29	-0.02	(0.006)	0.001
BMI (kg/m^2)	5.78	(4.89)	0.24	-0.98	(0.29)	0.001

* Fasting values, for used abbreviations see legends Table 1

(SE 0.009), p=0.04) (Table 3). Age- and gender-adjusted free IGF-I levels were not related to the carotid intima media thickness (regression coefficient per 0.01 nmol/L IGF-I: 0.0012 (SE 0.0016) mm, p=0.47). Mean free IGF-I levels were lower in subjects with a history of angina pectoris, and higher in subjects with hypertension but this value did not reach statistical significance after adjustment for age and sex (Table 3). Serum free IGF-I levels were significantly higher in subjects who had never smoked (p=0.02) than in ever (former and current) smokers. Mean free IGF-I levels were higher in subjects without any actual sign of cardiovascular disease (i.e. no angina pectoris, no myocardial infarction on the electrocardiogram and no atherosclerotic lesions in the carotid arteries), than in subjects with at least one symptom or sign of cardiovascular disease (i.e. angina pectoris and/or myocardial infarction on the electrocardiogram and/or atherosclerotic lesions in the carotid arteries) (0.107 nmol/L (SE 0.008) vs 0.086 nmol/L (SE 0.004), p=0.002 adjusted for age and sex; see Figure 1). This association remained significant after further adjustment for smoking and hypertension.

For total IGF-I, lower levels were observed in groups with presence of one cardiovascular disease and/or risk factor than in those without. The difference in total IGF-I levels was significant for angina pectoris; mean total IGF-I in subjects with angina pectoris: 15.0 (SE 2.1) nmol/L and 19.1 (SE 0.6) nmol/L in subjects without angina pectoris (adjusted for age and sex, p=0.04).

Mean IGFBP-1 levels were not significantly different in subgroups with or without cardiovascular diseases and risk factors, hypertension or smoking(Table 3).

Age-adjusted multiple logistic regression analysis performed with the presence of atherosclerotic plaques and the presence of coronary artery disease as dependent variable, respectively, and free IGF-I as independent variable- showed a significant decreased risk for the presence of plaques per 0.01 nmol/L increase in serum free IGF-I level: OR 0.94 (95 % CI: 0.89-0.99) per 0.01 nmol/L (P=0.03), and a decreased risk for the presence of coronary artery disease per 0.01 nmol/L increase in serum free IGF-I level: OR 0.91 (95 % CI: 0.84-0.99) per 0.01 nmol/L (p=0.02).

Table 3. Fasting free IGF-I(A) and IGFBP-1(B) levels (both expressed in nmol/L) in subjects with vs subjects without symptoms and signs of cardiovascular diseases, hypertension, and smoking.

	Present Mean	(SE)	Absent Mean	(SE)	p-value*
A. Free IGF-I					
History of Angina Pectoris	0.084	(0.012)	0.092	(0.004)	0.67
Coronary artery disease	0.077	(0.005)	0.096	(0.004)	0.04
Atherosclerotic plaques in Carotid arteries	0.088	(0.004)	0.101	(0.006)	0.02
Hypertension	0.096	(0.006)	0.091	(0.005)	0.73
Smoking	Ever 0.086	(0.004)	Never 0.110	(0.007)	0.02
B. IGFBP-1					
History of Angina Pectoris	0.657	(0.102)	0.785	(0.065)	0.66
Coronary artery disease	0.813	(0.091)	0.813	(0.099)	0.62
Atherosclerotic plaques in Carotid arteries	0.754	(0.062)	0.812	(0.121)	0.45
Hypertension	0.646	(0.080)	0.831	(0.077)	0.09
Smoking	Ever 0.802	(0.070)	Never 0.699	(0.112)	0.10

* p value for the differences in free IGF-I and IGFBP-1 levels, respectively, between subgroup with vs subgroup without cardiovascular diseases, hypertension and smoking adjusted for age and gender

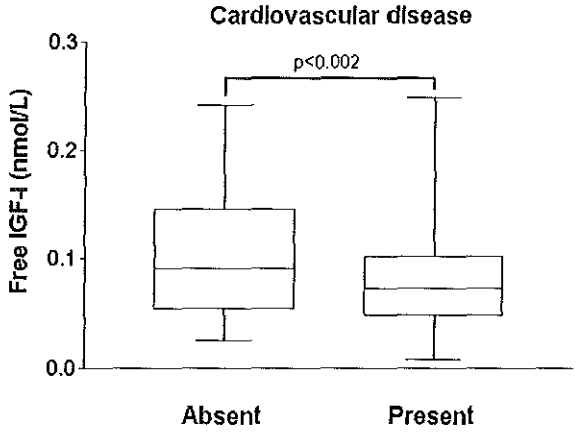

Figure 1. Mean free IGF-I levels (adjusted for age and gender) compared between subjects without signs and symptoms of cardiovascular diseases (left) and subjects with at least one cardiovascular symptom or sign (right). The line in the middle of the boxes represents the 50th percentile of the data. The boxes extend from the 25th percentile to the 75th percentile values, the whiskers extend from the 5th to the 95th percentile values of each group (difference 0.026 nmol/L (SE 0.008), p=0.002).

Discussion

Mean free IGF-I levels were significantly lower in subjects with atherosclerotic plaques in the carotid arteries and with coronary artery disease. When subjects without symptoms or signs of cardiovascular diseases (i.e. no angina pectoris, no myocardial infarction on the electrocardiogram and no atherosclerotic plaques in the carotid arteries) were compared with those with at least one symptom or sign of cardiovascular disease, mean free IGF-I levels were significantly higher in the former group. These findings suggest that lowered free IGF-I levels are associated with a higher prevalence of cardiovascular disease. Low circulating IGF-I levels have been previously associated with angiographically documented coronary artery disease (26). Other investigations have also suggested that low circulating IGF-I levels are associated with premature atherogenesis, cardiovascular morbidity and mortality (27, 28, 29). However, these latter studies need to be considered with

caution, because they were retrospective studies, while many subjects in these studies showed also deficiencies for other pituitary hormones, which might have been suboptimally replaced.

Serum free IGF-I is considered as an important biologically active IGF-I fraction (7). The serum free IGF-I levels in our study lie within the same range as previously reported for the affinity of the IGF-I receptor on both vascular endothelium as well as vascular smooth muscle cells (3, 30). Because endothelial cells are continuously exposed to IGF-I in vivo, it is conceivable that circulating (endocrine) IGF-I might be of importance in both the normal physiology of vascular endothelium and in disease states (e.g. atherosclerosis) (31). Evidence exists that IGF-I does cross vascular endothelium and in this respect might be comparable to insulin (30, 32). The importance of circulating (endocrine) IGF-I has been challenged by the autocrine/paracrine concept of IGF-I since most cells implicated in the pathogenesis of atherosclerosis are capable of expressing (auto- and paracrine) IGF-I, IGF-I receptors, and IGFBPs (1). There are many who even believe that there is no role for circulating IGF-I in the development of atherosclerosis. Some arguments in favour of this view are: that the expression of IGF-I in the vessel wall is independent of pituitary growth hormone secretion (33), and endothelial denudation of blood vessels in rats increases the accumulation of IGF-I in vascular smooth muscles without concomitant changes in hepatic IGF-I mRNA expression and serum total IGF-I levels (34). However, both observations can also be explained by an increased proteolysis of circulating IGFBPs by specific IGFBP- proteases present in serum (as plasmin and thrombin), contributing to an increased delivery of IGF-I at an injury of the vascular endothelium (35, 36). This mechanism may then result in increased association of IGF-I with IGF-I receptors on vascular smooth muscle cells during repair of injured arterial intima. This response to injury might be reflected in (transiently) lower serum free IGF-I levels as a consequence of consumption. In addition, the frequently used immunoreactive techniques to localize IGF-I ultrastructurally in atherosclerotic plaques do not demonstrate the origin of IGF-I (locally produced or endocrine). Other arguments in favour of a physiological effect of circulating IGF-I on the vessel wall are:

that administration of exogenous IGF-I to human retinal vascular endothelium cells in culture causes decreased IGF-I mRNA levels in these cells (37); and finally, several studies suggest that the majority of IGF-I secreted by vascular endothelial cells (in contrast to IGFBPs) results from IGF-I uptake from serum and not from local de novo synthesis (30, 38). These studies suggest that intact endothelium serves as a regional storage site for intravascular receptor-bound IGF-I .

Recent data suggest that IGF-I stimulates the production of NO from both the endothelium and vascular smooth muscles (39). Decreased NO production by vascular endothelium due to low free IGF-I levels, might be a mechanism, which could contribute to the observed relation between free IGF-I levels and the presence of cardiovascular symptoms and signs in our study (40).

In our study free IGF-I levels were inversely associated with fasting triglycerides. Indeed, administration of recombinant IGF-I to humans was reported to cause a decreasel in triglyceride levels (41).

Also in our study no relationships were found between (free and total) IGF-I and BMI or WHR. The total amount of excess fat on the body is roughly reflected in the BMI, wheras WHR is more strongly associated with the amount of abdominal fat. Several epidemiological studies suggest that abdominal obesity may especially be involved in atherosclerosis (42) and that a decreased growth hormone secretion is an endocrine characteristic of obesity (43). Growth hormone dependent IGF-I concentrations would thus be expected to be subnormal in obesity (44). However, total IGF-I levels have been reported not to be significantly different between obese subjects and lean control subjects (44, 45). In this latter study free IGF-I levels were reported to be higher in obese males while free IGF-I levels were not significantly elevated in obese females (45). The differences between these results and our are most likely due to difference in age, sex and degree of obesity in the study group as the subjects in our study were older, predominantly female and less obese. Moreover, in our study another method was used to measure free IGF-I levels.

Higher fasting age-adjusted IGFBP-1 levels were associated with decreased BMI, WHR, insulin and glucose levels, and increased HDL cholesterol levels. In a

previous study, it was demonstrated that reduced fasting IGFBP-1 levels correlate with an increased prevalence of cardiovascular risk factors in non-insulin dependent diabetes mellitus (46). Our study shows that this relationship similarly exists in a non-diabetic population and that the reverse is also true, i.e. higher IGFBP-1 levels are associated with an advantageous cardiovascular risk profile. Insulin is considered as the main regulator of IGFBP-1 levels, while it also modulates the action of IGFBP-1 (11): it also accelerates IGFBP-1 transport from the intravascular space through the endothelial walls of the capillaries to the target cells (11, 12). However, the associations between IGFBP-1 levels and BMI, WHR and HDL-cholesterol were independent of insulin levels. This suggests that a low IGFBP-1 level might be an independent marker for the metabolic disturbances, which are all associated with an increased risk of cardiovascular diseases.

Studies of the biological effect of IGFBP-1 have shown conflicting results. IGFBP-1 is capable of both inhibition and augmentation of IGF-I bioactivity (47). These conflicting observations may be explained by the recent findings that differential phosphorylation of IGFBP-1 could significantly alter its affinity for IGF-I, and therefore differently modulate IGF-I bioactivity (48). The IGFBP-1 assay used in our study cannot discriminate phosphorylated and non-phosphorylated IGFBP-1, but IGFBP-1 normally circulates mainly as a highly phosphorylated form (49). This latter form of IGFBP-1 would favor sequestration of IGF-I by IGFBP-1 resulting in a decreased IGF-I release to IGF-I receptors (47). The observed lower IGFBP-I levels in subjects with a disadvantageous cardiovscular risk profile, might be thus an adaptive mechanism to increase IGF-I bioactivity at the vascular endothelium.

Although most cells implicated in the pathogenesis of atherosclerosis are capable of expressing (autocrine and paracrine) IGF-I, IGF-I receptors, and IGFBPs, the results of our study suggest that the measurement of circulating free IGF-I and IGFBP-1 levels may be of clinical relevance and will help to unravel the role of the IGF-I/IGFBP-1 system in atherogenesis.

In conclusion, our findings indicate that low circulating free IGF-I levels are associated with an increased risk of presence of atherosclerotic cardiovascular disease. In addition, higher IGFBP-1 levels are related to a more favourable cardiovascular risk factor profile. These findings lend support to the view that the

IGF-I/IGFBP-1 system is related to cardiovascular risk in the elderly population.

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§ 3.3 Gender-specific relationship between serum free and total IGF-I and bone mineral density in elderly men and women.

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Summary

Objective: Little is known about the association between free IGF-I levels and bone mineral density (BMD).

Design: Cross-sectional study of 218 healthy subjects (103 men, 115 women, age 55-80 yrs)

Methods: fasting serum free, total IGF-I, estradiol and SHBG levels were measured. The ratio of estradiol to SHBG was used as an index of free estradiol. BMD measurements were performed by dual-energy X-ray absorptiometry (DXA) of the lumbar spine and the proximal femur.

Results: In multivariate analyses with BMD of the lumbar spine as the dependent variable and serum free IGF-I, age, BMI and the free estradiol index as independent variables- the free IGF-I was positively related to the BMD of the lumbar spine in men ($p=0.02$) but not in women. When the same analyses for the lumbar BMD were performed with total serum IGF-I the association also was only statistically significant in men ($p=0.05$). In multivariate analyses with the trochanter BMD as the dependent variable and serum free IGF-I, total IGF-I, age, BMI and the free estradiol index as independent variables- the associations between (free and total) IGF-I and the trochanter BMD in men was borderline significant.

Conclusions: In elderly men free and total IGF-I were positively related to lumbar BMD, while (free and total) IGF-I was borderline positively related to trochanter BMD. As these relations were not observed in elderly women, we suggest a weak gender-specific anabolic effect of IGF-I on BMD on trabecular bone.

Introduction

Insulin-like growth factor-I (IGF-I) and its specific Insulin-like growth factor binding proteins (IGFBPs) are postulated to play a key role in bone metabolism (1). It is generally agreed that IGF-I has anabolic effects on bone cells (2). It has been shown to be an important regulator of bone remodelling and growth (3) and has been found to stimulate collagen synthesis in bone, resulting in increased matrix production (4). Bone cells synthesize IGF-I and all of the six IGFBPs (1) and observations in osteoblast-like cell-lines have suggested that skeletal concentration of IGF-I is a reflection of its local synthesis by osteoblasts (3).

Recently a method was developed to measure free serum IGF-I (5, 6). Free IGF-I, analogous to free sex and adrenal steroids and thyroid hormones, is likely to be a major biologically active hormonal form of IGF-I (7).

Estrogens have a protective effect on bone mass and slow bone loss during aging (8). Moreover, it has been suggested that the estrogen status in women has a complex effect on serum concentrations of IGF-I and therefore has to be taken into account when evaluating levels of serum IGF-I (9).

In order to gain more insight into the relations between systemic IGF-I, estrogens and bone mineral density (BMD) in the elderly, we measured serum free and total IGF-I levels, estrogens and bone mineral density in 218 healthy elderly subjects.

Materials and methods

Study population

The population for the present study included 218 subjects aged 55 to 80 years, who had completed the baseline visit of the Rotterdam Study not more than six months earlier. The Rotterdam Study is a population based prospective cohort study of determinants of chronic disabling diseases in the elderly as described elsewhere (10). Briefly, all 10,275 inhabitants of a suburb of Rotterdam, aged 55 years and over, were invited to participate. From the 9161 independently living persons 77% participated in the interview and 71% also had extensive

examinations including bone density measurements.

The 218 people that took part in the present study were randomly selected from the 7983 participants of the Rotterdam Study after exclusion of subjects with acute, psychiatric disease or diabetes. Compared to the other participants of the Rotterdam Study without known diabetes mellitus, there were no differences in age and gender distribution, mean blood pressure, use of antihypertensive drugs, echocardiographic evidence of atherosclerotic plaques in the carotid arteries and electrocardiographic abnormalities. Only four women in this study population were on hormone replacement therapy.These four women were excluded from analysis. From all subjects informed consent was obtained and the study was approved by the medical ethics committee of Erasmus University Medical School.

Measurements

Biochemical measurement Participants were examined in the morning after an overnight fast. Fasting blood samples were taken by venipuncture between 8.00 and 9.00 a.m. and allowed to coagulate for 30 minutes. Subsequently serum was separated by centrifugation and quickly frozen in liquid nitrogen. Free IGF-I was measured with a commercially available two-site immunoradiometric assay (Diagnostic System Laboratories Inc., Webster, Texas, U.S.A.; intraassay and inter-assay coefficients of variation (C.V). : 10.3% and 10.7%, respectively) (5, 6). The free IGF-I assay needs no initial sample extraction as part of the standard procedure to measure IGF-I. Samples are added directly to tubes containing a dense coating of high-affinity free IGF-I antibody, incubated at 2 hr at room temperature, washed, incubated with ^{125}I labeled antibody directed to a second epitope, washed and counted. There is no cross-reactivity with IGF-II and no residual IGFBP-1 or IGFBP-3 is detectable after the first wash. It is likely that the free IGF-I fraction measured with the free IGF-I assay, is a combination of the true free and the fraction of IGF-I which can be readily dissociated from IGFBP-s under the specific assay conditions (5). Total IGF-I was determined by a commercially available radioimmunoassay (Medgenix Diagnostics, Brussels,

Belgium, intraassay and interassay C.V.: 6.1 and 9.9%) after an acidification/neutralization step. The purpose of this step is to convert the different forms of IGF-I present into free IGF-I. Estradiol was assayed with a radioimmunoassay (Diagnostic Products Corporation, Los Angeles, California, USA ; intraassay and interassay C.V. : 7.0% and 8.1%). SHBG was assayed with a commercially available immunoradiometric assay (Diagnostic Products Corporation; intraassay and interassay C.V. : 3.6% and 6.9%, respectively). The ratio of estradiol to SHBG was used as an index of free estradiol (11). Height and weight were measured wearing indoor clothes and without shoes. Body mass index (BMI) was defined as weight divided by the square of height (kg/m^2).

Measurement of bone mineral density (BMD) BMD measurements were performed by dual-energy X-ray absorptiometry (DXA), using a Lunar DPX-L densitometer (Lunar Radiation Corporation, Madison, WI, USA). Standard positioning was used with anterior-posterior scans of the lumbar spine and the right proximal femur. If there was a history of hip fracture or prothesis implantation, the left femur was scanned. Using standard software the vertebrae L2 to L4, and at the proximal femur, the femoral neck, Ward 's triangle and the greater trochanter were analyzed. Quality assurance included calibration with the standard of the machine and was performed routinely every morning. The in vivo coefficient of variation for BMD measurements was 0.9 % in the lumbar spine, 3.2 % in the femoral neck, 3.1% in the Ward 's triangle, and 2.5 % in the greater trochanter.

Statistical Analysis Pearson 's correlation coefficients were calculated to assess the associations between IGF-I and BMD, after adjustment for age, and other confounders when appropriate. Analyses in which the values were logarithmically transformed yielded results similar to those with untransformed data. Because interpretation of results from logaritmically transformed data is more difficult, the non-transformed results are presented. Linear regression analysis was used to calculate the statistical significance of differences in general characteristics

between men and women, as well as the associations between IGF-I and BMD, after adjustment for age, and other confounders when appropriate. A two-sided p-value of <0.05 was considered significant. All statistical analyses were performed with Stata statistical package (Computing Resource Center, Santa Monica, Ca, U.S.A).

Results

In Table 1 general characteristics of the study population are presented. Men were slightly older than women. Mean (free and total) IGF-I levels did not differ between the sexes. Bone mineral density was (age-adjusted) higher in men than in women at all four measured sites. Mean free estradiol index was significantly higher in men than in women.

Serum free IGF-I was positively associated with total IGF-I ($r=0.35$, $p<0.001$). Serum total IGF-I decreased with age in women, while this relation just missed significance in men (Table 2). Free IGF-I was not related to age in both sexes (Table 2). Free IGF-I was inversely related to BMI in men, but this latter relationship was lost after adjustment for age.Total IGF-I was not significantly related to BMI in both sexes (Table 2).

No (age-adjusted) relations were present between serum free IGF-I and the free estradiol index in both sexes (men:$r=0.13$, $p=0.21$; women: $r= 0.16$, $p=0.10$). Total serum IGF-I was (age adjusted) positively related to free estradiol index in men and women (men: $r=0.24$, $p=0.02$; women: $r=0.26$, $p=0.008$).

No relations were observed in the total study population between free or total IGF-I, and the BMD of the lumbar spine, femoral neck, Ward 's triangle and the greater trochanter, respectively, without further adjustments (to give an impression of the distribution of the data the relationships between (total and free) IGF-I and the lumbar spine and between (total and free) IGF-I and the greater trochanter are showed in Figure 1-2. Only in men free IGF-I and total IGF-I were significantly associated with BMD of the lumbar spine after adjustment for BMI and age (Table 3)

Table 1 Distribution of variables by sex

	Men n=103		Women n=111		p value
Age (years)	67.6	(0.6)	65.9	(0.6)	0.05
BMI (kg/m^2)	26.4	(0.3)	26.6	(0.4)	0.70 **
Total IGF-I* (nmol/L)	19.3	(0.83)	17.9	(0.70)	0.11 **
Free IGF-I* (nmol/L)	0.088	(0.005)	0.095	(0.005)	0.22 **
SHBG* (nmol/L)	50.9	(2.0)	60.2	(2.7)	0.006 **
Estradiol*(pmol/L)	106.4	(17.2)	82.1	(4.8)	0.10 **
Free estradiol Index* (pmol/nmol)	2.26	(0.25)	1.65	(0.13)	0.009 **
Bone mineral density (g/cm^2)					
Lumbar spine	1.19	(0.02)	1.03	(0.02)	< 0.001 **
Femoral neck	0.87	(0.01)	0.81	(0.01)	0.003 **
Ward' s triangle	0.71	(0.02)	0.73	(0.01)	0.027 **
Greater trochanter	0.83	(0.01)	0.73	(0.01)	< 0.001 **

Values are means with standard error between parentheses

* Fasting values

** Difference between men and women, adjusted for age.

BMI: body mass index; IGF-I: insulin-like growth factor-I; SHBG: sex-hormone binding globulin

Table 2. Association between fasting total and free IGF-I levels and age and BMI*

		r	p value
Total IGF-I			
Age	men	-0.17	0.09
	women	-0.28	0.003
Free IGF-I			
Age	men	0.14	0.15
	women	0.10	0.27
BMI			
Total IGF-I	men	-0.003	0.97
	women	0.09	0.35
Free IGF-I	men	-0.20	0.05
	women	-0.004	0.94

* Dependent variable is underlined

The relation between both free IGF-I and total IGF-I, respectively, and trochanter BMD in men reached borderline significance after the same adjustments. In multivariate analyses with BMD of the lumbar spine as the dependent variable and free IGF-I, age, BMI and the free estradiol index as independent variables- the association between free IGF-I and the BMD of the lumbar spine in men remained statistically significant (r=0.24, p=0.02). When the same analyses for the lumbar BMD were performed with total IGF-I rather than free IGF-I, the association was statistically significant in men (r=0.21, p=0.05). In analyses with the trochanter BMD as the dependent variable and free IGF-I, age, BMI and the free estradiol index as independent variables, the association between free IGF-I and the trochanter BMD in men just missed significance (r=0.19, p=0.06). When the same adjustments were performed for the relation between the trochanter BMD and total IGF-I, this association remained of borderline statistical significance (r=0.19, p=0.06).

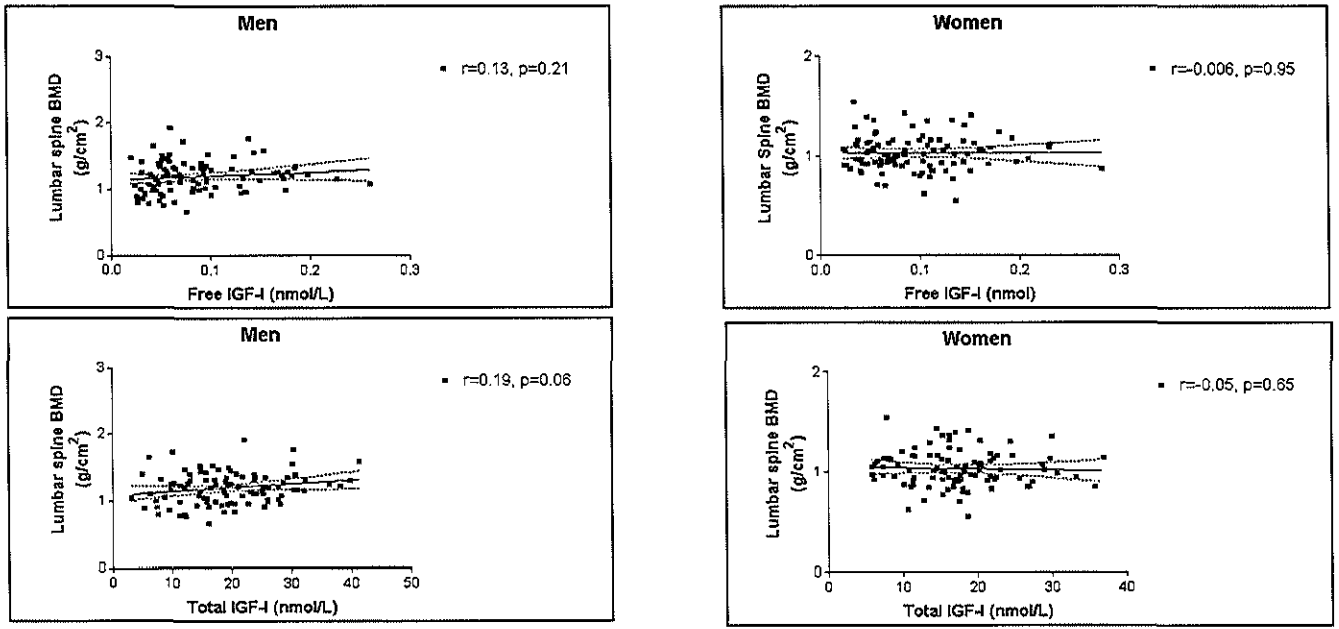

Figure 1. Univariate relationships between (free and total) IGF-I and BMD of the lumbar spine, respectively, in men and women. Top: Free IGF-I, Bottom: Total IGF-I. Left: men, Right: women.The solid line represents the regression line. The dotted lines represent the 95 % confidence interval.

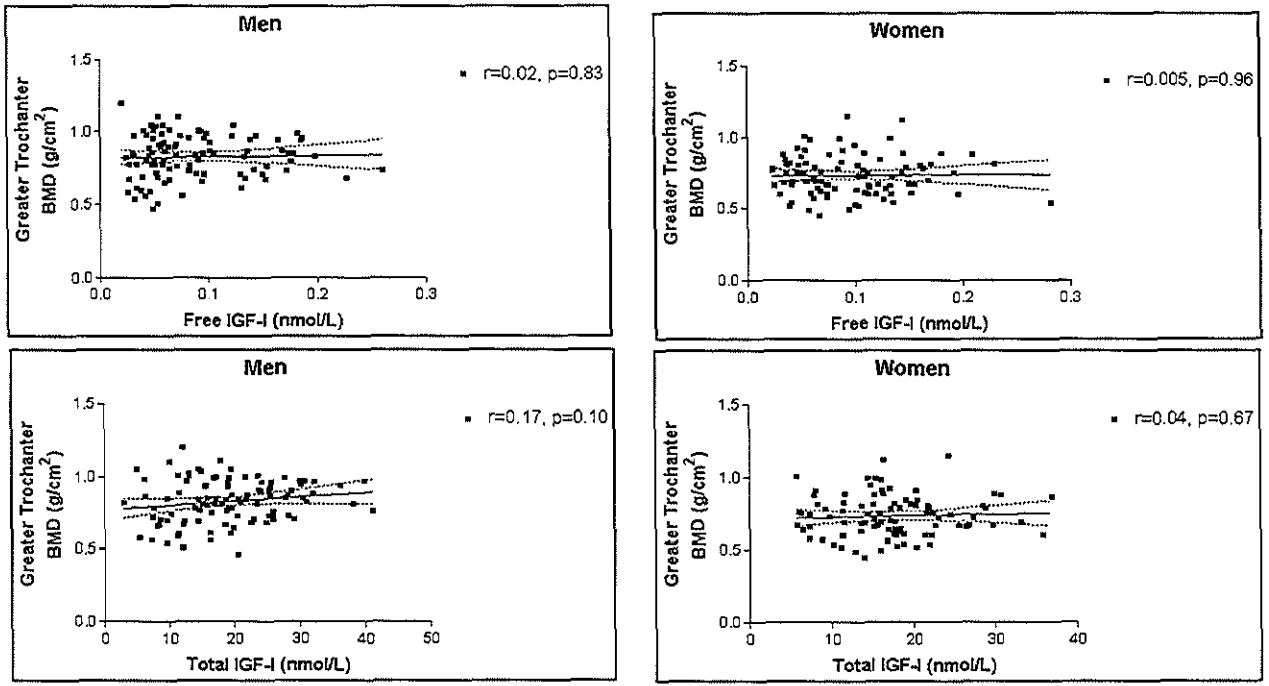

Figure 2. Univariate relationships between (free and total) IGF-I and BMD of the greater trochanter, respectively, in men and women. Top: Free IGF-I, Bottom: Total IGF-I. Left: men, Right: women. The solid line represents the regression line. The dotted lines represent the 95 % confidence interval.

Table 3. Associations between bone mineral density at various sites and fasting total and free IGF-I levels in men and women*

	Men r	p value	Women r	p value
Lumbar Spine				
Total IGF-I	0.22	0.03	-0.10	0.35
Free IGF-I	0.24	0.02	-0.009	0.93
Femoral Neck				
Total IGF-I	0.11	0.29	-0.12	0.27
Free IGF-I	0.09	0.36	-0.05	0.65
Ward 's Triangle				
Total IGF-I	0.15	0.15	-0.06	0.56
Free IGF-I	0.15	0.14	-0.04	0.72
Greater Trochanter				
Total IGF-I	0.19	0.06	0.007	0.94
Free IGF-I	0.18	0.08	0.02	0.89

Dependent variable is underlined
* adjusted for age and BMI.

Discussion

In this population derived sample of elderly subjects both free and total IGF-I were weakly positive related to BMD of the lumbar spine in elderly men, but not in women. In men also the relation between both total and free IGF-I and trochanter BMD reached borderline significance.

These findings suggest that IGF-I levels do not play a major role in protecting elderly from bone loss with aging. Serum free IGF-I levels are considered to be the major biologically active hormonal form of IGF-I (7). However, the assessment of serum free IGF-I levels in our study did not give much additional information than total IGF-I, since the relationships between free IGF-I and total IGF-I and bone mineral density were almost identical.

Previous studies have shown an age-related decrease in the serum level of total and free IGF-I (7, 12). Aging is also characterized by a decrease in bone density and a decrease in the IGF-I content of bone (13). Studies on osteoporosis in men have shown a relation between low bone mass and lower serum IGF-I levels as a consequence of decreased growth hormone secretion (14, 15). Other observations suggest that changes in the IGF-I content of bone may be related to changes in serum IGF-I concentration (16), which raises the possibility that the skeletal concentration of IGF-I reflects deposition from serum IGF-I, rather than local production (3). However, the relation between circulating (free and total) IGF-I levels and the lumbar and trochanter BMD in men, as observed in our study, contributes little to the issue whether skeletal IGF-I is predominantly derived from local production or from the circulation. This is still a very complex issue as circulating IGF-I is a marker of growth hormone activity and may simply reflect the direct effects of growth hormone on local skeletal IGF-I production. Moreover, growth hormone and circulating IGF-I may have indirect effects on bone mass, which may have nothing to do with skeletal IGF-I production. For example, there are many interactions between growth hormone/IGF-I and estradiol in men and women and between calcium and growth hormone. The issue is even more complex if one considers that the interindividual differences in skeletal IGF-I concentrations in old age may have already existed in young age and correlations

concentrations in old age may have already existed in young age and correlations between IGF-I and bone mass may be due to mixtures of genetic and/or environmental influences (17). It is therefore possible that any association between IGF-I and bone mass in elderly individuals may be due to events which occurred long before the actual measurements were performed.

No relations could be disclosed between free and total IGF-I and BMD in women. This latter relationship is similar to that reported in postmenopausal women by Kassem et al. (18), Bennett et al. (19) and Rosen et al.(20). Although there are some studies which have shown a positive relationship between BMD and IGF-I in women (21, 22), the patients in the study of Pun et al. (21) had also a concomitant vitamin D deficiency, while in the study of Wuster et al. (22) both pre- and postmenopausal women were included in the study. In this latter study there also was a considerable difference in age and body weight between patients and controls. Finally, both these studies were not population based.

The above mentioned associations between (free and total) IGF-I and (lumbar and trochanter) BMD in men turned out to be present only after adjustment for BMI. It has previously been demonstrated that weight and BMI are some of the strongest predictors of bone mass at all ages in both sexes (23, 24), and this effect is especially present at weight-bearing sites such as the lumbar spine and the femur. We observed strong associations between BMD and BMI in both sexes. In men this result might be related to the contribution of lean body mass to BMI, as it has been suggested that lean body mass is the principal determinant of BMD in men (25).

In conclusion, a weak positive relation between serum IGF-I and BMD was observed in men but not in women, possibly indicating a gender-specific anabolic effect of IGF-I on trabecular bone.

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§ 3.4 Serum free and total IGF-I, IGFBP-1 and IGFBP-3 levels in healthy elderly: relation to self-reported quality of health and disability.

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Summary

Background: Little is known about the influence of the free insulin-like growth factor-I/insulin-like growth factor binding protein (IGF-I/IGFBP) system on the quality of health and on disability in the elderly population.

Design: In a cross-sectional population based study of 218 healthy elderly subjects (age 55-80 yrs) fasting free and total Insulin-like growth factor (IGF-I), insulin-like growth factor binding protein-1 (IGFBP-1) and insulin-like growth factor binding protein-3 (IGFBP-3) levels were measured.

Subjective quality of health was assessed by asking all participants whether they judged the quality of their health as better, the same or worse than that of their peers. Disability was determined by the Disability Index of the Stanford Health Questionnaire.

Results: Mean serum free IGF-I levels were significantly lower in the 21 subjects, who experienced their health as worse, than that of their peers, compared to the 181 subjects who experienced their health as better or the same as their peers 0.069 nmol/L (SE: 0.009) vs 0.093 nmol/L (SE: 0.004)) ($p=0.04$). Mean IGFBP-1 levels were significantly higher in subjects, who felt less healthy than their peers 1.23 nmol/L (SE: 0.26) vs 0.73 nmol/L (SE: 0.82) ($p=0.01$).

Free and total IGF-I, IGFBP-1 and IGFBP-3 levels, were not different in subjects with the lowest and the highest Disability Index Score.

Conclusion: Low free IGF-I and high IGFBP-1 levels are associated with a decreased self-reported quality of health, but are not related to physical disability in the elderly.

Introduction.

Pituitary secretion of growth hormone (GH) and circulating levels of total insulin-like growth factor-I (IGF-I) decline physiologically with age (1).This gradual age-related decline in IGF-I levels has been related to loss of lean body mass, atrophy of muscles, accumulation of body fat, loss of bone mass and reduced aerobic work activity, as they occur during natural aging (2,3).

No population-based data are available in elderly adult individuals about the relationship between the IGF/IGFBP system and psychological well-being.

Non-elderly adult patients with pathological GH deficiency are also characterized by loss of lean body mass, atrophy of muscles, loss of bone mass, and low IGF-I levels (4). Studies of adults with GH-deficiency, a state characterized by low total IGF-I levels, have indicated a decreased psychological well being (5). Replacement therapy of GH deficient patients with GH results in normalisation of total IGF-I levels and improved psychological well-being in most patients (6).These data together suggest that low total IGF-I levels contribute or are related to decreased physical and psychological well-being during aging. In all these studies only the link between total serum IGF-I levels and physical and psychological well-being was investigated. Recently a well-validated method has been developed to measure free serum IGF-I levels (7, 8, 9). Serum free IGF-I level, analogous to sex and adrenal steroids and thyroid hormones, is likely to be a more biological active hormonal form of IGF-I (4). Measurement of serum free IGF-I levels may thus give more information on the biological effects of the GH-IGF-I axis than serum total IGF-I.

Since little is known about the relationships between the IGF-I/IGFBP system and the quality of health and disability in the healthy aging elderly population, the current population based study was designed to investigate whether in healthy elderly men and women serum circulating levels of total and free IGF-I and IGFBPs are associated with the self-reported level of perceived health and with disability.

Subjects and methods

Study population For the present study, a sample of participants from the

Rotterdam Study was invited for an additional examination. The Rotterdam Study is a population-based cohort study of the determinants of chronic disabling diseases in the elderly. All approximately 10,000 inhabitants of a suburb of Rotterdam, aged 55 years and over were invited to participate as described elsewhere (10). Overall 7983 participants were examined in the Rotterdam Study (response rate 78 %).

The population for the present study included subjects aged 55 to 80 years, who had completed the baseline visit of the Rotterdam Study not more than six months earlier. Subjects with acute, psychiatric or endocrine diseases, including diabetes mellitus treated with medication, were not invited. From these subjects a random sample of 218 persons was examined. Compared to the other participants of the Rotterdam Study without known diabetes mellitus, there were no differences in age and gender distribution, mean blood pressure, use of antihypertensive drugs, echocardiographic evidence of atherosclerotic plaques in the carotid arteries and electrocardiographic abnormalities. From all subjects informed consent was obtained and the study was approved by the medical ethics committee of Erasmus University Medical School.

Measurements Information on self assessed health was obtained by a trained research assistant using a computerized questionnaire, who was unaware to the blood results. Participants were asked if they experienced their health as better, the same or worse than subjects of their own age. Sixteen subjects did not answer this question. Disability was assessed by the Disability Index of the Stanford Health Assessment Questionnaire (11). The disability index was composed of the mean score (0 indicating no impairment, 3 unable to perform) for six component questions on arising, walking, bending, and getting in and out of a car.

Serum samples Participants were examined in the morning after an overnight fast. Fasting blood samples were taken by venipuncture between 8.00 and 9.00 a.m. and allowed to coagulate for 30 minutes. Subsequently serum was separated by centrifugation and quickly frozen in liquid nitrogen. Free IGF-I was measured with a commercially available two-site immunoradiometric assay (Diagnostic System Laboratories Inc., Webster, Texas, U.S.A.; intraassay and interassay C.V.: 10.3% and 10.7%, respectively) (7,8). Total IGF-I was determined by a commercially

available radioimmunoassay (Medgenix Diagnostics, Brussels, Belgium, intraassay and interassay coefficients of variation (C.V.): 6.1%; 9.9%). Commercially available immunoradiometric assays were also used for measurement of IGFBP-1 and IGFBP-3 (Diagnostic System Laboratories Inc; intraassay and interassay C.V. for IGFBP-1: 4.0% and 6.7 %, respectively; and for IGFBP-3: 1.8 % and 1.9%, respectively). Serum glucose levels were determined, using a standard glucose hexokinase method. Height and weight were measured wearing indoor clothes and without shoes. Body mass index was defined as weight divided by the square of height (kg/m^2).

Statistical Analysis Linear regression analyses were used to calculate gender differences in the baseline clinicical characteristics between men and women, after adjustment for age. Linear regression analysis was also used to test for trends in the IGF-I/IGFBP system index across groups with differences in quality of health adjusting for confounding variables. In this latter analysis the self-reported quality of health was used as independent variable in the model. The free IGF-I levels of those who scored zero on the Disability Index were compared with those of the remainder of the sample. Pearson 's correlation coefficient was calculated to assess the association between self-reported quality of health and the Disability Index score. A two-sided p-value of <0.05 was considered significant. Analyses in which the values for the IGF-I/IGFBP system were logarithmically transformed yielded results similar to those with untransformed data. Because interpretation of results from logarithmically transformed data is difficult, the non-transformed results are presented. All statistical analyses were performed with the Stata statistical package (Computing Resource Center, Santa Monica, Ca, U.S.A).

Results

In Table 1 the general characteristics of the study population are presented. Men were slightly older than women. Mean (free and total) IGF-I and IGFBP-1 levels did not differ between the sexes. IGFBP-3 levels were (age-adjusted) significantly lower in men, than in women 103.5 nmol/L (SE: 2.5) vs 115.7 nmol/L (SE: 2.4), $p=0.002$.

There were no sex- and age-specific differences in subjectively perceived health and disability score.

Fasting total IGF-I and IGFBP-3 levels decreased with age (total IGF-I: 0.23 nmol/L per year (SE 0.09), p=0.04; IGFBP-3: 0.95 nmol/L per year (SE 0.29), p=0.001), while free IGF-I and IGFBP-1 levels remained stable.

Mean serum free IGF-I levels were significantly lower in the 21 subjects (12 men, 9 women) who felt less healthy than subjects of their own age, compared to the 181 subjects (85 men, 96 women), who perceived their health as better or the same 0.069 nmol/L (SE:0.009) vs 0.093 nmol/L (SE:0.004), p=0.04 (Figure 1 A). This difference remained significant after adjustment for gender, age, BMI, WHR, fasting insulin and glucose levels. Mean total IGF-I and IGFBP-3 levels did not differ between these two groups. Mean IGFBP-1 levels were significantly higher in subjects who felt less healthy than their peers (1.23 nmol/L (SE: 0.26) vs B: 0.73 nmol/L (SE: 0.82), p=0.01 (Figure 1B). This difference remained significant after adjustment for gender, age, BMI, WHR, fasting insulin and glucose levels.

Free IGF-I levels were not significantly different in subjects with or without any disability 0.091 nmol/L (SE: 0.005) vs 0.093 nmol/L (SE: 0.005). For total IGF-I, IGFBP-1, and IGFBP-3 levels also no differences in serum levels were present in this respect (data not shown). There also was no relationship between free IGF-I, total IGF-I, IGFBP-1, and IGFBP-3 levels and Disability Index score when only subjects were analyzed who scored higher than zero for the Disability Index. The self-reported quality of health was (age-adjusted) significantly correlated with the Disability Index score ($r=0.39$, $p<0.001$)

Table 1. Distribution of variables in 218 elderly subjects (115 women and 103 men)

	mean	(SE)
Age (years)	66.7	(0.4)
BMI (kg/m^2)	26.4	(0.2)
WHR	0.92	(0.006)
Total IGF-I* (nmol/L)	18.7	(0.5)
Free IGF-I* (nmol/L)	0.092	(0.004)
IGFBP-1* (nmol/L)	0.77	(0.06)
IGFBP-3* (nmol/L)	110.0	(1.8)
Insulin* (mIU/L)	13.5	(0.5)
Glucose* (mmol/L)	5.9	(0.06)
Subjectively experienced Quality of Health		
Better than peers (%)	55.3	
Comparable to peers (%)	34.5	
Worse than peers (%)	10.2	
Disability Index Score		
No disibality (no of subjects)	97	
With any disability (no of subjects)	115	

Values are means with standard error (SE) between parentheses,

* Fasting blood samples
BMI, body mass index; WHR, waist-to-hip ratio; IGF-I: insulin-like growth factor I; IGFBP: Insulin-like growth factor binding protein; See Methods for Disability Index Score

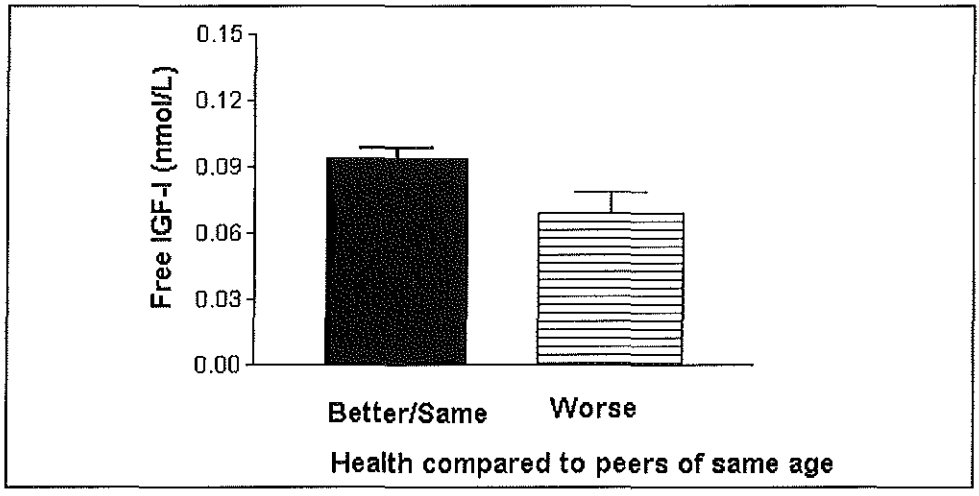

Figure 1A. Free IGF-I levels (vertical axis) divided by self-reported perceived health in men and women aged 55-80 yrs.The bars represent the mean and the error bars represent the standard error. The difference between both groups was statistically significant (p=0.04)

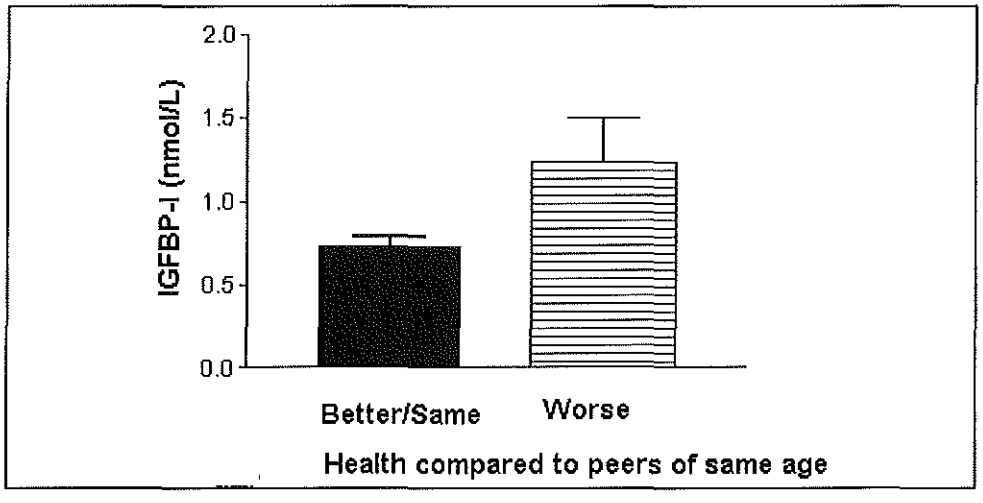

Figure 1B. IGFBP-1 levels (vertical axis) by self-reported perceived health in men and women aged 55-80 yrs.The bars represent the mean and the error bars represent the standard error. The difference between both groups was statistically significant (p=0.01).

Discussion

Mean free IGF-I levels were lower in subjects who reported a decreased quality of general health compared to their peers. This relation was independent of age and gender. Although there was much overlap in the actual range of free IGF-I levels between the three categories of perceived quality of general health, our study indicates that free serum IGF-I levels tend to be lower in elderly, who feel unhealthy. Increases in IGFBP-1 levels result in increased binding of free IGF-I, thereby decreasing the amount of IGF-I available at the target cells (12). Our observation of increased IGFBP-1 levels in subjects who felt less healthy, is interesting in this context as these changes may be the cause of the lowered free IGF-I levels. Catabolism is generally characterized by low IGF-I and high IGFBP-1 levels (13). This might indicate that subjects who feel less healthy are in a more catabolic state, or vice versa. However, at present it is not clear, whether the observed changes in free IGF-I and IGFBP-1 levels reflect cause, effect, or are mere correlates of (as yet undiagnosed) disease in this study population.

In an earlier intervention study of elderly individuals, dehydroepiandrosterone administration induced an increase in the bioavailability of IGF-I (14). This increase in bioavailable IGF-I was accompanied by a an improvement of self-reported physical and psychological quality of health in both sexes (14). Studies of adult patients with GH deficiency, a state characterized by low free IGF-I levels (4), have indicated a decreased self-reported quality of health (5). Replacement therapy of GH deficient patients with GH results in normalisation of free IGF-I levels (15) and improved self-reported quality of health in most patients (6). These data, together with our observations suggest that low free IGF-I levels contribute or are related to decreased self-reported physical and psychological quality of health with aging.

In our study, free and total IGF-I levels were not associated with physical disability. The quality of life of elderly individuals is more determined by their level of cognitive functioning and their ability to remain independent than by specific disability (16). This may explain for the observed difference between free IGF-I, disability and (self-rated) perception of health status. In accordance with our observation, it was recently reported that an age-related decline in IGF-I secretion

is not responsible for functional decline with age (16).

In conclusion, low free IGF-I and high IGFBP-1 levels in both sexes are associated with a decreased perceived quality of health, but are not related to physical disability in the elderly.

Acknowledgement

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4

The diagnostic value of the IGF-I/IGFBP system in growth hormone deficiency and growth hormone excess states

§ 4.1 Practical aspects of the diagnosis of growth hormone deficiency in adults

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Introduction

As most if not all of the circulating growth hormone (GH) is produced by the pituitary gland, it is most likely that growth hormone deficiency (GHD) must be present in any patient without an anterior pituitary. The problem is however, that many patients still have some pituitary remnant after selective pituitary surgery, while GHD is often incomplete in those diagnosed during childhood. In these patient–groups, one therefore needs more information in order to determine whether GHD does exist. This has become more important after human GH became available for long–term treatment of adult GHD patients. Simultaneously, the role of GH as an important metabolic regulator during life is being gradually unraveled. Many studies have demonstrated that GH replacement therapy to GHD adults has significant beneficial effects on the quality of life, body composition, metabolic status and lipid spectrum (3, 5, 7, 30, 31, 50, 52, 58, 60, 61, 66, 94). However, the costs of this therapy remains an important problem. It is therefore mandatory to treat only those patients with proven GHD. The procedure of making the diagnosis of GHD remains controversial, however in the following pages we will try to formulate the most important practical tests and parameters, which can be used as diagnostic tools for GHD in adult patients.

What is GHD?

Now the important regulatory role of GH for many tissues and metabolic processes has been revealed, it is not surprising that the signs and symptoms of GHD are versatile (See figure 1). According to the available literature and based upon clinical experience, the signs and symptoms of a typical patient with GHD have their origins in the following characteristics (7, 26, 47, 76, 79, 89):

Increased fat mass (mainly localized around the hip)
Reduced lean body mass, muscle mass and muscle strength
Decreased extracellular water
Lowered bone mineral density
Impaired cardiac function
Poor physical performance

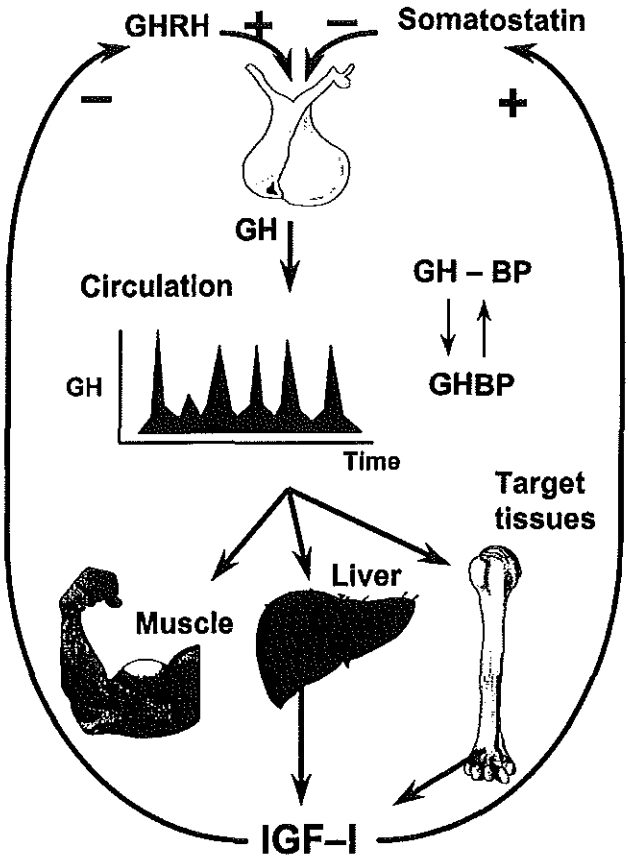

Figure 1 Overview of metabolic pathways of GH

Many of these features are also present in patients with other diseases. However, there are also many patients with a defect in anterior pituitary function after pituitary surgery, who do not have the clinical signs and symptoms related to long-standing GHD. The discriminatory value of these characteristics is therefore limited in the diagnosis of GHD. Apart from this, most patients with anterior pituitary insufficiency

are on replacement therapy with other pituitary hormones. A variety of symptoms encountered in GHD patients might be related to over or undertreatment with corticosteroids, thyroxine or sex-steroids. The clinical picture described above is important however for the selection of those patients in whom it will be necessary to perform a biochemical test to prove the presence of GHD. As GH secretion and insulin-like growth factor–I (IGF–I) levels decrease with increasing age, it is crucial to have available carefully collected age-and sex-specific control data both for GH stimulation tests, as well as for IGF–I levels in the circulation (99). This is especially true for elderly patients with a history of pituitary disease but with a normal production of the anterior pituitary hormones other than GH. These factors all together did raise the need for the development of reliable sensitive and specific tests to assess GH reserve.

Growth Hormone reserve tests.

As shown in Figure 2, both physiological and pharmacological GH secretion stimulatory tests have been developed, which can be used to distinguish between normal and GHD patients. These tests can be divided into tests which act directly

GH : **physiological**

- 12 or 24 h profiles
- exercise stimulation
- sleep
- urinary GH
- GH-binding protein

pharmacological stimulation

- insulin
- glucagon
- arginine
- GHRH
- clonidine
- L-DOPA/propranolol
- Pyridostigmine

IGF-I and/or IGFBP-3

Figure 2 Biochemical methods of diagnosis of GHD in adulthood

upon the pituitary gland, such as the GHRH test and tests which act via supra–pituitary mechanisms, such as the Insulin Tolerance Test (ITT). There are reports that peak GH concentrations after provocative testing correlate poorly with 24-h mean concentrations in GHD, which suggests that provocative GH testing frequently does not correlate with endogenous GH secretion (10).

Insulin Tolerance Test

In the early seventies the first reports about GH stimulatory test methods, using insulin became available (32, 33). In 1986, Kaplan et al. described the changes in hGH levels during insulin–induced hypoglycemia in a large group of short and normal children (68). The ITT remained the golden standard for the diagnosis of GHD. Rahim et al. (109) compared the GH response to insulin-induced hypoglycemia, intravenous arginine administration, oral clonidine administration (100 and 200 μg) and intramuscular glucagon in normal individuals. They found that the peak GH response in the ITT was significantly greater than for any other agent, followed by glucagon, arginine, clonidine 200 and 100 μg and placebo respectively. Hoffman et al reported that hypopituitary subjects have significantly lower stimulated peak GH levels, mean 24 h. GH, IGF–I and insulin-like growth factor binding protein-3 (IGFBP–3) concentrations than normal subjects. Only the ITT however could separate GHD patients from normal individuals, while the other parameters (spontaneous GH secretion, IGFI and IGFBP–3) did show considerable overlap with normals (49). One has to keep in mind, however, that the reproducibility of the ITT (and other tests) is limited (48). The cut-off value of the maximal GH concentration after appropriate hypoglycemia is considered to be < 3 μg/L for "absolute" and ≤ 5 μg/L for relative GHD. The ITT is an uncomfortable test to the patient and whether it is a safe test when used in adult patients with hypopituitarism is still a matter of debate. The fact that these patients frequently also lack the increase in cortisol secretion as counteracting mechanism during hypoglycemia makes this test contra-indicated in patients with coronary heart disease, while it should only be carried out in experienced endocrine units under close supervision. seems not to be a major problem in every day practice.

Arginine and pyridostigmine

The intravenous administration of arginine stimulates pituitary GH secretion (1). The GH-releasing effect of both arginine and pyridostigmine is mediated via the same mechanism, namely, by suppression of endogenous somatostatin release. Combined administration of either arginine or pyridostigmine with GHRH has a striking powerful GH-releasing effect, which is considerably higher than that after GHRH alone (38, 39, 42, 43, 70). These test methods have been used for many years, especially in pediatric centers, mainly because they have been proven to be safe (55, 87, 95,101,105-107). Masuda et al. suggested that some factor (or factors) other than GHRH and somatostatin are involved in the mechanism by which the ITT stimulates GH secretion, because the effect of insulin was not fully blocked in the presence of a somatostatin analog (75).

GHRH and GHRP tests

In normal man, several analogues of GHRH have been developed, which are safe, sensitive and specific stimuli to GH release. Continuous infusion of GHRH leads to a decrement in responsiveness, due at least in part to changes in hypothalamic somatostatin. The GH response to GHRH is also modulated by obesity, blood glucose levels, free fatty acids, and GH itself. (2, 11, 12, 37,44, 45, 80, 84, 97,100). The GH-releasing activity of hexarelin (GHRP–6), a new synthetic hexapeptide, after i.v. administration has a low variability and has been shown to be superior to the GH response to GHRH. The GH-releasing activity appeared to be dose dependent. Thus, hexarelin can be clinically useful to stimulate GH secretion in humans (36, 80). Especially if the ITT is contraindicated, or cannot be carried out under experienced and close supervision a combined arginine + GHRH or a combined arginine + pyridostigmine stimulation test are excellent and probably equally valuable alternatives.

IGF–I

As mentioned above, a decrease in circulating IGF–I levels occurs. This is the reason that the assessment of IGF–I alone is of limited value in elderly patients, as

long as one does not have carefully collected control values for all ages, as well as both sexes. If lowered IGF–I levels are found in a patient with otherwise disturbed anterior pituitary function, chances that this patient has GHD are high. IGF–I concentrations within the normal range do not rule out GHD, however. This necessitates further biochemical investigation, especially if other factors make GHD likely. Recently it has become possible to determine serum free IGF–I concentrations. Whether or not this free IGF–I plays a major metabolic role, or only reflects just a small non-significant amount of unbound IGF–I is not yet known. Hasegawa et al. demonstrated that patients with complete GH deficiency had low levels of both free IGF-I and total IGF-I, with 94% and 100% of the levels below the 5ht percentile for age, respectively. They concluded that in the evaluation of childhood GHD the clinical utility of plasma free IGF-I measurements is similar to measurements of total IGF-I (46). Important confounders in the evaluation of serum IGF-I levels are malnutrition, cirrhosis of the liver, poorly controlled diabetes mellitus and hypopituitarism.

IGFBP–3

GH and e.g. sex-steroids, but not IGF–I, stimulate IGFBP–3 production. GHD therefore results in decreased IGFBP–3 levels. Serum levels of total IGF–I and IGFBP–3 reflect endogenous GH secretion in healthy children and exhibit little diurnal variation, which makes them good diagnostic markers for screening of GHD in short children, although some controversy still exists. The IGFBP–3 concentrations in adult GHD, however, demonstrate a considerable overlap with values that are found in the lower ranges of IGFBP–3 levels in normal individuals (17, 18, 22, 63, 64). In our study involving 173 patients with GHD IGF-I and IGFBP-3 levels were lower than the 5^{th} percentile in 86 % and in 74 % of those patients with childhood onset GHD, while this was the case in 73 % and 45 % of the 99 patients with adult onset GHD (Figure 3). For this reason, the assessment of IGFBP–3 levels in adult GHD seems of limited value. Juul et al. reported however that in childhood onset GHD, both IGF–I and IGFBP–3 determinations predict the

outcome of GH provocative tests in adults suspected of GHD and these authors suggest that IGF-I, as well as IGFBP-3 serum concentrations are valuable diagnostic parameters in the evaluation of GHD in adults with childhood-onset disease (67).

Other test methods

Other ways to assess GH reserve in patients are e.g. the administration of clonidine (95) and the measurement of GH secretion during sleep (34), but these test methods have not been widely used in the diagnostic work-up of GHD in adulthood. Recently, highly sensitive assays for the detection of GH have become

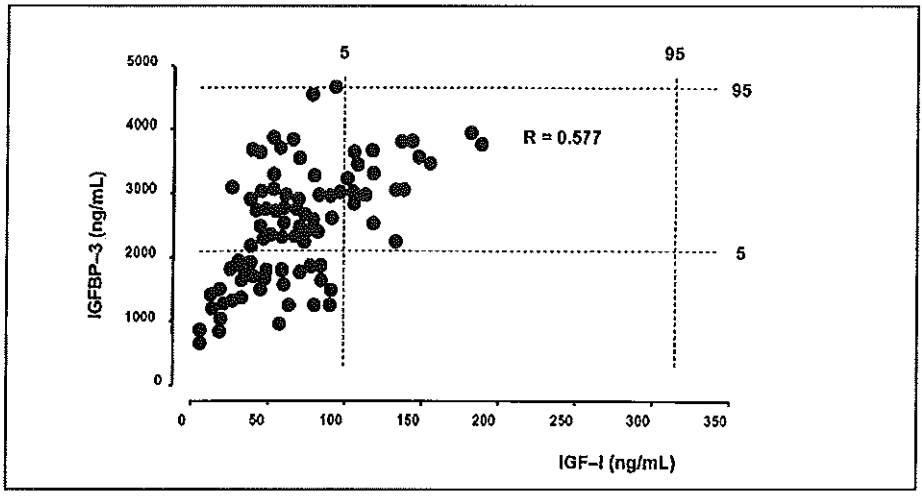

Figure 3 Baseline correlation between serum IGF-I and IGFBP-3 in untreated adult-onset GHD. Note that a relatively large number of patients have normal IGFBP-3 concentrations in comparison with IGF-I levels.

available. Reutens et al. did demonstrated that a sensitive Enzyme Linked Immunosorbent Assay (ELISA), used to quantify 24 hr. integrated GH concentrations in normal and hypopituitary subjects did not clearly separate GHD patients from normal individuals, however, despite their impaired GH responses to ITT (85,86).

One might therefore conclude that the highly– and ultra sensitive assays have no additional value over the conventional Radio Immuno Assay (RIA's) and Immuno Radiometric Assay (IRMA's), despite their superior sensitivities. However, one should keep in mind that the cut-off values of GH responses to ITT mentioned above, refer to the use of polyclonal RIA for GH determination; these values should be amended if ultrasensitive GH assays are used.

The optimal biochemical diagnosis of GHD.

Hoffman et. al. reported that the ITT was the only test method, when compared to spontaneous GH secretion, IGFI and IGFBP–3, that was able to provide a 100 % diagnostic accuracy in defining GHD (49). The risk of inducing severe hypoglycemia in hypopituitaristic patients because of their enhanced sensitivity for insulin however, remains an actual danger. Ghigo et al. reported extensively about the applicability of pyridostigmine (PD; a muscarinic cholinergic agonist), arginine and GHRH alone or in combination (38,41,74). PD + GHRH and arginine + GHRH tests are reliable tests for the diagnosis of primary GHD in childhood (25,40).

The same group of investigators recently confirmed in a study in 45 patients with GHD and 326 healthy controls that IGF-I measurements had no value in the diagnosis of GH deficiency in adults aged over 40 years. IGF–I assessment was reliable however, in the group of young adults of 20-40 years of age. The ranges of peak GH responses to PD + GHRH and ARG + GHRH tests were clearly different between GHD patients and normal individuals. They concluded that both PD + GHRH and ARG + GHRH testing should be considered as reliable biochemical measurements of GH deficiency in adults. In the same study, both combinations of tests were slightly better than the ITT in stimulating GH secretion. In contrast to the PD + GHRH test, the ARG + GHRH test turned out to be reliable throughout the adult lifespan and appeared to be the most appropriate for patient compliance and safety (35).

Important characteristics of GHD.

Body composition

There are many reports which demonstrated that untreated adult patients with GHD have an increase in total body fat mass (both in visceral and subcutaneous fat). Their lean body mass is decreased, as well as their bone mineral density, their muscle strength and exercise performance (6, 8, 9, 15, 19, 23, 26, 28, 59, 62, 72, 88, 90, 92, 93). There are many techniques to measure body composition. Widely used are Bioelectric Impedance Analysis (BIA), isotope dilution techniques, total body potassium assessment by ^{40}K, total body water (TBW) by tritium dilution, total body nitrogen (TBN) by neutron activation, sum of skinfold assessment and finally dual-energy X-ray absorptiometry (DEXA), which has become more or less the golden standard. Apart from this multitude of methods for measuring body composition, one has to take into account that for mathematical purposes there are two-compartment and multicompartment body composition models. In a study by Bosaeus et. al. a comparison of several methods to estimate body fat in 25 patients with known GHD was made. At baseline and at group level, the results of DEXA fat mass measurements correlated well with measurements based on TBW or TBK alone, in a four-compartment model based on TBK and TBW, and a multi–compartment model based on bone mineral (by DEXA), TBN and TBW. The frequently used body fat measurement by BIA agreed less well. They repeated these measurement after 12 months of GH treatment and found that body fat decreased by all methods used. This decrease was smaller by DEXA than by the other methods. The four-compartment model based on TBK and TBW, and TBW alone, showed the best agreement with changes in DEXA fat. They concluded that all methods demonstrate a decrease of body fat with GH replacement therapy, but variation between methods is considerable (19). On the average, total body fat in GHD adults is 7 kg higher in GHD patients than in normal controls (15, 88). Computer Tomography (CT) studies have revealed that muscle mass is decreased in GHD (24, 28, 65). GH significantly affects body water homeostasis: TBW content in GHD is decreased, while GH treatment, using the D_2O dilution method induces an increase in TBW, with a maximal increase within 4–6 weeks after start of

therapy (9).

Although at present the role of GH in maintaining bone mineral density (BMD) during adult life is not completely understood, results obtained from many controlled and uncontrolled studies show that BMD in (adult) GHD is significantly reduced. Changes in BMD with GH replacement therapy can be observed. After a transient decrease in BMD, many studies reported an increase in BMD when patients are treated longer than 6 months (4, 5, 13, 14, 16, 20, 27, 29, 31, 50-52, 56, 57, 61, 69, 82,102).

Quality of Life (QOL)

As GHD patients frequently complain about fatigue, lack of energy, social and sexual problems, validated questionnaires were developed that could quantify the degree of loss of quality of life. Frequently used are e.g. Nottingham Health Profile, the Self–Esteem scale, the Hospital Anxiety and Depression scale, the mental Fatigue Questionnaire, while nowadays more and more disease specific questionnaires are being developed (21, 53, 54, 73, 77, 78, 83, 90, 96, 104,108). Almost all questionnaires demonstrate that GHD and loss of QOL go hand in hand. In figure 4, the QOL score measured by the Nottingham Health Profile in GHD patients in comparison to other invalidating diseases is shown. This clearly shows that GHD is a true invalidating entity and needs treatment if possible.

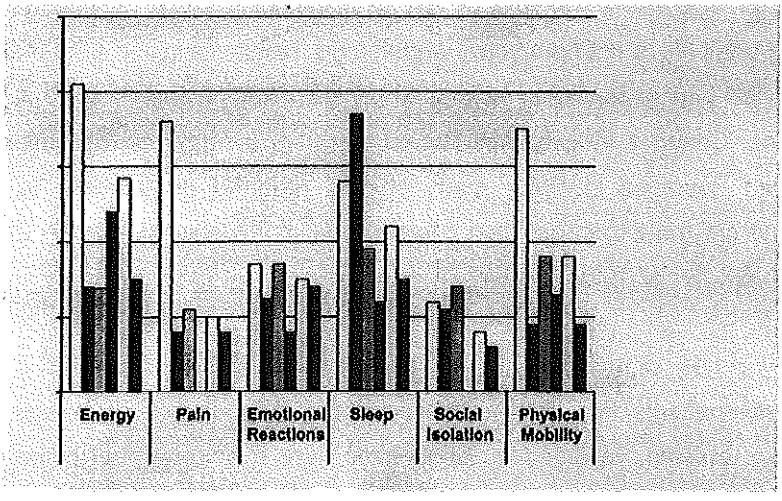

Figure 4 Comparison between QOL scores (Nottingham Health Profile Questonnaire) for several chronic diseases. Note that GHD is an incapacitating condition. From left to right per parameter: rheumathoid arthritis, breast cancer, coronary heart disease, diabetes, adult-onset GHD, childhood-onset GHD.

Childhood onset GHD

Several reports indicate that in childhood onset adult GHD patients, especially those with idiopathic GHD, the GH status has to be reinvestigated to confirm GHD (71,81,91,103). Clearly Nicolson et al. demonstrated, using the ITT as stimulatory test, that childhood onset adult GHD patients, due to previous irradiation of the skull showed an increase in the incidence of GHD during adulthood (48.8 versus 55.8%). Patients diagnosed in the past as idiopathic GHD, however, did show a decrease in incidence of GHD during adulthood (78.1 versus 53.1%). Roughly 47 % of these patients with an initial maximal GH concentration of less than 5 μg/L in the ITT showed a maximal GH level of higher than 5 μg/L during a second test in adulthood (81).

Another important factor is the number of other concomitant anterior pituitary insufficiencies that is present in a patient, suspected to have GHD. Toogood et al. demonstrated in a group of 190 patients with pituitary disease that GHD was

present in 91% of the patients with two or three other anterior pituitary insufficiencies. When one pituitary hormone insufficiency was present, only 55% of these patients had also GHD. When no pituitary insufficiencies were found, only 24% of these individuals turned out to be GHD (98).

Recommendations

Even more important than to decide which test, or which parameter to use when GHD is suspected, is the issue of whom should be tested in the first place.

We propose the following guide–lines:

In the absence of other signs and symptoms of hypothalamic or pituitary dysfunction, or previous GH therapy during childhood persons do not need to undergo tests for GHD. For this purpose, some sort of score has to be developed, which gives the physician an indication whether a patient is at risk for GHD. This will lead to a more uniform approach of the diagnostic work–up of GHD.

This score must preferentially include data obtained from a (disease specific) QOL questionnaire, the assessment of the number of other pituitary insufficiencies present in the patient and the clinical signs and symptoms, which are known to frequently occur after long-term GHD. Also helpful at this stage of the diagnostic process is the measurement of body composition of the patient.

If this score indicates that the chances of a given patient having GHD is high, an assessment of total (or free) IGF–I concentrations should be performed in patients younger than 40 years of age. An IGF–I level less than 2 standard deviations (< – 2SD) below the age specific normal value should be considered as proof for the existence of GHD. Only in cases when absolute evidence must be obtained (for example for health care authorities, authorizing the prescription of GH), one single stimulatory test to assess GH reserve could be performed.

Patients older than 40 years of age and younger patients with a IGF–I concentration higher than – 2 SD below the age specific normal value should undergo at least one, but preferentially two stimulatory tests. It is anticipated that the combined arginine + GHRH test (or combined arginine + pyridostigmine test),

promoted by Ghigo et al. will proof itself as a test method with sufficient potency to be used as a single test (35). Figure 5 shows this proposed diagnostic route.

At present the post–marketing studies carried out by the major GH producing companies, include at least one QOL scale, some body composition measurement and at least one GH stimulatory test. Hopefully these studies will enable us to eventually manufacture an easier diagnostic flowchart, which can be used in future patients.

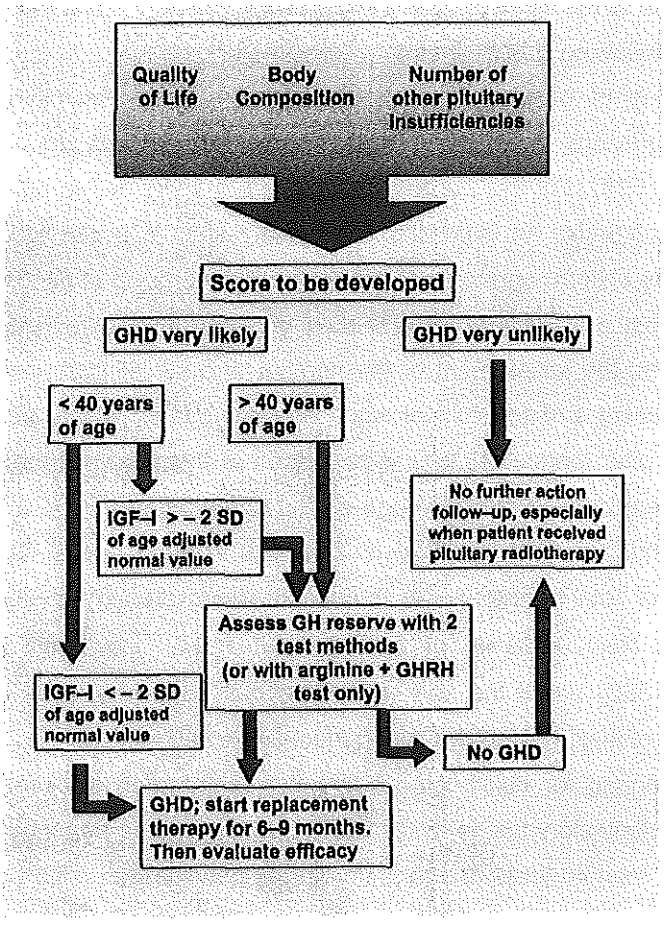

Figure 5 Proposal for a flow chart for diagnosing GHD. Preferentially, a quantitative score must first be developed, consisting of at least three parameters: QOL, body composition and number of other pituitary insufficiencies.

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§ 4.2 IGFBP-3 IS A POOR PARAMETER FOR CLINICAL ACTIVITY IN ACROMEGALY

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Summary

Objective: Elevated serum IGF-I and IGF binding protein-3 (IGFBP-3) levels have been found in patients with active acromegaly. We have studied the relative diagnostic merits of measurements of IGFBP-3 over IGF-I as a parameter of disease activity in these patients.

Design/Patients: Thirty untreated patients with acromegaly were compared with 30 healthy adults.

Measurements: 24 h sampling for serum GH in patients with acromegaly, serum IGF-I and IGFBP-3.

Results: Mean IGF-I levels amounted to 22.0 nmol/L (range: 6.5 - 38.4 nmol/L) in the healthy adults and to 118.7 nmol/L (range: 67.7 - 206.0 nmol/L) in patients with acromegaly. Mean IGFBP-3 levels amounted to 3.5 mg/L (range: 2.1 - 4.8 mg/L) in controls and to 5.4 mg/L (range: 4.2 - 6.6 mg/L) in patients with acromegaly. Mean IGF-I/IGFBP-3 ratios amounted to 6.5 nmol/mg (range: 1.9 - 14.5 nmol/mg) in the healthy adults and to 22.0 nmol/mg (range: 14.3 - 32.7 nmol/mg) in patients with acromegaly. There was a considerable overlap for IGFBP-3 levels between normals and acromegalics, but not for IGF-I levels. Also, the IGF-I/IGFBP-3 ratio showed overlap between normals and acromegalics. There was a significant correlation between the mean 24 h GH and IGFBP-3 levels ($p = 0.036$) and between the IGF-I and IGFBP-3 levels ($p < 0.002$) in acromegaly. In patients with acromegaly, the IGFBP-3 levels showed a decrement, but the IGF-I/IGFBP-3 ratio did not change significantly with age.

Conclusions: IGFBP-3 has no additional discriminitative value over IGF-I measurements for the assessment of clinical activity in acromegaly. In acromegaly, IGFBP-3 decreases with increasing age. In acromegaly, IGFBP-3 levels significantly correlate with mean 24 h GH levels and IGF-I levels.

Introduction

Acromegaly is a disorder caused by an excessive GH production, most commonly from a somatotroph adenoma located within the pituitary gland. Under normal conditions, GH regulates circulating IGF-I levels (1). However, unlike GH levels, IGF-I levels do not exhibit pulsatility or circadian patterns. Serum IGF-I levels correlate well with the 24 h GH secretion and, therefore, are usually elevated in active acromegaly (2-5). However, serum total IGF-I concentrations do not reflect disease activity in some cases of acromegaly. IGF-II levels remain in the normal range in active acromegaly, which is in agreement with the contention that GH is not a major regulator of IGF-II in adulthood (6-8). Most of the IGF-I and IGF-II in the circulation and throughout the extracellular space is bound to members of a recently characterized family of high affinity binding proteins (IGFBPs)(9). Under normal conditions, the 264-amino acid, 46-53-kDa glycoprotein IGFBP-3 is the most abundant IGFBP in human serum. After complexing with the IGF, this protein binds an additional 80-88-kDa acid-labile subunit (ALS), and the thus constituted 150-kDa ternary complex is the major storage form of IGFs in the circulation. When associated with the ternary complex, the IGFs do not readily leave the vascular compartment and their half lifes are prolonged from less then 10 min to 12-15 h. Subsequently, the IGFs can be released after cleavage of the ternary complex by specific proteases. The production of IGFBP-3 is increased in response to an increase in GH, insulin, IGF-I, or a change in nutritional status (9-10). Therefore, elevated IGFBP-3 levels have been found in patients with active acromegaly (7, 8, 10-12).

The aim of our study was to evaluate the relative diagnostic merits of measurements of IGFBP-3 over IGF-I as a parameter of disease activity in acromegaly.

Patients and Methods

Patients with active acromegaly

Thirty previously untreated patients (14 females, 16 males; mean age 50.6 years)

with newly diagnosed active acromegaly were studied. The diagnosis of acromegaly had been based on a typical clinical presentation, persistently elevated GH levels over 24 h, elevated IGF-I levels and nonsuppressible GH following oral glucose loading. A pituitary tumor was demonstrated in all patients by computed axial tomography, or T1-weighted MR images of the sellar region in the coronal or sagital plane. Patients were euthyroid and none of the patients had disturbed renal function. All patients had a somatotroph pituitary adenoma removed at subsequent surgery. Serum GH was sampled from 0800 h to 1800 h at hourly intervals, followed by 2 hourly sampling to 0600 h and a final sample was taken at 0700 h. A single non-fasting blood sample was drawn for IGF-I and IGFBP-3.

Healthy adults

Thirty healthy adults (14 females, 16 males; mean age 56.6 years) were studied as controls. A single non-fasting blood sample was drawn for IGF-I and IGFBP-3.

Immunoassays

Serum GH was measured by immunoradiometric assay (IRMA) supplied by CIS Bio International, Gif-sur-Yvette Cedex, France (intraassay CV, 2.8%; interassay CV, 4.4%). Total serum IGF-I was determined by radioimmunoassay (RIA), using kits obtained by Medgenix Diagnostics, Fleurus, Belgium (intraassay CV, 6.1%; interassay CV 9.9%). Serum IGFBP-3 levels were determined by IRMA supplied by Diagnostic System Laboratories, Webster, Texas (intraassay CV 3.9%, interassay CV 1.9%).

Statistics

Differences between the sexes were calculated using a one way ANOVA. Results are given as means. Correlations were made by the Pearson two-tailed bivariate correlation test. All statistics were prepared using the commercial available software program SPSS for Windows, release 6.0 (SPSS Inc., USA). Statistical significance was defined as $p < 0.05$.

Results

Table 1 shows shows the individual parameters as sex, age, mean 24h GH, IGF-I and IGFBP-3 levels and the calculated ratio between serum levels of IGF-I and IGFBP-3 in the 30 patients with acromegaly. There were no statistical significant differences between the sexes with regard to mean GH, IGF-I and IGFBP-3 levels, or the IGF-I/IGFBP-3 ratio. Mean IGF-I levels were 22.0 nmol/L (range: 6.5 - 38.4 nmol/L) in the healthy adults (Table 2) and were 118.7 nmol/L (range: 67.7 - 206.0 nmol/L) in patients with acromegaly. Mean IGFBP-3 levels were 3.5 mg/L (range: 2.1 - 4.8 mg/L) in controls and 5.4 mg/L (range: 4.2 - 6.6 mg/L) in patients with acromegaly. Mean IGF-I/IGFBP-3 ratios were 6.5 nmol/mg (range: 1.9 - 14.5 nmol/mg) in the healthy adults and 22.0 nmol/mg (range: 14.3 - 32.7 nmol/mg) in patients with acromegaly.

Table 1 Characteristics and biochemical parameters of 30 patients with acromegaly

Patient number	Sex	Age (years)	Mean 24-h GH (mU/L)	Basal IGF-I (nmol/L)	Basal IGBP-3 (mg/L)	IGF-I/IGFBP-3 (mmol/mg)
1	f	39	157	134.7	5.3	25.4
2	f	43	51	147.3	5.6	26.3
3	f	65	50	107.0	6.0	17.8
4	f	37	32	206.0	6.4	32.2
5	f	68	56	178.0	5.7	31.2
6	f	59	44	87.0	5.4	16.1
7	f	51	55	175.0	6.5	26.9
8	f	59	176	159.7	6.1	26.2
9	f	58	48	129.7	6.4	20.3
10	f	77	14	67.7	4.6	14.7
11	f	40	108	121.6	5.3	22.9
12	f	58	30	83.1	4.7	17.7
13	f	33	34	108.0	5.8	18.6
14	f	47	56	101.7	4.4	23.1
15	m	73	98	137.3	4.2	32.7
16	m	55	52	93.7	4.6	20.4
17	m	70	82	98.0	4.7	20.9
18	m	59	36	126.6	5.1	24.8
19	m	54	84	97.0	5.7	17.0
20	m	47	42	91.8	5.2	17.7
21	m	46	26	101.9	4.5	22.6
22	m	49	22	103.6	4.8	21.6
23	m	40	17	111.6	5.7	19.6
24	m	51	71	117.5	5.9	19.9
25	m	41	325	142.6	6.6	21.6
26	m	29	222	140.7	6.0	23.5
27	m	55	16	71.7	5.0	14.3
28	m	52	39	94.5	5.0	18.9
29	m	30	95	101.9	4.9	20.8
30	m	34	41	123.4	5.2	23.7
Mean		50.6	74	118.7	5.4	22.0
Range		29-77	14-325	67.7-	4.2-6.6	14.3-32.7

Table 2 General characteristics and biochemical parameters of 30 healthy controls

	Age (years)	Basal IGF-I (nmol/L)	Basal IGFBP-3 (mg/L)	IGF-I/IGFBP-3 (mmol/mg)
	56.6	22.0	3.5	6.5
	53-61	6.5-38.4	2.1-4.8	1.9-14.5

In Figures 1a, b and c, the IGF-I and IGFBP-3 levels and the IGF-I/IGFBP-3 ratios in the individual healthy adults and patients with acromegaly are shown. These figures show that there was a considerable overlap for IGFBP-3 levels between normals and acromegalics, but not for IGF-I levels. Also, the IGF-I/IGFBP-3 ratio showed overlap between normals and patients with acromegaly.

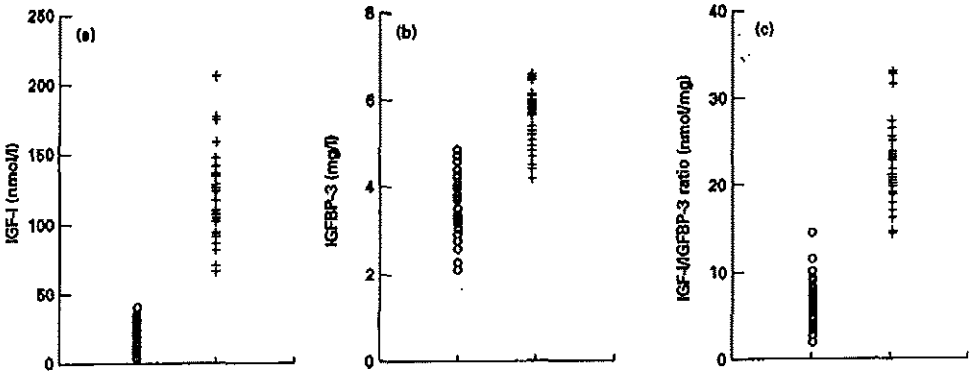

Figure 1 a, IGF-I; b, IGFBP-3 and c, IGF-I/IGFBP-3 ratio in o, 30 healthy adults and +, 30 patients with acromegaly

Figures 2a and b show the relationships between IGFBP-3 and mean 24-hour GH levels and between IGFBP-3 and IGF-I in acromegalics. There was a significant correlation between the mean 24-hour GH and IGFBP-3 levels ($p=0.036$) and between IGF-I and IGFBP-3 levels ($p<0.002$) in these patients.

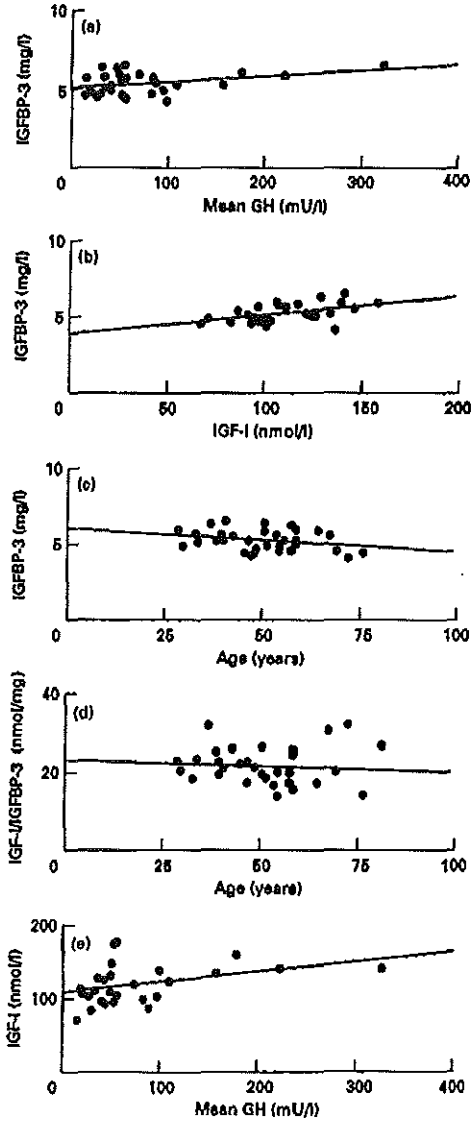

Figure 2 Relationships between IGFBP-3 and mean 24 h GH levels (Figure 2a), between IGFBP-3 and IGF-I levels (Figure 2b), between IGFBP-3 levels and age (Figure 2c), between the IGF-I/IGFBP-3 ratio and age (Figure 2d) and between IGF-I and mean 24 h GH levels (Figure 2e) in 30 patients with acromegaly. To convert GH values from μg/L to mU/l multiply by 2.0.

Figures 2c and d show the relationships between age and IGFBP-3 and the IGF-I/IGFBP-3 ratio in acromegalics. The figures show that the IGFBP-3 levels decrease with increasing age, but the IGF-I/IGFBP-3 ratio does not change significantly with age in these patients. Figure 2e shows the relationship between IGF-I and mean 24 h GH levels in our patient group. The correlation between the mean 24 h GH and IGF-I levels was not statistically significant ($p = 0.089$).

Discussion

Using a specific IGFBP-3 Radioimmunoassay (RIA), Grinspoon et al. recently demonstrated that IGFBP-3 is a sensitive physiological marker of somatotroph hyperplasia, because there was no overlap between the IGFBP-3 levels in 18 patients with untreated acromegaly and age-comparable controls or the age-adjusted normative range (12). Even though the mean age in our control group was slightly higher than in our acromegaly patients, there was a considerable overlap of IGFBP-3 but not of IGF-I levels between the patients and normals. In normal healthy adults, IGFBP-3 levels decrease with increasing age, which is in line with a decline in GH secretion (6, 11, 13). Therefore, our data suggest that IGFBP-3 has no additional value over IGF-I estimations for the assessment of GH excess in adults.

Elevated serum IGF-I and IGFBP-3 levels normalize after successfull therapy in acromegaly (8). In acromegaly, serum IGF-II levels are within the normal range (6-8). As IGFBP-3 binds both IGF-II and IGF-I, the ratio between (IGF-I (+ IGF-II)) and IGFBP-3 has been proposed as an index of free, biologically active IGF-I (7, 8). Juul et al. have recently shown that the IGF-I/IGFBP-3 ratio is significantly higher in acromegalics than in controls (7). They could also demonstrate a significant correlation between this ratio and urinary GH secretion or serum GH levels. However, Jorgensen et al. observed that the (IGF-I + IGF-II)/IGFBP-3 ratio was similar when comparing active and succesfully treated acromegaly and control subjects (8). In our study, the IGF-I/IGFBP-3 ratio in patients with acromegaly was generally higher than in controls. However, as compared to the individual IGF-I levels, we could not demonstrate an additional discriminitative value of this ratio for

the diagnosis of acromegaly.

In acromegalic patients, IGF-I levels tend to decrease with aging, which is in line with a decline in GH secretion (14). We could also demonstrate a decrease in serum IGFBP-3 levels with aging in these patients. In normal adults, the IGF-I/IGFBP-3 ratio decreases with increasing age (7). We could not demonstrate a decrease in the IGF-I/IGFBP-3 ratio with increasing age in our group of acromegalic patients. This might be explained by an in parallel decrement of IGF-I and IGFBP-3 in these patients.

We have found a significant correlation between the mean 24 h GH levels and IGFBP-3 and between the IGF-I and IGFBP-3 levels in patients with untreated acromegaly. In our study, all patients presented with elevated IGF-I levels.

Grinspoon et al. found a correlation only between IGF-I and IGFBP-3, but not between random GH levels and IGFBP-3 in their group of acromegalic patients (12). Their series included 2 out of 18 patients with clinical acromegaly and elevated IGFBP-3 levels, but normal IGF-I levels and GH suppression to 2 μg/L or less with glucose. These authors concluded that GH stimulation of IGFBP-3 is at least partially mediated through its effects on IGF-I (12).

In conclusion, our data suggest that IGFBP-3 alone, or in a IGF-I/IGFBP-3 ratio has no additional discriminitative value over IGF-I measurements for the assessment of clinical activity in acromegaly. IGFBP-3 levels, like IGF-I levels tend to decrease with increasing age in acromegaly. In acromegaly, IGFBP-3 levels significantly correlate with mean 24 h GH levels and IGF-I levels.

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§ 4.3 Acromegaly: the significance of serum total and free IGF-I and IGFBP-3 in the diagnosis.

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Summary

We have studied the physiological and clinical relevance of measurements of serum total and free IGF-I, and IGFBP-3 in 57 previously untreated patients with active acromegaly (32 males, 25 females; mean age 47 years) as compared to sex and age-matched normal healthy controls. Serum total and free IGF-I, but not IGFBP-3 are suitable biochemical parameters for screening for acromegaly. In acromegalics, the mean 24-hour serum GH, total IGF-I and IGFBP-3 levels tend to decrease with aging. However, in our series of patients, mean 24-hour serum GH levels, IGFBP-3, total and free IGF-I do not correlate with disease activity in acromegaly.

Introduction

In normal healthy adults, spontaneous GH secretion shows a diurnal pattern and is influenced by age and sex. More than 99% of growth hormone (GH) is secreted in a pulsatile fashion. In patients with active acromegaly, only 50% of GH secretion is excreted in a pulsatile manner, but the GH pulse frequency is increased as compared to normal healthy adults (1). Because of the variability in GH secretion over the day and the short half-life of GH (approximately 20 min.) in the circulation, random GH measurements have only limited value for the differential diagnosis of acromegaly. Generally, "undetectable" GH levels measured with a standard commercially available radioimmunoassay (RIA) or immunoradiometric assay (IRMA) in a patient suspected of having acromegaly exclude the diagnosis. However, it should be noted that the actual GH level in this situation may not really be zero, but below the sensitivity level of the assay. In contrast, spontaneous, exercise-induced, or postprandial GH pulses during the day may occasionally result in an abnormally high random GH determination in a normal subject. Using 24-hour GH levels, a good separation can be made between patients with active acromegaly and normal subjects, but only after matching for age (2). In normal subjects, serum GH levels (measured with a commercially available RIA) are suppressed to < 2 μg/L (< 4 mU/L) within 1-2 hours following the administration of an oral load of 75-100 g glucose after overnight fasting. However, in acromegaly there is a failure to suppress GH levels to < 2 μg/L (< 4 mU/l) during oral glucose tolerance testing (OGTT) or even shows a paradoxical rise (3, 4).

Insulin-like growth factor I (IGF-I), formerly known as somatomedin-C, mediates many of the growth hormone actions. GH induces IGF-I synthesis and release, especially from the liver. In contrast to serum GH levels, serum (total) IGF-I levels show little fluctuation during the course of the day. Serum (total) IGF-I levels are invariably elevated in active acromegaly (5). Pregnancy and late puberty are also associated with elevated serum (total) IGF-I levels. A highly significant correlation exists between serum (total) IGF-I levels and the area under the serum GH curve estimated during OGTT ($r = 0.66$) (6). A positive correlation has also been demonstrated between the mean 24-hour serum GH levels and serum (total) IGF-I

levels. The relationship between mean serum GH levels during either OGTT, or in 24-hour profile and serum (total) IGF-I levels shows a curvilinear pattern. A linear relationship exists between GH levels < 10 μg/L (< 20 mU/L) and serum (total) IGF-I. Serum (total) IGF-I levels reach their maximum at serum GH levels > 20 μg/L (> 40 mU/L) (6, 7).

The majority of IGF-I (and IGF-II) in the serum are complexed with binding proteins (IGFBPs). In the circulation, about 75% of the IGFs are complexed with IGFBP-3 and an acid-labile subunit (ALS) in a 150-200 kDa ternary complex. The remainder of the circulating IGFs is either bound to lower molecular mass IGFBPs or circulate in the free form. IGFBP-3 appears to be the primary regulator of IGF bioavailability in response to changes in circulating GH levels (8). The ternary complex does not cross the vascular endothelium and, therefore, serves as a reservoir for IGFs. When associated with the ternary complex, the half-life of the IGFs is prolonged.

Breakdown of the IGFBPs is regulated by IGFBP proteases. An increase in IGFBP-3 proteolysis will lead to increased formation of lower molecular mass IGF-IGFBP complexes, which are capable of crossing the endothelium. Elevated IGFBP-3 levels have been found in patients with active acromegaly (9, 10). The 28 kDa IGFBP-1 appears to be the primary regulator of IGF bioavailability in response to the changes in the circulating insulin level (8). Serum levels of IGFBP-5, but not IGFBP-4, show significant positive correlation's with the IGFs. IGFBP-1 and IGFBP-4 in serum have predominant IGF-I inhibitory activities, whereas IGFBP-5 in serum has IGF-I stimulatory activities (11).

By analogy with steroid and thyroid hormones, the unbound fraction may be the dominating fraction for the biological activity of IGF-I. Frystyk et al have shown that fasting serum free IGF-I levels are increased in acromegaly and showed no overlap with levels found in healthy controls (12).

We have studied the physiological and clinical relevance of measurements of serum total and free IGF-I and IGFBP-3 in acromegaly as compared to normal healthy adults.

Patients and Methods

Fifty-seven previously untreated patients (32 males, 25 females; mean age 47 years) with active acromegaly were studied. Disease activity was scored according to 5 clinical parameters: excessive sweating, headaches, fatigue, paresthesias and arthralgias (see Table 1). Fifty-seven sex and age-matched adults were studied as controls.

Table 1 Clinical activity score in acromegaly. Total score: <3, mild; 3-5, moderate; >5, severe

Score	Headaches	Fatigue	Perspiration	Paresthesias	Arthralgia
0	None	None	None	None	None
1	Mild	Mild	Mild	Mild	Mild
2	Moderate	Moderate	Moderate	Moderate	Moderate
3	Severe	Severe	Severe	Severe/CTS	Severe

CTS, carpal tunnel syndrome

In patients with acromegaly, serum GH was sampled from 0800 to 1800 h at hourly intervals, followed by 2-hourly sampling to 0600 h. A final sample was taken at 0700 h. Fasting samples for measurements of IGF-I, IGFBP-3 and free IGF-I were taken from acromegalics and healthy controls. Serum GH was measured by IRMA supplied by CIS Bio International, Gif-sur-Yvette Cedex, France (intraassay CV, 2.8%, interassay CV, 4.4%). Serum total IGF-I was determined by RIA using kits obtained by Medgenix Diagnostics, Fleurus, Belgium (intraassay CV, 6.1%, interassay CV, 9.9%). Serum free IGF-I was measured by IRMA supplied by Diagnostic Service Laboratories, Webster, Texas (intraassay CV 10.3%, interassay CV, 10.7%). Serum IGFBP-3 levels were determined by IRMA supplied by Diagnostic Service Laboratories, Webster, Texas (intraassay CV, 3.9%, interassay CV, 1.9%).

Results

In figures 1a, b and c, serum total IGF-I, free IGF-I and IGFBP-3 levels in normal healthy controls and patients with active acromegaly are shown. The figures show that there was a considerable overlap for serum IGFBP-3 levels between normals and acromegalics, but not for serum total and free IGF-I. Figures 2a, b, c and d show the relations between age and mean 24-hour serum GH, total IGF-I, free IGF-I and IGFBP-3 levels in patients with active acromegaly. Mean 24-hour serum GH, serum total IGF-I and IGFBP-3 levels decrease with increasing age. However, there is no relation between serum free IGF-I levels and age in acromegaly.

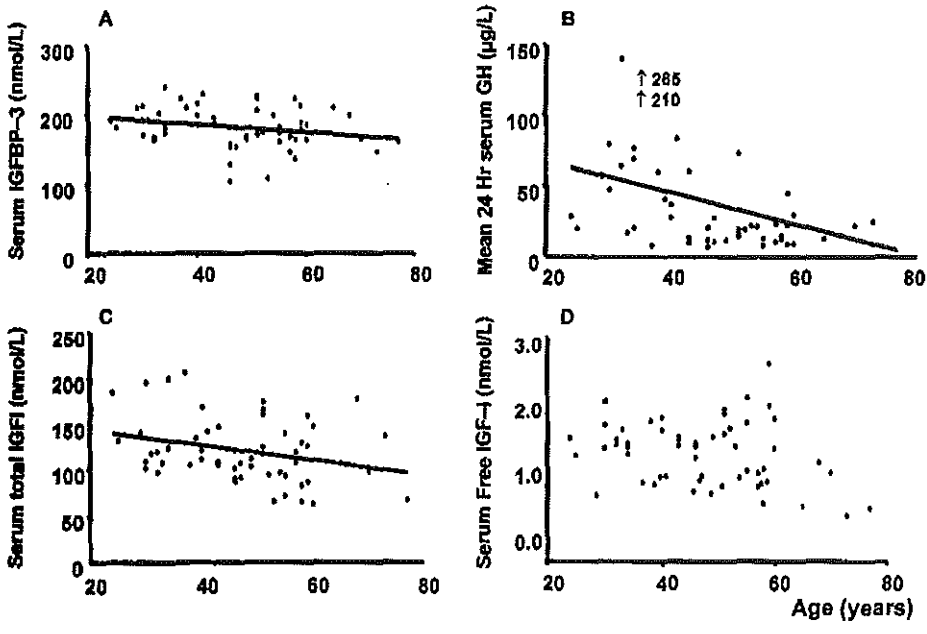

Figure 2 Relations between (A) serum IGFBP-3 and age ($r=-0.25$, $p=0.02$), (B) mean 24 h serum GH and age ($r=-0.29$, $p=0.002$), (C) serum total IGF-I and age ($r=-0.30$, $p=0.02$) and (D) serum free IGF-I and age (not significant) in 57 patients with active acromegaly, (non-parametric correlation (Spearman). Statistical significance was defined as $p<0.05$.

In Table 2, the correlations between mean 24-hour serum GH levels and serum total IGF-I, free IGF-I and IGFBP-3 are shown. There was a significant correlation between mean 24-hour serum GH levels and serum total IGF-I levels ($r = 0.38$, $p = 0.005$, non-parametric correlation (Spearman)). There was no significant correlation between mean 24-hour serum GH and serum free IGF-I, or serum IGFBP-3 levels. There was a significant correlation between serum total IGF-I and serum IGFBP-3 levels ($r = 0.55$, $p < 0.001$, non-parametric correlation (Spearman)), but not between serum total and free IGF-I levels. Also, no significant correlation was found between serum free IGF-I and IGFBP-3 levels.

Table 2 Correlations between the various clinical and biochemical parameters in 57 patients with ac acromegaly (non-parametric correlation (Spearman)). Statistical significance was defined as $p < 0.05$.(NS, significant)

	Total IGF-I	IGFBP-3	Free IGF-I	Disease activity	Mean 24 h GI
Age	-0.28 (p=0.04)	-0.28 (p=0.04)	-0.13 (NS)	-0.46 (p<0.001)	-0.40 (p=0.00
Total IGF-I		0.55 (p<0.001)	0.12 (NS)	0.18 (NS)	0.38 (p=0.00
IGFBP-3			0.01 (NS)	0.06 (NS)	0.21 (NS)
Free IGF-I				0.01 (NS)	0.26 (NS)
Disease Activity					0.10 (NS)

Figures 3a, b, c and d show the correlation's between disease activity and mean 24-hour serum GH, free and total IGF-I, and IGFBP-3 levels. These figures show that mean 24-hour serum GH, free and total IGF-I, and IGFBP-3 levels did not correlate with disease activity.

Discussion

By comparing a large series of patients with untreated acromegaly with sex and age-matched controls, the present study has shown a considerable overlap of serum IGFBP-3 levels, but not of free and total IGF-I levels between the controls and acromegalics. This is in line with our previous findings in a smaller group of patients with acromegaly (10).

Therefore, IGFBP-3 is not a marker for the assessment of GH excess in adults. Both serum free and total IGF-I measurements can serve better for this purpose.

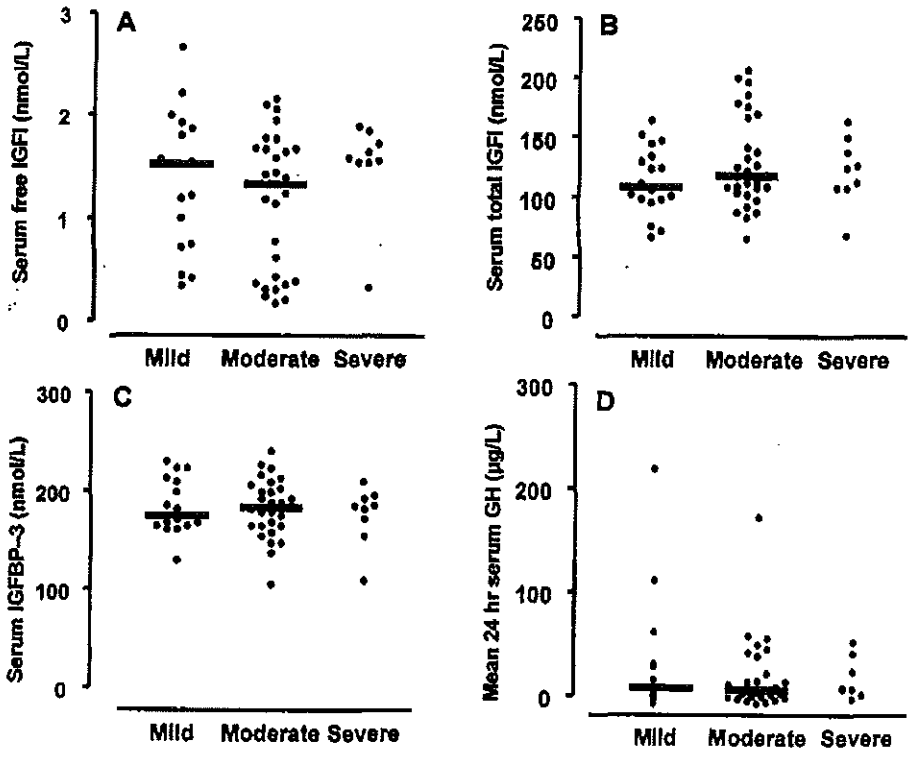

Figure 3. Relations between (A), serum free IGF-I and clinical activity (not significant), (B) serum total IGF-I and clinical activity (non significant), (C) serum IGFBP-3 and clinical activity (non significant), (D) mean 24-hour serum GH and clinical activity (non significant) in 57 patients with active acromegaly (non-parametric correlation (Spearman)). Statistical significance was defined as $p < 0.05$.

Our results show that in acromegalics, mean 24-hour serum GH, total IGF-I and IGFBP-3 levels tend to decrease with aging. This is also in line with our previous observations (10, 13). We could not demonstrate a decrease in serum free IGF-I with age. This might be explained by a parallel decrement of total IGF-I and IGFBP-3. We have shown that mean 24-hour serum GH levels, IGFBP-3, total and free IGF-I does not correlate with disease activity in acromegaly.

Conclusions

- Serum total and free IGF-I, but not IGFBP-3 are suitable biochemical parameters for screening for acromegaly.
- In acromegalics, mean 24-hour serum GH, total IGF-I and IGFBP-3 levels tend to decrease with aging.
- Mean 24-hour serum GH, total and free IGF-I and IGFBP-3 levels do not correlate with disease activity in acromegaly.

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5

The IGF-I/IGFBP system in IDDM and congenital partial lipodystrophy

§ 5.1 Free and Total IGF-I, IGFBP-1 and IGFBP-3 and their relationships to the presence of diabetic retinopathy and glomerular hyperfiltration in insulin-dependent diabetes mellitus (IDDM).

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Summary

The existing literature on serum insulin-like growth factor I (IGF-I) levels in insulin dependent diabetes mellitus (IDDM) is conflicting. Free IGF-I may have greater physiological and clinical relevance than total IGF-I. Recently a validated method has been developed to measure free IGF-I levels in the circulation.

Serum free and total IGF-I, insulin-like growth factor binding protein-1 (IGFBP-1) and insulin-like growth factor binding protein-3 (IGFBP-3) levels were measured in 56 insulin-treated IDDM patients and 52 healthy sex- and age-matched controls. Diabetic retinopathy was established by direct fundoscopy. In 54 IDDM patients glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) were calculated from the clearance rate of ^{125}I-iothalamate and ^{131}I-. iodohippurate sodium.

Fasting free IGF-I, total IGF-I and IGFBP-3 levels were significantly lower in IDDM patients than in age- and sex-matched healthy controls (free IGF-I: $P<0.005$; total IGF-I: $P<0.001$; IGFBP-3: $P=0.001$), while IGFBP-1 levels were higher ($P<0.001$).

In IDDM subjects a decrease of free IGF-I, total IGF-I and IGFBP-3 levels with age was observed (free IGF-I: $r=-0.27$, $P=0.05$; total IGF-I: $r=-0.52$, $P<0.001$; IGFBP-3: $r=-0.37$ $P=0.005$). Free IGF-I was inversely related to fasting glucose in IDDM subjects ($r=-0.35$, $P=0.01$) whereas the relationhip between total IGF-I and fasting glucose did not reach significance ($r=-0.27$, $P=0.06$).

Age-adjusted free IGF-I levels were significantly higher ($P<0.05$) in IDDM subjects with retinopathy than in subjects without retinopathy after adjustment for age.

Total IGF-I and IGFBP-3 levels were positively related to GFR (Total IGF-I: $r=0.35$ and $P<0.05$; IGFBP-3: $r=0.28$ and $P<0.05$). Both of these differences lost significance after adjustment for age.

Free IGF-I, total IGF-I levels and IGFBP-3 levels were lower and IGFBP-1 levels were higher in insulin-treated IDDM subjects when compared to age- and sex-matched controls. Free IGF-I, total IGF-I and IGFBP-3 levels decreased significantly with age in IDDM subjects. Age-adjusted free IGF-I levels in subjects with diabetic retinopathy were higher than those in subjects without diabetic retinopathy. Total IGF-I and IGFBP-3 levels were positively related to GFR in IDDM subjects, but

these relations were lost after adjustment for age. Measurement of free IGF-I levels in IDDM subjects did not have clear advantages compared that of total IGF-I, IGFBP-1, and IGFBP-3 levels. Serum IGF-I and IGFBPs reflect their tissue concentrations to a various degree. Consequently, extrapolations concerning the pathogenetic role of the IGF-I/IGFBP system in the development of diabetic complications at the tissue level remain speculative

Introduction

The literature on insulin-like growth factor-I (IGF-I) in patients with diabetes is conflicting. Flyvbjerg reported that in IDDM patients the serum levels of IGF-I were within the normal range for the entire population (age 20-80 years) compared with age- and sex-matched controls (1). Therefore, Lamberton et al. have concluded that serum IGF-I, measured as total IGF-I levels, probably do not play an important role in the pathogenesis of diabetic microvascular disease (2). On the other hand Merimee et al. have reported a transient rise in total serum IGF-I levels in patients with diabetes mellitus in the early phase of neovascularisation of the retina (3). These findings could not be confirmed by Hyer et al. (4) or Wang et al. (5).
Blood-born IGF-I may also contribute to the glomerular hyperfiltration (GFR) observed in early diabetic nephropathy (6). However, circulating levels of total IGF-I do not closely correlate with renal hypertrophy, and there is a lack of correlation between kidney size and the progressive decline in GFR in diabetic patients, suggesting that factors which are important for growth might not necessarily be involved in renal hemodynamics (7, 8).

Part of the controversy on serum IGF-I levels in IDDM may be explained by methodological problems in the IGF-I assay. Until recently the assay methods used to measure IGF-I in serum were not able to distinguish among unbound, free IGF-I, and IGF-I bound to insulin-like growth factor binding proteins (IGFBPs) (9). IGFBP-3 appears to be the primary regulator of IGF levels in response to changes in circulating GH levels and serves as a storage pool for IGF-I (10) whereas IGFBP-1 appears to be the primary regulator of IGF-I levels in response to changes in circulating insulin levels (11).

Determination of free IGF-I in stead of total IGF-I might be an important step to get more insight in the concentrations and the role of the IGF-I/IGFBP system in IDDM (7). Free IGF-I, by analogy with sex and adrenal steroids and thyroid hormones, may be the major biological active hormonal form of IGF-I(12).

Here we report the results of a study, in which we investigated the relationships of circulating free IGF-I, total IGF-I, IGFBP-1 and IGFBP-3 levels and with the presence of diabetic retinopathy and renal hemodynamics in patients with IDDM.

Subjects and Methods

Subjects

The study group consisted of 56 consecutive patients with IDDM which visited our outpatient-clinic. Inclusion criteria were: IDDM, age18 yr or above at the time of the study, age at diagnosis below 40 years and insulin dependency within 6 months of diagnosis, no other medication than insulin, glycosylated hemoglobin ($HbA1_c$) less than 11 %, normal serum creatinine (50-110 μmol/l) and no overt nephropathy (albuminuria less than 300 mg/24 h in two out of three measurements). About half of these patients were part on a previous study in which the dynamics of GH secretion were studied (13). All patients were on an insulin-dosing schedule of 2-4 times daily, using combinations of short-acting and intermediate long-acting insulin. Fifty-two, age- and sex-matched, controls without evidence of disease were recruited from the normal urban population and staff members of our hospital to measure free and total IGF-I levels. The study was approved by the hospital ethical review committee and all patients and volunteers gave their informed consent.

Methods

Blood sampling

Blood was collected between 0800-0900 h in the morning after an overnight fast in both IDDM patients as well as in the control group. The last insulin injection on the day before blood sampling was adminstered at 2200 h by IDDM subjects on intensive insulin therapy (4 daily injections, injection of an intermediate long-acting insulin at 2200 h) and at 1800 h by IDDM subjects on conventional therapy (2 daily injections of a mixture of short and medium acting insulins).

Hemodynamic and renal function tests

In 54 of 56 subjects with IDDM the renal and hemodynamic function tests were performed. The glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) were calculated from the clearance of ^{125}I-iothalamate and ^{131}I-iodohippurate sodium, respectively, as described in detail previously (14).

Blood glucose measurements were performed every 30 min. Glucose (50 mg/kg per hour) and insulin (daily dose in U divided by 100/hour) was given iv.
To maintain the blood glucose concentration between 6.0-10 mmol/L the rate of glucose infusion was adjusted according to the blood glucose levels. The mean GFR in a group of normal persons (n=18,10 men and 8 women, mean age 39 yr; range, 25-61) yr) was 107 (range, 88-130) ml/min.1.73 m^2. A GFR greater than 130 ml/min.1.73 m^2 indicated hyperfiltration.
Blood pressure was measured every 5 min during a 60-min period in the supine position using an automatic device (Datascope, Accutor 1a, Parasmus, NJ). The mean of at least six values between 30-60 min was calculated.

Analytical methods

The free IGF-I immunoradiometric assay (Diagnostics System Laboratories Inc, Webster, TX) used in the present study needs no sample extraction. The assay has been previously described (15, 16). In short, serum samples (100 μl) or recombinant human IGF-I standards are added to tubes containing a dense coating of the primary high-affinity IGF-I antibody, incubated at 2 h at room temperature. No residual IGFBP-1 or IGFBP-3 is detectable after the first wash according to the manufacturer 's data (15).The tubes were washed, and incubated for 2 h at room temperature with the secondary radiolabeled antibody, washed three times with deionized water and counted. The lower limit of detection is 4 pmol/L. There cross-reactivity with IGF-II is less than 0.01 %. The intra-assay and inter-assay coefficients of variation (CVs) for the free IGF-I assay are 10.3 and 10.7 %, respectively at a plasma level of 0.01 nmol/L (n=8).
Total IGF-I was determined by a commercially available RIA (Medgenix Diagnostics, Brussels, Belgium, intra-assay and interassay CVs: 6.1% and 9.9%). Immunoradiometric assays were used for measurement of IGFBP-1 and IGFBP-3 (Diagnostics System Laboratories Inc, Webster; intra-assay and inter-assay CVs for IGFBP-1 5.2 and 6.0 %; intra-assay and inter-assay CVs for IGFBP-3: 0.56 and 1.9 %). HbA_{1c} was measured by HPLC (normal values: 5.0-6.3 %) (Variant HPLC-

Biorad, Veenendaal, The Netherlands), and blood glucose concentrations were measured in venous whole blood by an automatic hexokinase method (Boehringer Mannheim Gmbh, Mannheim, Germany). Urinary albumin was measured with a radial immunodiffusion on agarose gel that contained 0.4 μL anti-albumin antiserum (Dako A 001, Dakopatts, Copenhagen, Denmark) (17). The lower limit of detection was 2 mg/L and the interassay variability 8%. The means of the results of three collection periods are presented.

Diabetic retinopathy

Diabetic retinopathy (DRP) was established by direct funduscopy as judged by an experienced opthalmologist and was graded as no DRP, background DRP, or proliferative DRP (18).

Statistical Analysis.

Baseline clinical characteristics are presented as mean and range. Free IGF-I, total IGF-I, IGFBP-1, IGFBP-3, and urinary albumin excretion have a log normal distribution and are therefore presented as the geometric mean. IDDM subjects were compared with healthy controls. For the healthy control group we did not match on individual basis but performed frequency matching, id est selecting our controls in such a way that age- and sex distribution were similar in cases and controls. For diabetic subgroups mean and 95 % confidence intervals are presented. Baseline differences between variables were analyzed with one way ANOVA. Pearson 's partial correlation coefficients were calculated to analyze the associations between variables and to test significance. Free IGF-I, total IGF-I, IGFBP-1, and IGFBP-3 did not met the criteria for normality and were logarithmic transformed before analysis. After this transformation, a normalization of the distribution was achieved. The results presented in Table 3 were adjusted for age by using linear regression analysis with IGF- I(etc.) as dependent and age and retinopathy (yes/no) as independent variables.The results presented in Table 4 were adjusted for age by using linear regression analysis with IGF-I(etc.) as dependent and age and hyperfiltration (yes/no) as independent variables. A two

sided P-value of <0.05 was considered significant. All statistical analyses were performed with Stata statistical package (Computing Resource Center, Santa Monica, Ca).

Results

General characteristics

The baseline clinical characteristics of 56 IDDM subjects (23 women, 33 men) are presented in Table 1. In the 52 sex- and age-matched controls the female/male ratio was 21/31 and not significantly different from the IDDM patients; The mean age in the control group was 34.5 yrs (SD 13.8, range 18-64) and did not differ from the IDDM group. 22 out of the 54 IDDM patients had a GFR above 130 mL/min/1.73 m^2 and were classified as hyperfiltrators.

Serum free and total IGF-I levels in the IDDM patients and control subjects are shown in Figure 1 and Table 2. Serum free and total IGF-I levels were significantly lower in the IDDM patients than in the control group. The ratio between free IGF-I and total IGF-I levels was lower but not statistically different between IDDM patients and healthy controls. Serum IGFBP-3 levels were significantly lower and IGFBP-1 significantly higher in the IDDM patients than in the control group (Fig 1 and Table 2).

Free IGF-I serum levels were positively related to total IGF-I concentration (IDDM: r=0.52 and P<0.001; control group: r=0.47 and P<0.001) and IGFBP-3 (IDDM: r=0.33 and P<0.05; control group: r=0.19 and P=0.17) and inversely related to IGFBP-1 concentration (IDDM: r=-0.40 and P<0.005; control group: r=-0.47 and P<0.001).The plasma concentration of total IGF-I was positively related to IGFBP-3 (IDDM: r=0.72 and P<0.001; control group: 0.47 and P<0.001) and inversely to IGFBP-1 levels (IDDM: r=-0.42 and P<0.005; control group: r=-0.56 and P<0.001).

Table 1. Clinical characteristics of patients with insulin-dependent diabetes mellitus

	Mean	Range
Age (yrs)	36.3	(18-63)
BMI (kg/m^2)	23.8	(16.1-37.9)
Duration Diabetes (yrs)	17.6	(3-44)
Fasting glucose (mmol/L)	10.5	(3.2-19.6)
HbA1c (%)	8.7	(5.4-10.5)
Serum creatinine (μmol/L)	79	(50-119)
Albuminuria* (mg/24 hr)	12	(2-167)
Systolic blood pressure (mm Hg)	130	(100-197)
Diastolic blood pressure (mm Hg)	75	(60-101)
Insulin dose (IU/day)	53	(30-78)
GFR (mL/min/1.73 m^2)	126	(59-206)
ERPF (mL/min/1.73 m^2)	554	(298-917)
Retinopathy (number of patients)		
None	29	
Background	13	
Proliferative	14	

* Results are presented as arrythmic mean and range between brackets; albumin is expressed as geometric mean and range
BMI: body mass index was defined as weight divided by the square of height (kg/m^2);
GFR: glomerular filtration rate; ERPF: effective renal plasma flow

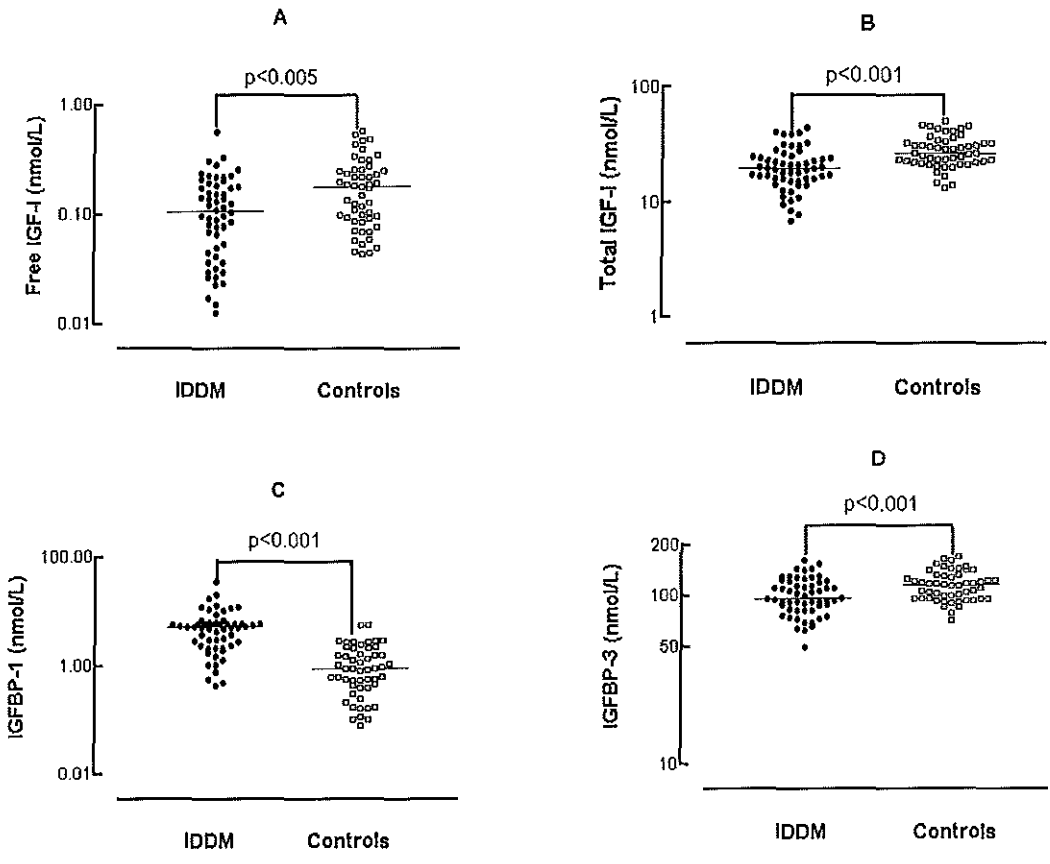

Fig 1A-D

Free (A) and total IGF-I(B), IGFBP-1(C), and IGFBP-3 (D) levels in IDDM subjects (left) and healthy controls (right). Note the logarithmical scale of the vertical axes. The bar represents the median value in each group.

Table 2. Comparison of fasting total and free IGF-I, IGFBP-1, and IGFBP-3 levels between IDDM patients and (age- and sex-matched) healthy controls

	IDDM		Controls		P-value[a]
	Mean	Range	Mean	Range	
Free IGF-I (nmol/L)	0.09	(0.01-0.57)	0.15	(0.04-0.58)	**<0.005**
Total IGF-I (nmol/L)	19.4	(6.9-44.3)	27.6	(13.3-51.4)	**<0.001**
Free IGF-I over Total IGF-I ratio x 100%	0.59	(0.11-1.99)	0.67	(0.13-2.37)	ns
IGFBP-1 (nmol/L)	4.1	(0.4-35.3)	0.78	(0.08-5.46)	<0.001
IGFBP-3 (nmol/L)	99.0	(50.2-163.3)	114.6	(79.6-170.7)	=0.001

Results are expressed as geometric mean and range between brackets. The results of the free IGF-I/total IGF-I are presented as the mean.
[a] By Anova

Both free IGF-I, as well as IGFBP-3 levels decreased significantly with age in diabetic patients, whereas IGFBP-1 levels did not change with age (Fig 2, A and C). In the normal individuals such a decrease in free IGF-I and IGFBP-3 with age was statistically not significant (Fig 2, A and C), while total IGF-I levels were inversely related with age in both IDDM and healthy controls (Fig. 2 B). Free IGF-1 and IGFBP-3 levels tended to be disproportionately lower in IDDM than healthy controls with increasing age (Fig 2, A and C).

Free IGF-I was inversely related to fasting glucose in IDDM subjects, but not to HbA_{1c} (Fig 3). The relation between total IGF-I and fasting glucose did not reach significance(r=-0.27, P=0.06) and fasting IGFBP-1 levels were positively related to fasting glucose levels in IDDM subjects (Fig 3). Fasting glucose levels were positively related to HbA_{1c} (r=0.36, P=0.01).

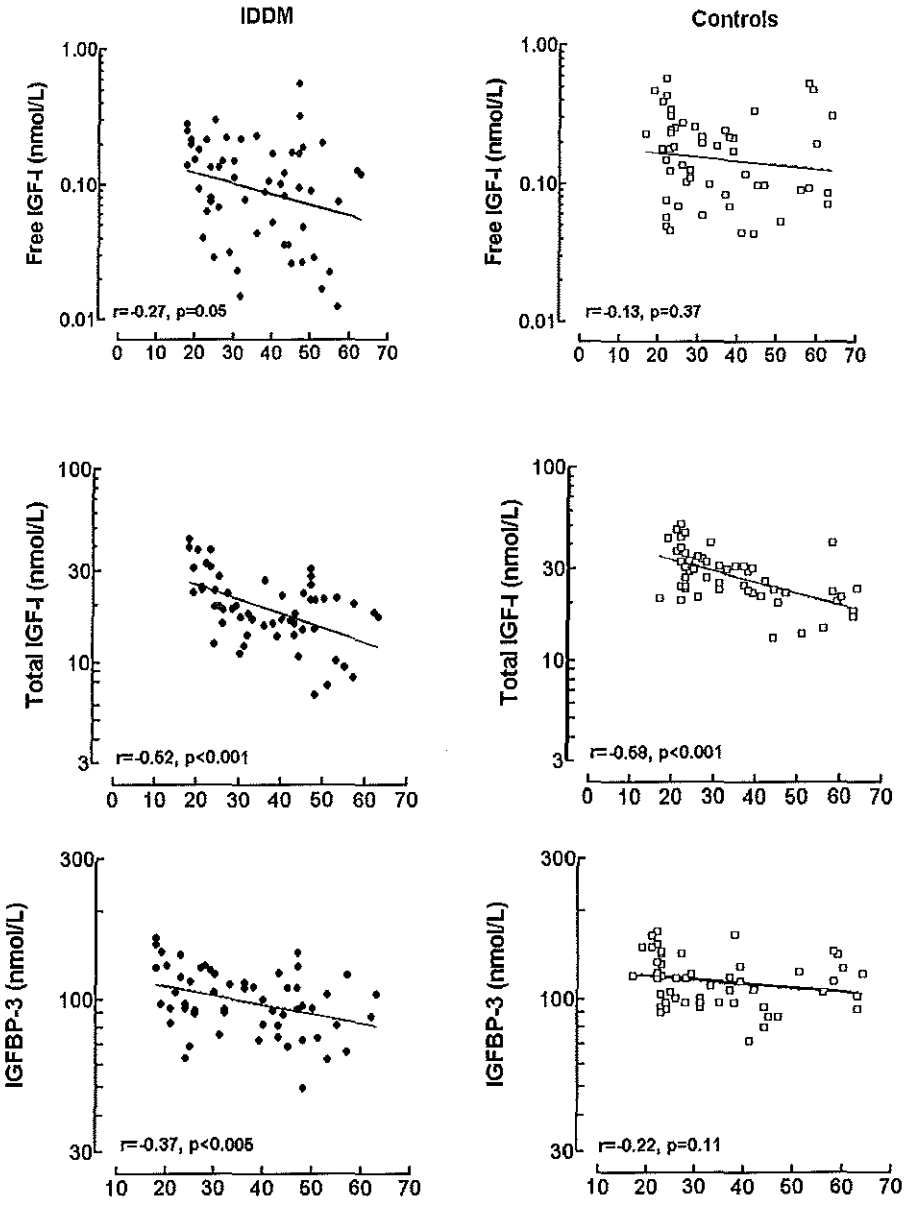

Figure 2 Relation between age and free IGF-I levels (A), total IGF-I levels (B), and IGFBP-3 levels (C) respectively, in healthy controls (top) and IDDM patients (bottom) matched for age- and sex. Note the logarithmical scale of the vertical axes.

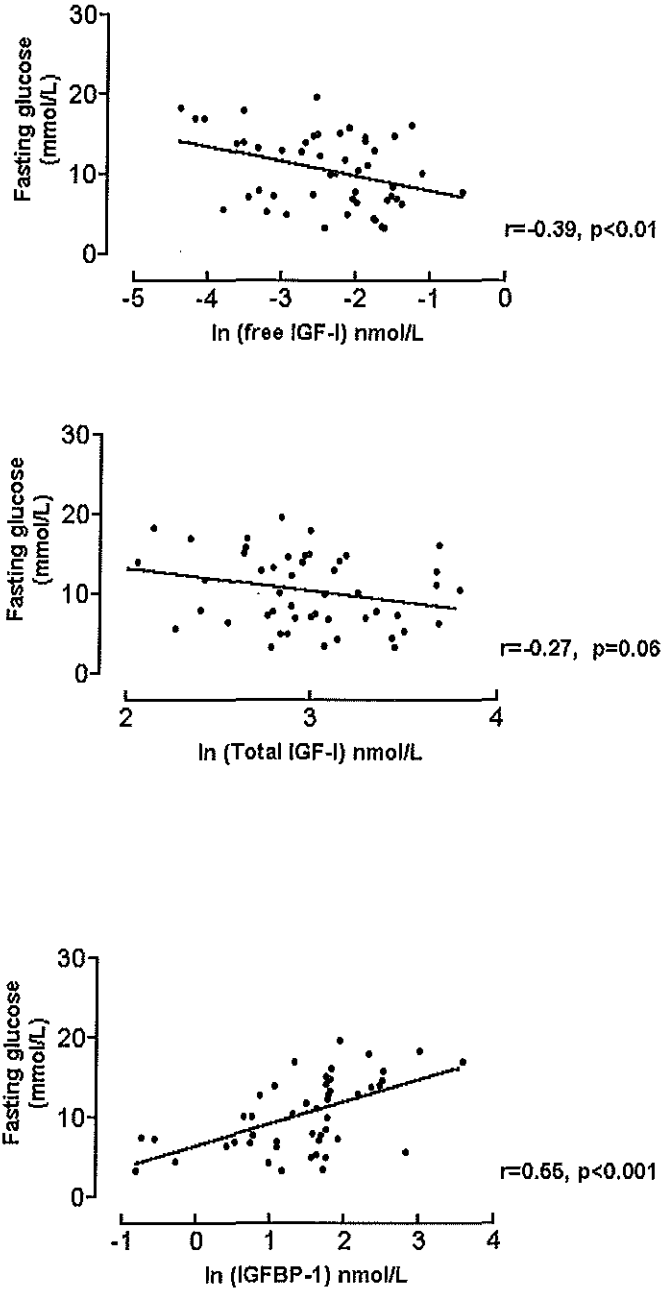

Figure 3

Relation between fasting glucose levels and free IGF-I (upper panel), total IGF-I(center panel) and IGFBP-1 levels (lower panel), respectively, in IDDM subjects after adjustment for age. Note the logarithmical scale of the horizontal axes.

Table 3. Serum free IGF-I levels and other clinical characteristics of diabetic patients with and without clinically manifest diabetic retinopathy

	No Retinopathy (N=29)		Retinopathy[a]		
	Mean	95 % CI	Mean	95 % CI	P-value[b]
Free IGF-I (nmol/L)[c]	0.09	(0.04-0.20)	0.10	(0.04-0.23)	ns
Total IGF-I (nmol/L)[c]	21.2	(10.0-45.1)	17.6	(8.3-37.3)	ns
IGFBP-1 (nmol/L)[c]	3.98	(2.85-5.57)	4.22	(2.86-6.22)	ns
IGFBP-3 (nmol/L)[c]	104.4	(94.8-115.1)	93.5	(84.6-103.3)	ns
Age (yr)	29.1	(25.3-33.0)	44.1	(39.7-48.5)	**<0.001**
Duration Diabetes (yr)	12.4	(10.4-14.5)	23.2	(19.4-27.0)	**<0.001**
Systolic blood pressure (mm Hg)	125	(120-130)	134	(127-143)	**<0.05**
Diastolic blood pressure (mm Hg)	73	(69-77)	77	(73-81)	ns
Serum creatinine (μmol/L)	75	(70-80)	83	(74-91)	ns
GFR (mL/min/1.73 m^2)	140	(131-148)	111	(102-121)	**<0.001**
ERPF (mL/min/1.73 m^2)	594	(557-631)	514	(473-555)	**<0.005**
Albuminuria (mg/24hr)[c]	10	(4-23)	16	(6-41)	ns
Fasting Glucose (mmol/L)	10.67	(8.87-12.48)	10.27	(8.24-12.29)	ns
Hemoglobin A_{1c} (%)	8.77	(8.21-9.33)	8.57	(8.07-9.06)	ns
Insulin dose (IU/day)	55	(50-60)	52	(46-58)	ns
BMI (kg/m^2)	24.0	(21.9-26.2)	24.5	(22.7-26.3)	ns

For abbreviations, see Table 1

[a] n=27; 13 background and 14 proliferative

[b] By Anova, comparing subjects with and without retinopathy, $P<0.05$ was considered statistically significant

[c] Geometric mean

Table 4. Serum free IGF-I levels and other clinical characteristics of diabetic patients with glomerular hyperfiltration (GFR >130 ml/min/1.73 m^2) vs subjects without glomerular hyperfiltration (GFR<130 mL/min/1.73 m^2).

	A. Subjects (N=32) without hyperfiltration		B. Subjects (N=22) with hyperfiltration		
	Mean	95 % CI	Mean	95 % CI	P-value[a]
Free IGF-I (nmol/L)[b]	0.08	(0.03-0.18)	0.11	(0.05-0.26)	ns
Total IGF-I(nmol/L)[b]	17.7	(8.4-37.4)	21.9	(10.2-46.8)	**0.06**
IGFBP-1 (nmol/L)[b]	5.04	(3.66-6.93)	3.51	(2.42-5.09)	ns
IGFBP-3 (nmol/L)[b]	95.5	(87.8-103.8)	103.8	(92.0-117.2)	ns
Serum creatinine (μmol/L)	83	(76-90)	73	(67-79)	**< 0.05**
ERPF (mL/min/1.73 m^2)	515	(481-550)	610	(567-653)	< 0.001
Albuminuria (mg/24hr)[b]	13	(5-32)	12	(5-29)	ns
Systolic blood pressure (mm Hg)	132	(124-139)	127	(123-131)	ns
Diastolic blood pressure (mm Hg)	75	(72-79)	74	(70-79)	ns
Age (yr)	41.1	(36.7-45.5)	30.0	(25.6-35.8)	**<0.005**
Duration Diabetes (yr)	19.6	(16.2-23.0)	15.0	(11.2-18.7)	ns
Fasting Glucose (mmol/L)	10.25	(8.43-12.07)	11.59	(9.79-13.39)	ns
Hemoglobin A_{1c} (%)	8.50	(8.00-8.99)	8.90	(8.33-9.48)	ns
Insulin dose (IU/day)	51	(46-56)	57	(51-62)	ns
BMI (kg/m^2)	23.9	(22.1-25.7)	24.8	(22.5-27.0)	ns

For abbreviations, see Table 1

[a] Anova comparing subjects with and without hyperfiltration, $p<0.05$ was considered statistically significant

[b] Geometric mean

IGF-I/IGFBP system in IDDM patients with retinopathy

The 27 IDDM patients with retinopathy were older than the 29 patients without retinopathy, the duration of diabetes was longer, blood pressure was higher, and the GFR and ERPF were lower (Table 3). Free and total IGF-I, IGFBP-1, IGFBP-3, HbA_{1c} and fasting glucose levels were not different between the 2 groups. When adjusted for age the patients with retinopathy tended to have higher free IGF-I levels (P=0.04).

Renal hemodynamics

Using 130 mL/min/1.73 m^2 as a cut-off, 22 patients had hyperfiltration and 32 had a normal GFR (Table 4). The patients with hyperfiltration were younger, and the blood pressure was not different between the two groups. In IDDM subjects, GFR decreased significantly with age (r=-0.53, P<0.001). Free IGF-I, IGFBP-1 and IGFBP-3 levels tended to be higher in the IDDM patients with hyperfiltration than in subjects with a glomerular filtration below 130 mL/min/1.73 m^2 (Table 4), but the differences were not significant.

Total IGF-I and IGFBP-3, but not free IGF-I levels were positively related to GFR. (r=0.35 and P<0.05, and r=0.28 and P<0.05, respectively). The relationships between total IGF-I and IGFBP-3 levels and GFR were lost after adjustment for age. After adjustment for fasting glucose IGFBP-1 levels were inversely related to GFR (r=-0.30, P<0.05). Total and free IGF-I levels were not related to ERPF.

Discussion

There are many contradictory studies on IGF-I in patients with diabetes and used methodologies are highly variable even between studies showing similar findings. Some studies have previously found low to lowered total IGF-I and IGFBP-3 levels and high fasting IGFBP-1 levels in IDDM, despite hypersecretion of GH (19-21). Our study shows that the fasting serum levels of total IGF-I (the sum of free IGF-I and IGF-I bound to an array of IGF-binding proteins) and IGFBP-3 are lower and IGFBP-1 levels are higher in insulin-treated IDDM patients than in an age- and sex-matched healthy control group. We observed also lower fasting free

IGF-I levels in adult IDDM patients, despite insulin treatment. It is likely that the measured free IGF-I levels in our study, represent a combination of the true free IGF-I levels and a fraction of IGF-I which can be readily dissociated from IGFBPs under the specific assay conditions (15). Recently, free IGF-I concentrations were found to be low in untreated IDDM children using the same assay, but these concentrations tended to normalize after start of insulin treatment (22).

We observed a significant decrease of free IGF-I and IGFBP-3 levels during aging in IDDM subjects, but not in healthy controls. Therefore free IGF-I and IGFBP-3 levels tended to be disproportionately lower in IDDM than healthy controls. A similar difference between IDDM subjects and healthy controls was not observed for total IGF-I levels with increasing age. In accordance with this, Graubert et al. reported that in the streptozocin-induced diabetic rat serum free IGF-I concentrations were more depressed than total IGF-I(23). Reduced free IGF-I concentrations probably account for reduced IGF-I bioactivity in diabetic serum, as has been reported in earlier studies (24).

Free IGF-I levels were inversely related to fasting glucose levels in IDDM subjects while fasting glucose levels were positively related to HbA_{1c}. IGF-I exerts a tonic hypoglycemic effect in the circulation (25) and decreased IGF-I effect may induce an increase in serum glucose levels. Since we observed a significant decrease of free IGF-I levels during aging in IDDM subjects, this decrease might contribute to gradually higher serum glucose levels and thereby to the development of diabetic microvascular complications (26).

IGFBP-1 levels were positively related to fasting glucose levels. A previous study in IDDM subjects reported also a positive relationship between IGFBP-1 and fasting plasma glucose levels (27). IGFBP-1 administration causes an acute rapid increase in plasma glucose levels (25), which suggest that increased IGFBP-1 levels results in a decreased availability of free IGF-I levels (28). IGFBP-1 is thought to modulate the free fraction of IGF-I.The inverse relationship between free IGF-I and IGFBP-1 in our study supports the hypothesis that circulating free IGF-I levels are low(er) when IGFBP-1 levels are high (15).

Free IGF-I levels tended to be higher in IDDM patients with retinopathy after

adjustment for age. However, this result needs to be interpreted with caution since retinopathy scoring was performed with direct ophtalmoscopy, a technique that may result in some misclassification of subjects and could, therefore, materially alter the results and the conclusions of our study.

An increased GFR is considered as an early symptom in diabetes mellitus (29). We observed lower values for total serum IGF-I in IDDM patients than in sex- and age-matched controls, which suggests at first glance no important role for IGF-I in the pathogenesis of hyperfiltration in IDDM. Nevertheless, in our study total IGF-I levels were positively related to the GFR of the kidney. The observed correlation coefficient between total IGF-I and GFR is in agreement with previous data showing, that IGF-I leads to an augmentation of GFR by 10 - 20 % (30, 31). In an experimental animal model of diabetes which was characterized by an increased GFR, an increased binding of IGF-I to its renal IGF-I receptors was found in comparison to controls (32). Increased binding of IGF-I to the IGF-I receptor in the IDDM subjects, despite low serum total IGF-I levels, might also give an explanation for the observed relationship between total IGF-I and GFR in our study. However, this relationship was lost after adjustment for age, which suggest that the relationship between GFR and serum total IGF-I levels is age-dependent and lost with the physiological age-dependent decrease of serum total IGF-I levels during aging.

In conclusion, free and total IGF-I and IGFBP-3 levels are lower and IGFBP-1 levels are higher in IDDM subjects than in controls. Free IGF-I and IGFBP-3 levels decrease significantly with age in IDDM subjects between 20 and 65 years. Higher (age-adjusted) free IGF-I levels were observed in IDDM subjects with diabetic retinopathy than in subjects without retinopathy.Total and IGFBP-3 levels were related to glomerular filtration rate in IDDM subjects but this relation was lost after adjustment for age.

Measurement of serum free IGF-I levels in IDDM subjects did not provide clear advantages compared to that of total IGF-I, IGFBP-I, and IGFBP-3 levels. Serum free IGF-I and IGFBPs reflect their tissue concentrations to various degrees. Consequently, extrapolations concerning the pathogenetic role of the IGF-I/IGFBP

system in the development of diabetic complications at the tissue level remain speculative.

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§ 5.2 **The IGF-I/IGFBP system in congenital partial lipodystrophy.**

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Summary

Background & Objectives: Insulin and IGF-I interact at many levels. Little is known about the insulin-like growth factor-I/insulin-like growth factor binding proteins (IGF-I/IGFBP) system in congenital partial lipodystrophy, a syndrome characterized by insulin resistance, hyperinsulinemia and absence of truncal and limb subcutaneous fat. Some cases have acromegaloid features with thick skin and large hands and feet in association with normal levels of circulating growth hormone.

Methods: In four females known with congenital partial lipodystrophy, hyperinsulinemia with acromegaloid features, the number and affinity of the IGF-I receptors on peripheral blood mononuclear cells (PBMCs), and the concentration of circulating insulin, total and free IGF-I, IGFBP-1 and IGFBP-3 levels were measured in the fasting and the fed state.

Cultures of PBMCs of the patients with lipodystrophy were also used to study the effect of IGF-I stimulation on thymidine uptake *in vitro*.

Measurements: In the subjects with lipodystrophy the affinity and the number of the IGF-I receptors on peripheral mononuclear cells (PBMCs) and erythrocytes did not significantly differ from controls in the fasting state. Insulin levels were significantly higher in subjects with lipodystrophy both in the fasting as well in the fed state.

Total IGF-I, free IGF-I and IGFBP-3 levels did not differ but serum IGFBP-1 levels were lower in lipodystrophy subjects than in healthy controls. The free IGF-I/IGFBP-1 ratio was increased in lipodystrophy subjects both in the fasting and fed states.The effects of IGF-I stimulation on thymidine uptake by PBMCs of lipodystrophy subjects in the absence of IGFBP-1 were not different from healthy controls cultures in vitro. When a combination of IGFBP-1 (in a concentration comparable to the fasting serum IGFBP-1 levels in lipodystrophy patients found in our study) and IGF-I was added to PBMC cultures from lipodystrophy patients no decrease in thymidine uptake by PBMCs was found.

Conclusions: In the four subjects with lipodystrophy hyperinsulinemia, lowered free IGF-I and IGFBP-1 levels, but increased free IGF-I/ IGBP-1 ratios were observed. Low IGFBP-1 concentrations in culture media did not reduce the stimulating

Our data suggest that the observed increased IGF-I/IGFBP-1 ratio in lipodystrophy patients contributes to an unopposed biological effect of IGF-I on IGF-I receptors, thereby inducing the development of acromegaloid features, acanthosis nigricans and polycystic ovaries in some patients with congenital partial lipodystrophy.

Introduction

The acromegaloid features in type A insulin resistance have been suggested to result from excessive insulin action (hyperinsulinemia) because the levels of growth hormone (GH) and total IGF-I seemed too low to explain these features (1). There are striking similarities between the clinical features of the acromegaloid variant of type A insulin resistance and congenital partial lipodystrophy (2). Congenital partial lipodystrophy (Köberling-Dunningan syndrome) is a syndrome, that is characterized by the absence of subcutaneous fat from the trunk and limbs (3). The faces of affected subjects often shows broad and acromegaloid features (2). Associated clinical features which are variably expressed are severe hyperlipidemia, acanthosis nigricans, hepatosplenomegaly, tubero-eruptive xanthomata, elevated basal metabolic rate (especially in the postprandial state), insulin-resistant diabetes without ketoacidosis and carpal tunnel syndrome (2, 4, 5). Polycystic ovaries and menstrual irregularity are common in affected females, while in the majority of cases essential hypertension is also present (6). An X-linked dominant mode of inheritance has been suggested, which explains the female predominance (7).

Hyperinsulinism and insulin resistance are striking features of congenital partial lipodystrophies (8). The cause of lipodystrophy is unknown. While it is apparent that insulin action is diminished in congenital partial lipodystrophy, only a few isolated case studies have addressed the potential role of defects at the level of the insulin receptor. Recently a homozygous mutation of the insulin receptor gene has been described in an extremely insulin-resistant subject with partial lipodystrophy (9). In partial lipodystrophy, it has been demonstrated that, using in vitro clonogenic assays of primary cultures of erythroid progenitors, there is a blunted response to insulin stimulation, while there is a normal response to insulin-like growth factor-I (IGF-I) (10). The insulin receptor and the IGF-I receptor are structurally similar. Each is a heterotetrameric glycoprotein comprising two alpha and two beta subunits. The latter subunits contain tyrosine kinase activity in their cytoplasmic portion (11). Under normal physiological conditions insulin stimulates the insulin receptor, but insulin might also stimulate the IGF-I receptor at higher concentrations (12, 13). IGF-I in serum is mainly bound to circulating IGF-binding

proteins. These binding proteins are an important regulatory system and modulate the action of IGF-I at its target cells (14). As there is little known concerning the IGF-I/IGFBP system in partial lipodystrophy, the aim of the present study was to investigate the relationships between serum (total and free) IGF-I, IGFBP-1, and IGFBP-3 levels and the number and affinity of the IGF-I receptors on blood cells in four known subjects with partial lipodystrophy, hyperinsulinemia and insulin resistance, and acromegaloid features.

Subjects and Methods

Subjects

Patients with partial lipodystrophy

Since partial lipodystrophy is a rare disease we were only able to study four patients which were visiting our outpatient clinic. All four female patients with partial lipodystrophy showed absence of subcutaneous fat on their trunk and limbs, and broad faces with short thick necks. Patient A was 27 yrs old, patient B 36 yrs old, patient C 47 yrs old, and patient D 26 yrs old. Patient C, the mother of patient A and D, had a recent history of precordial angina and intermittent claudication. Patient B was not closely related to the other three subjects. Patient A and D had known acanthosis nigricans, hirsutism, polycystic ovaries, and menstrual irregularities. All four subjects have acromegaloid features as well as soft tissue swelling of their hands and feet. Patient A had complaints of paresthesiae due to a bilateral carpal tunnel syndrome. All four subjects had hyperlipidemia (fasting cholesterol: range 5.8-7.8 mmol/L, HDL cholesterol: range 0.42-0.72 mmol/L and triglycerides range 3.6-12.8 mmol/L) and three of them were treated for essential hypertension.

No lipodystrophic patient showed symptoms or signs of liver disease.

Patient A had developed NIDDM, the other three subjects were normoglycemic.

For the IGF-I receptor studies two lipodystrophic subjects (A and B) were investigated in the fasting state; Three lipodystrophic subjects (A, C and D) were

investigated in the fed state. The measurement of thymidine uptake by PBMCs after addition of IGF-I and/or IGFBP-1 to the cell culture were performed on fasting blood samples.

Healthy Controls

For the experiments performed in the fasting state, eighteen healthy human volunteers (15 men and 3 women, aged 23-47 yr) entered the study, while for the experiments in the fed state, fourteen healthy human volunteers (10 men and 4 women, aged 26-47 yr) were investigated.

The measurement of thymidine uptake by PBMCs after addition of IGF-I and/or IGFBP-1 to the cell culture were performed on fasting blood samples both in the patients with partial lipodystrophy as well as the healthy controls.

The study was performed according to the rules of the hospital medical ethics committee. All subjects entered the study after informed consent.

Blood

For the fasting experiments blood was obtained between 08.00-10.00 after an overnight fast started at midnight.

For the experiments in the fed state blood was obtained between 09.00-11.00 am after a non-standardized breakfast between 07.30 and 8.00 am.

Cells

Human erythrocytes were isolated from freshly taken heparinized blood from each subject. Isolation was performed by density centrifugation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) at 400 G for 30 min at room temperature. The interphase containing the mononuclear cells and granulocytes was collected, and prepared as described below. The pellet containing the erythrocytes was resuspended in 0.9 % saline and centrifuged again on Ficoll-Paque under the same conditions. Erythrocytes were recovered from the pellet after the second centrifugation, and washed with two volumes of ice-cold assay buffer (For composition see Peptides and Medium). Next the erythrocytes were centrifuged at 400 G for 10 min

at 4 °C. After this centrifugation step the erythrocytes were resuspended in ice-cold assay buffer (two volumes cells, one volume assay buffer). This resulted in a cell concentration above 5 x 10^9 cells/mL. The remaining part of the IGF-I receptor assay was performed at 4 °C. The number of cells was determined with the aid of an electronic cell counter model AD-260 (Sysmex, Kobe, Japan).

Human peripheral blood mononuclear cells (PBMCs) were isolated from the same freshly taken heparinized blood from all subjects. Isolation was performed by density centrifugation on Ficoll-Paque as already described above. The centrifugation on Ficoll-Paque yields an interface of mononuclear cells in which 95 +/- 5 % are mononuclear lymphocytes. Mononuclear cells were recovered from the Ficoll medium interface, washed twice with 0.9% saline and after each washing, centrifuged during 5 minutes at 670 G at room temperature. The cells were resuspended in 1.75 mL assay buffer, which resulted in a cell concentration above 15 x 10^6 /mL. The number of cells per ml was counted with the aid of a hematocytometer. After resuspension in assay buffer cells were kept at 4 °C.

Peptides and Medium

3-[^{125}I]iodotyrosyl IGF-I was purchased from Amersham International Inc. (Amersham, Buckinghamshire, United Kingdom) and used as radioligand in the competitive IGF-I receptor assay.The radiolabeled IGF-I had a specific activity of 2000 Ci/mmol. Competitive binding studies were performed with approximately 0.05 nM ^{125}I-labeled hIGF-I and, without and with increasing concentrations of unlabeled purified human recombinant IGF-I (hIGF-I). hIGF-I was purchased from Saxon Biochemicals GmBH (Hannover, Germany).The assay buffer in the IGF-I receptor assay consisted of 50 mM Hepes, 50 mM Tris,10 mM dextrose, 50 mM $MgSO_4$, 2 mM EDTA, 10 mM $CaCl_2$, 45 mM NaCl, 4.5 mM KCl, and 0.1% BSA (15). The pH of assay buffer was adjusted to 8.0 at room temperature. For reduction of non-specific binding, bacitracin was added in a concentration of 2 mg/mL to the incubation medium (16).

Competitive IGF-I receptor assay

Competitive binding studies were carried out in Eppendorf tubes. For the IGF-I receptor assay the methods described by Hizuka et al. (17) and Izumi et al. (15) were used with three modifications: Firstly, the final incubation volume was decreased to 250 μL, for measurement of IGF-I binding to erythrocytes, and to 100 μL, for measurement of IGF-I binding to PBMCs. Secondly, bacitracin was added to the assay buffer. Thirdly, after 20 h incubation bound and unbound ligand were separated by three washing steps with saline in stead of separation with dibutylphthalate as described in the original method.

Erythrocytes: In a final volume of 250 μL, 200 μL erythrocytes (> 1.0 x 10^9 erythrocytes / mL final concentration), were incubated with ^{125}I-IGF-I (10-30,000 cpm) in assay buffer and in the absence or presence of increasing concentrations of unlabeled hIGF-I at 4 °C for 20h. Each experiment was performed in duplicate. For the competition studies, added unlabeled concentrations of hIGF-I ranged from 10^{-11} to 10^{-7} M.

PBMCs: In a final volume of 100 μL 60 μL PBMCs (cell concentration > 15 million mononuclear cells/mL), were incubated with ^{125}I-IGF-I (10-30.000 cpm) in assay buffer and in the absence or presence of increasing concentrations unlabeled recombinant hIGF-I at 4 °C for 20h. Each experiment was performed in duplicate. For the competition studies, the same added unlabeled concentrations of hIGF-I were added, as earlier described for the IGF-I receptor assay of erythrocytes (see above).

After 20 h incubation, bound and unbound radioligand were separated by three washing steps with saline at 4 °C. In each washing step, the cells were centrifuged at 10,000 G during 2 minutes and the supernatant was removed. After the third centrifugation step the cell-bound radioactivity was measured in a gamma counter. Non-specific binding was defined as the amount of radioligand bound in the presence of excess of unlabeled IGF-I (10^{-7} M). Specific binding of ^{125}I-IGF-I was determined by subtracting the amount of radioactivity bound in the presence of excess of unlabeled IGF-I (10^{-7}M).

The binding data were analyzed according to the method of Scatchard (18). The number of binding sites is expressed as sites per cell. The dissociation constant (Kd) is given in nmol/L. Specificity of the IGF-I receptor assay was established as follows: 1] unlabeled IGF-I, unlabeled IGF-II, and unlabeled insulin were able to displace labeled and bound IGF-I to erythrocytes and PBMCs, although with striking differences in affinity. 2] Binding of ^{125}I-IGF-1 to erythrocytes and PBMCs was inhibited by the unlabeled peptides in the following order of potency: IGF-I>IGF-II>>>insulin. 3] after preincubation with αIR-3 (a specific anti-IGF-I receptor antibody) the specific binding of ^{125}I-IGF-I in the IGF-I receptor assay was totally lost.

Hormone assays

Blood samples were obtained at the same time as those for the IGF-I receptor study and allowed to coagulate for 60 minutes. Subsequently serum was separated by centrifugation and quickly frozen to -20 ^{o}C. Free IGF-I was measured with a commercially available two-site immunoradiometric assay (Diagnostic System Laboratories Inc., Webster, Texas, U.S.A.; intra-assay and inter-assay C.V. : 10.3% and 10.7%, respectively) (19, 20).Total IGF-I was determined by a commercially available radioimmunoassay (Medgenix Diagnostics, Brussels, Belgium, intra-assay and inter-assay coefficients of variation (C.V.): 6.1%; 9.9%) after an acidification/neutralization step.
Commercially avalaible immunoradiometric assays were also used for measurement of IGFBP-1, and IGFBP-3 (Diagnostic System Laboratories Inc., Webster, Texas, U.S.A.; intra-assay and inter-assay C.V. for IGFBP-1: 6.0% and 3.5%, respectively; and for IGFBP-3: 3.9% and 1.9%, respectively). Insulin was determined by a commercially available radioimmunoassay (Medgenix Diagnostics, Brussels, Belgium, intra-assay and inter-assay C.V.: 8.0%; 13.7%). For plasma GH an immunoradiometric assay was used (CIS bio international, Gif-sur Yvette Cedex, France, detection limit 0.4 μg/L, intra-assay and inter-assay CV 2.8 and 4.4%, respectively; normal value < 5 μg/L).

Thymidine Uptake of PBMCs in culture.

hIGF-I was purchased from Saxon Biochemicals GmBH (Hannover, Germany). RPMI-1640 (nr 042-02511) was purchased fom Gibco BRL (Life Technologies BV, Breda, the Netherlands). Bovine Serum Albumin (BSA) was from Sigma (Instruchemie BV, Hilversum, the Netherlands), The BSA did not contain any detectable IGF-I activity as determined by radioimmunoassay (Medgenix diagnostics, Brussels, Belgium). [^{3}H] radiolabeled thymidine, ([^{3}H]-dTd], TRK 686, 1mCi/mL) was obtained from Amersham International (Amersham, UK). 48 well cell culture flat-bottom plates (catalog number 3548) were obtained from Costar (Cambridge, USA).

IGFBP-1 was purified from midgestational human amniotic fluid using an ammonium-sulphate precipitation. The pellet was discarded and the supernatant adjusted to 40% methanol. Using hydrophobic interaction chromatography with methanol gradient, the IGFBP-1 was eluted as a single peak. At that point, using gel analysis, no impurities can be detected.

For the thymidine assay we primarily used mostly male controls for our female patients because we could only obtain permission to study a small number of healthy controls (less than 30 subjects) to perform this sort of study.

We were very careful in reaching an optimal match for age between the lipodystrophic subjects and the healthy controls. Age is to our knowledge a far more important factor than gender when studying IGF-I effects on thymidine uptake (own unpublished observations and 21-23).

Cell Culture Medium

The culture medium contained RPMI 1640 with 4 mM glutamine, 0.1% BSA, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1% BSA. The pH of the medium was adjusted to 7.4.

Cell culture

For the cell culture experiments blood was drawn in the fasting state. Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood. Blood was initially taken for this purpose from four subjects with congenital partial lipodystrophy and from five healthy subjects. Isolation was performed by density centrifugation on Ficoll-Paque as above already described. PBMCs were cultured in flat-bottom 48-well polystyrene plates. To each well 320,000 PBMCs were added. PBMCs were cultured in a final volume of 250 μL medium in combination with IGF-I (range 0-10^{-7} M). After 96 h culture at 37 °C in 5% CO_2, 100% relative humidity, the cells were pulsed with 0.5 μCi [^{3}H]thymidine and then cultured for another 4 h. The cells were then harvested using standard scintillation procedures and [^{3}H] thymidine radioactivity was measured in a liquid scintillation counter. Blood was taken from two subjects with congenital partial lipodystrophy and from two healthy subjects on a second occasion to investigate the effect of IGF-I on thymidine uptake by PBMCs in the presence of IGFBP-1.
These latter experiments were performed under the same conditions and using the same procedures as described above. IGFBP-1 was added in this last experiment at a concentration of 0.15 x 10^{-9} M (a concentration of IGFBP-1 comparable to fasting serum IGFBP-1 levels observed in lipodystrophy patients in vivo).

Statistical Analysis

Results are expressed as the mean +/- SE. The Mann-Whitney test was used to test for differences between groups. Partial Pearson 's correlation coefficients were used to calculate the correlations between Kd and number of IGF-I receptors. A two-sided p value of < 0.05 was considered significant.

Results

^{125}I-IGF-I specifically bound to human erythrocytes and PBMCs of the healthy volunteers and the binding increased linearly as a function of the number of cells (data not shown). Scatchard analysis for IGF-I binding to erythrocytes and PBMCs showed in both instances a linear plot (data not shown). Table 1A shows the results of IGF-I receptor assays carried out on erythrocytes and PBMCs from 18 healthy subjects in the fasting state.Table 1B shows these results in the two subjects with partial lipodystrophy. The IGF-I receptor characteristics (number and Kd) on erythrocytes and PBMCs from subjects with lipodystrophy did not differ from those of normal healthy volunteers (Figure 1).

In the fasting state there were no differences in mean total and free IGF-I levels, insulin levels were significantly higher in the patients with lipodystrophy in comparison to healthy controls, while IGFBP-1 levels were lower and the free IGF-I/IGFBP-1 ratio was significantly higher in lipodystrophy patients than in healthy controls (Table 1).

In the fed state the IGF-I binding sites and the Kd of the IGF-I receptor on blood cells were lower in lipodystrophy patients than in healthy controls (Table 2). Free IGF-I and IGFBP-1 levels were significantly lower in lipodystrophy patients than in healthy controls while insulin levels and the free IGF-I/ IGFBP-1 ratio were significantly higher in lipodystrophy patients than in healthy controls (Table 2) .GH values in the subjects with partial lipodystrophy were normal (maximal 0.6 μg/L).

Thymidine uptake by PBMCs after 100 nmol/ L IGF-I (10^{-7} M) stimulation in the absence of IGFBP-1 in the culture medium was not significantly different in subjects with lipodystrophy compared to healthy controls (Figure 2). When basal thymidine uptake was set at 100%, the maximal percentage stimulation of thymidine uptake after IGF-I (10^{-7} M) stimulation was for lipodystrophy subjects (n=4): 46, (-1-91)% (mean, (range)) and in healthy controls (n=5): 51, (29-88) % (Mann-Whitney test: not significant) (Figure 2). Also the baseline incorporation of radioactivity did not differ between both groups.

Table 1. Characteristics of IGF-I binding on Erythrocytes and PBMCs, circulating IGF-I and IGFBPs, and insulin in the fasting state in 18 healthy volunteers (A) and 2 patients with partial lipodystrophy (B).

	A mean	±SE	(range)	B mean	±SE	(range)	p-value*
Age subjects (years)	32.6	±1.8	(23-47)	31.5	±1.8	(29-34)	ns
Erythrocytes							
No of specific IGF-I binding sites/cell	34.3	±4.0	(11-57)	30	±2.1	(27-33)	ns
Kd (nM)	2.26	±0.29	(0.42-4.75)	1.90	±0.12	(1.73-2.07)	ns
PBMCs							
No of specific IGF-I binding sites/cell	3631	±524	(430-8774)	6779	±1101	(5222-8336)	ns
Kd (nM)	1.98	±0.26	(0.22-3.73)	2.38	±0.08	(2.26-2.50)	ns
Total IGF-I (nmol/L)	28.6	±1.7	(13.3-46.4)	26.6	±2.8	(22.7-30.5)	ns
Free IGF-I (nmol/L)	0.14	±0.02	(0.03-0.26)	0.09	±0.01	(0.07-0.11)	ns
IGFBP-1 (nmol/L)	1.38	±0.30	(0.17-5.46)	0.11	±0.001	0.11	0.02
Free IGF-I/IGFBP1 ratio	0.22	±0.07	(0.008-1.42)	0.80	±0.10	(0.66-0.93)	0.04
IGFBP-3 (nmol/L)	100.9	±2.7	(80.0-121.7)	113.1	±18.4	(87-139.1)	ns
Insulin (mU/L)	13.1	±1.1	(5.2-21.8)	65.7	±35.3	(30.3-101.0)	0.02

For used abbreviations, see text
* Statistics by Mann-Whitney test comparing mean values of variables in healthy subjects vs variables in subjects with lipodystrophy.
ns not significant

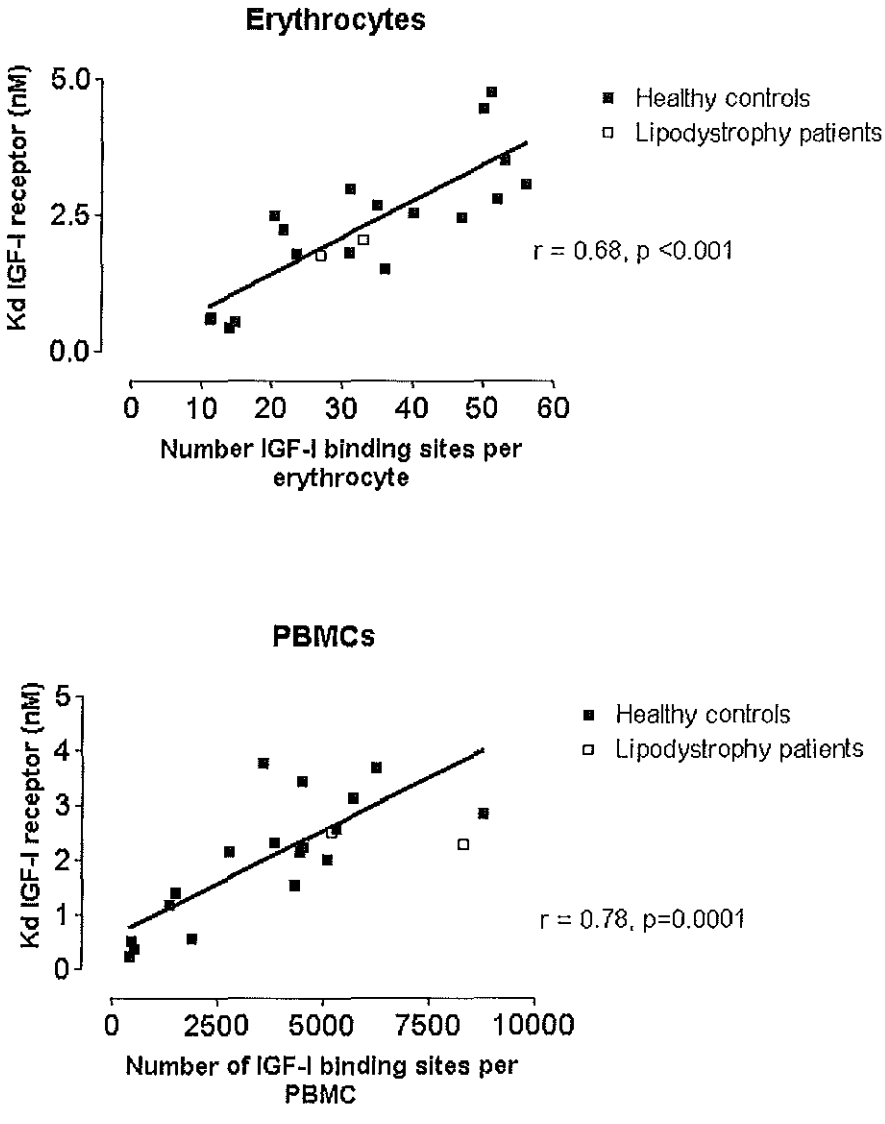

Figure 1

The relationships between Kd and number of IGF-I binding sites per erythrocyte (Top panel) and per PBMC (Bottom panel) did not differ between lipodystrophy patients and healthy controls.The Pearson 's partial correlation coefficients are calculated from the data of the healthy controls.

Table 2. Characteristics of IGF-I binding on Erythrocytes and PBMCs, circulating IGF-I and IGFBPs, and insulin in the fed state in 14 healthy volunteers (A) and 3 patients with partial lipodystrophy (B).

	A mean	±SE	(range)	B mean	±SE	(range)	p-value
Age subjects (years)	34.4	±1.9	(26-47)	34	±5.3	(27-47)	ns
Erythrocytes							
No of specific IGF-I binding sites/cell	9.2	±1.34	(5-25)	4.7	±0.5	(4-6)	0.02
Kd (nM)	0.62	±0.11	(0.19-1.6)	0.26	±0.01	(0.23-0.29)	ns
PBMCs							
No of specific IGF-I binding sites/cell	1057	±161	(402-2135)	779	±62	(691-867)	ns
Kd (nM)	0.53	±0.08	(0.19-1.23)	0.185	±0.004	(0.18-0.19)	0.03
Total IGF-I (nmol/L)	28.8	±2.0	(17.9-40.9)	26.1	±1.8	(23.5-30.5)	ns
Free IGF-I (nmol/L)	0.33	±0.03	(0.13-0.60)	0.16	±0.05	(0.10-0.27)	ns
IGFBP-1 (nmol/L)	1.01	±0.03	(0.18-4.75)	0.02	±0.004	(0.02-0.03)	0.02
Free IGF-I/IGFBP1 ratio	2.25	±6.64	(0.05-25.3)	10.68	±3.24	(5.41-13.82)	0.02
IGFBP-3 (nmol/L)	107.5	±5.6	(70.6-137.4)	123.6	±5.46	(110.3-131.1)	ns
Insulin (mU/L)	14.0	±3.2	(3.5-55.5)	132.5	±43.0	(32.2-210.7)	0.01

For used abbreviations, see text

* Statistics by Mann-Whitney test comparing mean values of variables in healthy subjects vs variables in subjects with lipodystrophy.

ns not significant

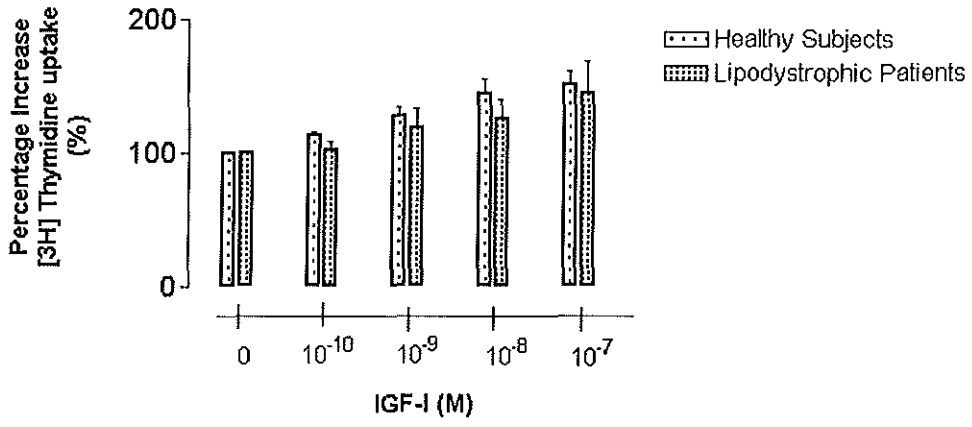

Figure 2 Effect of IGF-I stimulation (range 10^{-10}-10^{-7} M) stimulation on [^{3}H] thymidine uptake by 320,000 PBMCs during 96 h culture at 37 ° C and 5% CO_2 in RPMI-1640-0.1% BSA. Left: effect in healthy controls, Right: effect in lipodystrophic patients. The mean and SEM values of quadruplo wells are presented. Basal thymidine uptake was set at 100 %. Results are expressed as percentage stimulation of thymidine uptake by 320,000 cells per culture (vertical axis).The mean basal thymidine uptake and thymidine uptake after stimulation with IGF-I did not differ between healthy controls and lipodystrophy patients (See also text).

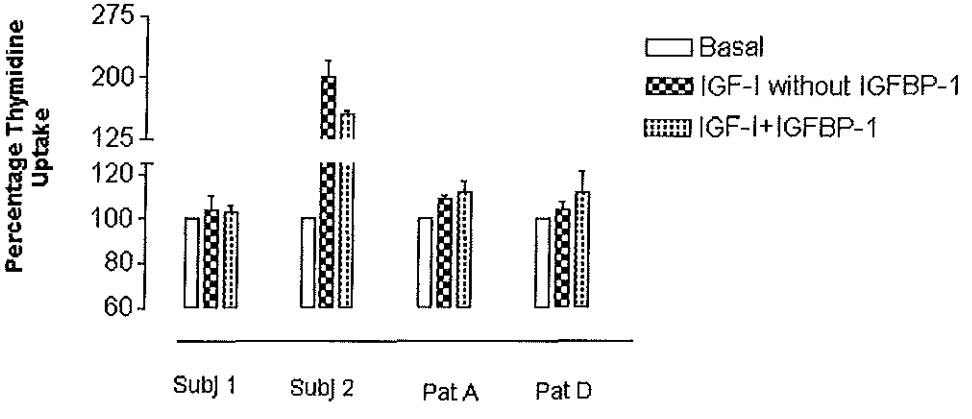

Figure 3 From left to right, basal thymidine uptake, effect of IGF-I (10^{-7} M), and the combination of IGF-I (10^{-7} M) and IGFBP-1 (0.15 x 10^{-9} M) on thymidine uptake by PBMCs in two healthy controls (Subj 1 and 2) and two lipodystrophy patients (Pat A and D). Basal thymidine uptake (= PBMCs cultured during 96 h culture at 37 ° C and 5% CO_2 in RPMI-1640-0.1% BSA) was put equal to 100 %. The Mann-Whitney test was used to test for statistical significant differences in thymidine uptake comparing the results of experiments in which only IGF-I was added with the experiments after addition of the combination of IGFBP-1 and IGF-I. (See also text)

When IGFBP-1 was added to cultures in a concentration of 0.15 x 10^{-9} M, no statistically significant effect on thymidine uptake in comparison to basal (=unstimulated) thymidine uptake was seen in either lipodystrophic subjects or healthy controls (data not shown). When both IGF-I (10^{-7} M) as well as IGFBP-1 (0.15 X 10^{-9} M; a concentration comparable to the fasting serum IGFBP-1 concentrations observed in lipodystrophy patients in vivo) were added to cell cultures, no fall in thymidine uptake was observed in lipodystrophic subjects (in comparison to thymidine uptake after stimulation with IGF-I (10^{-7} M)), while such an effect could be observed in one of the two healthy controls under the same culture conditions (Figure 3, Subj 2).

Discussion

Our patients showed acromegaloid features, large hands and feet. In patients with partial lipodystrophy acromegaloid features along with thick skin and large hands and feet have been described in association with normal levels of circulating growth hormone (8, 24). As in other syndromes of insulin resistance, the occurrence of acromegaloid features, acanthosis nigricans and polycystic ovaries in the (partial) lipodystrophy syndrome has been previously attributed to cross stimulation of the IGF-I receptors by the existing hyperinsulinemia (25, 26). However, the affinity constant of insulin to the normal IGF-I receptor is about 10 μmol/L, a concentration which even under conditions of extreme insulin resistance is not reached in human subjects under physiological conditions (e.g. even at circulating levels of 2,000 IU/L insulin, the insulin concentration in blood is not higher than 12 nmol/L).

Total IGF-I and IGFBP-3 levels in lipodystrophy patients were comparable to the values observed in healthy controls, while fasting insulin levels were significantly elevated both under fasting and fed conditions. IGFBP-1 levels were lower in lipodystrophy patients than in healthy controls both in the fasting as well in the fed state. Free IGF-I in lipodystrophic tended to be lower in the fed state. Despite the lower levels of free IGF-I and IGFBP-1 in lipodystrophy subjects, the free

IGF-I/IGFBP-1 ratio was higher than in the healthy controls. Variation in serum IGF-I bioactivity appears to be largely due to variations in IGFBPs (27). It has previously been suggested that a higher total IGF-I/IGFBP-1 ratio may reflect a higher bioavailability of IGF-I (28). The elevated free IGF-I/IGFBP-1 ratio found in our lipodystrophy patients therefore suggests that the occurence of acromegaloid features, acanthosis nigricans and polycystic ovaries in the (partial) lipodystrophy syndrome is mediated by a direct effect of IGF-I on the IGF-I receptor. Untreated acromegalic subjects also show an increased free IGF-I/IGFBP-1 ratio (unpublished observations).

At present the relevance of studying the IGF-I receptor on erythrocytes and PBMCs is unclear. We know of only one paper from a Japanese group which has studied IGF-I receptor characteristics on erythrocytes and liver microsomal membranes in one patient with typical leprechaunism (29). The IGF-I receptors on erythrocyte and microsomal membranes in this patient showed a lower affinity and a higher capacity on both cell types than that observed in infants of the same age (28). We do not have own data or other data from the literature in which IGF-I receptor numbers have been compared on blood cells and on other tissues. Recently we have published about the relationships of IGF-I receptors, IGF-I and IGFBPs on erythrocytes and PBMCs in another study (30). One of the conclusions of this paper was that studies of IGF-I receptor characteristics on erythrocytes and PBMCs in the fasting state are cell specific and cannot be extrapolated without restrictions to other cell types. This latter phenomenon might be due to the fact that IGF-binding proteins, which are considered important modulators of IGF-I action, are synthesized in a tissue- and cell-specific pattern (31).

Although the lowered serum IGFBP-1 levels in lipodystrophy subjects may be the consequence of an increased inhibitory effect of insulin on m-RNA production of IGFBP-1 in the liver (32), chronic hyperinsulinemia might also have caused a permanent increased movement of IGFBP-1 from the vascular compartment to extravascular tissues (33). Thus chronic hyperinsulinemia in lipodystrophy subjects may result in an increased extravascular transport of IGFBP-1. As IGFBP-1 is also considered a "shuttle" for IGF-I (34), this will result in an increased IGF-I effect on

the IGF-I receptor in lipodystrophic subjects secondary to the increased availability of IGF-I in the extracellular space to interact with the IGF-I receptor.

We did not observe an additional effect of IGF-I on the increase in thymidine uptake of PBMCs drawn from lipodystrophy patients during experiments performed in the absence of IGFBP-1. The lack of normal IGF-binding proteins in vitro may alter the biological activity of IGF-I. Under these circumstances in vitro, all the IGF-I would have been present as free IGF-I. After addition of both IGFBP-1 (in a concentration which was comparable to the serum IGFBP-1 levels observed in lipodystrophy patients in vivo) and IGF-I to the PBMCs cultures, no decrease in thymidine uptake by PBMCs was observed in lipodystrophy subjects, while at this IGFBP-1 concentration a significant decrease in thymidine uptake could be observed in PBMCs cultures of one healthy subject. Normally the addition of IGFBP-1 to in vitro cell cultures has been reported to result in the inhibition of IGF-I within these experimental systems (35, 36). However, one of the requirements for this effect is that IGFBP-1 must be added in molar excess to IGF-I (31). Therefore, our study suggests no inhibiting action of IGFBP-1 on IGF-I in subjects with partial lipodystrophy as a consequence of the lowered serum IGFBP-1 levels.

In conclusion, we observed in lipodystrophy subjects normal to lowered serum free IGF-I levels, as well as lowered serum IGFBP-1 levels, but an increased free IGF-I/IGFBP-1 ratio in the fasting and the fed state. In addition, the lipodystrophy subjects showed normal functioning IGF-I receptors in vitro and no decrease in thymidine uptake by PBMCs after addition of a combination of IGF-I and low concentations of IGFBP-1 to the PBMCs cultures.

We hypothesize that increased free IGF-I/IGFBP-1 ratios may result in an unopposed effect of IGF-I on IGF-I receptors, contributing to the development of acromegaloid features, acanthosis nigricans and polycystic ovaries as seen in congenital partial lipodystrophy.

Acknowledgement

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6

Conclusions and General Discussion

The aim of this thesis was to investigate the (patho)physiologic role of the IGF-I/IGFBP system in adult subjects in health and disease under several conditions like aging, GH deficiency, acromegaly, diabetes mellitus and congenital partial lipodystrophy.

In chapter 2 we have studied whether it is possible to measure IGF-I receptors on blood cells in vitro and what its (clinical) importance might be. We have adapted and further developed a method to measure the IGF-I receptor characteristics on blood cells in vitro. The observed affinity constants for the IGF-I receptor on blood cells using this method showed a concentration range that was in the same range as that measured for serum free IGF-I levels. In the fasting state the Kd of IGF-I receptors on erythrocytes was positively related to circulating free IGF-I levels while for the IGF-I binding sites on PBMCs no relation with free IGF-I levels were found. Serum IGFBP-3 levels were inversely related to the number of IGF-I binding sites on erythrocytes and to the Kd of the IGF-I receptor on PBMCs. These observations suggest that studies of the IGF-I receptor characteristics are cell-specific, and cannot be extrapolated without restrictions to other cell types in the body. We therefore concluded that measurement of IGF-I receptors on blood cells does not give a good impression of the action of IGF-I on other (relevant) target tissues in the body.

In Paragraph 2.4 we have studied whether in vivo changes occur in the IGF-I/IGFBP system and the IGF-I receptor characteristics on blood cells during an oral glucose load.The circulating free IGF-I levels and the IGF-I receptors on PBMCs showed no changes during a period of 120 minutes after the oral glucose load. Serum IGFBP-1 and serum IGFBP-3 levels also did not change significantly during this period, despite the observation of a transient increase of serum insulin levels after the glucose load. The absence of changes in the IGF-I receptor characteristics after an oral glucose load suggest that the changes in circulating insulin and IGFBP-1 levels acutely regulate IGF-I bioactivity.

The "free hormone hypothesis" has been a basic tenet of endocrine physiology for many decades (1). The assumptions of this hypothesis can be summarized as follows (1): 1] hormones are physiologically active only in the unbound, free state,

2] measurements of free hormone levels are more reliable indicators of biological action than measurements of total hormone levels, and 3] normal, physiologic mechanisms preferentially regulate concentrations of the free, rather than total, hormone. IGF-I is present in body fluids in association with specific IGFBPs, which regulate and, for the most part, inhibit the bioactivity of IGF-I. Measurement of free IGF-I, theoretically the bioactive fraction of IGF-I, seems an important step to get more information on the biological actions of the IGF-I/IGFBP system in vivo. Free IGF-I concentrations reported in the literature vary widely and range from <1% to 19% of total IGF-I in the circulation (2). Several assay methods to measure free IGF-I exist, many of which utilize a chromatographic or filtration step prior to the assay (1). Matrix changes in the sample matrix using these both methods, could change the dynamic equilibrium between the free and bound IGF-I fractions (1). We have used an IRMA to measure free IGF-I levels in our studies. This latter assay can be used directly on unaltered serum samples and the sample matrix is not disturbed. Recently, it was suggested that the free IGF-I assay used in our study, is the best that is available at present and appears to generate experimental data that were similar to "expected" free IGF-I concentrations (2). This supports the view that the free IGF-I levels measured in our studies reflect indeed the majority of the biologically active fraction of IGF-I. Free IGF-I levels might in this respect be comparable to free thyroxine and free cortisol levels. However, free IGF-I levels need to be interpreted with more caution, since the biological action of IGF-I presumably is far more complex than observed previously for thyroxine and cortisol levels. The bioactivity of IGF-I is modified by at least six binding proteins, many of which are modified in their activity by protease activity. Another methodological reservation of our free IGF-I assay is that the amount of free IGF-I measured depends on the affinities of the IGFBPs vs. the affinities of the IGF-I antibodies used in this assay. The effect of this latter phenomenon may be that our free IGF-I assay actually measures a combination of the true free IGF-I and a portion of IGF-I, which is capable of being competitively dissociated from the IGFBPs. On the other hand, this may also be the strength of this assay since one can argue that the free IGF-I fraction, which is measured in our assay, may represent the free IGF-I fraction

that is available to the tissues.

As judged from the observed affinity of the IGF-I receptor for IGF-I (Kd ± 1-2 nmol/L) serum free IGF-I levels as determined by our assay may elicit biological effects via the IGF-I receptor. Cross-reaction with the insulin receptor is unlikely because of its low affinity for IGF-I at this concentration. Besides receptor affinity and the concentration of free IGF-I, IGF-I receptor abundance determines the cellular response (3). Tissues with more abundant IGF-I receptors may therefore respond to lower IGF-I levels than tissues with a lower number of IGF-I receptors.

The question whether the measurement of serum free IGF-I levels gives additional and/or other information than the total IGF-I assay was studied in Chapter 3.

In Chapter 3 (Paragraph 3.1) we observed that free IGF-I levels did not decrease with age in a healthy elderly population (studied age range 55-80 yrs). In the same study population and age range we observed the expected physiologically occurring decrease in total IGF-I levels during aging. These results suggest that changes in serum free IGF-I levels do not always parallel those of total IGF-I and that the measurement of free IGF-I levels might give other information than total IGF-I levels.

The free IGF-I levels were actually even higher in individuals above the age of 70 years when compared with subjects between 55 and 70. However, one needs to realize that a cross-sectional study as we carried out in Chapter 3 has limitations. For example the actual rate of change of IGF-I during aging within one individual was not measured. Nevertheless, we hypothesize that the higher free IGF-I levels in the subjects above 70 years observed in our study may be the consequence of a selective survival of the members of this subgroup. This may imply that subjects with higher free IGF-I levels live longer. Long-term prospective and population based studies are needed to confirm this intriguing possibility. If in such prospective studies subjects with the highest free IGF-I levels demonstrate a selective survival, this would be the mirror-image of previous findings in growth-hormone deficient adults (a condition characterized by low IGF-I levels), that showed an increased total and cardiovascular mortality (4-6).

We did not observe a relationship between free IGF-I levels and free androgen and

free oestrogen indices in this healthy aging population. This suggests that the changes in the biological activity of the IGF-I/IGFBP system and that of sex hormones occur mainly independently during aging.

In Chapter 3 (Paragraph 3.2) we observed that higher free IGF-I levels were associated with a lowered prevalence of cardiovascular disease. For total IGF-I levels no relation with the prevalence of cardiovascular disease was found. Higher fasting IGFBP-1 levels were associated with a more favourable cardiovascular risk profile. As mentioned above, previous studies have suggested that GH deficiency and low IGF-I levels are associated with premature and/or increased atherogenesis (4-6). The response-to-injury hypothesis of the pathogenesis of atherosclerosis suggests that injury of the vascular endothelium leads to an inflammatory-fibroproliferative response which if occurring chronically, may culminate in the occlusive, advanced lesions of atherosclerosis (7). IGF-I is a well-known stimulator of DNA synthesis and proliferation of vascular smooth muscle cells and it is this effect that is generally considered to contribute to the development of atherosclerosis (8). Such inflammatory-fibroproliferative responses seem initially designed to be protective for the vascular endothelium (8). It was observed that in hypophysectomized rats with low IGF-I levels the proliferation of vascular smooth muscles is markedly delayed after endothelial damage, suggesting an impaired adaptive growth response in the proces of healing of the injured arterial wall, when compared to normal control animals (9). However, these hypophysectomized animals showed preserved local induction of IGF-I mRNA in the vessel wall after balloon injury, suggesting undisturbed autocrine and/or paracrine IGF-I secretion (10). Low circulating IGF-I levels have also been reported to be associated with angiographically documented coronary artery disease in humans (11). Moreover, the highest IGF-I levels during life are normally found at puberty (12, 13), a period that is characterized by the absence of atherosclerosis. All these findings together challenge both the view that high IGF-I levels contribute to the development of atherosclerosis as well as an important role for the paracrine/autocrine IGF-I/IGFBP system in the development of atherosclerosis. On the contrary, these findings might even be compatible with a protective effect of high (endocrine)

IGF-I levels on the development of atherosclerosis (comparable to the known effects of IGF-I in wound healing) and give an explanation for the observed decreased prevalence of cardiovascular disease in elderly subjects with the highest free IGF-I levels in our study.

High fasting IGFBP-1 levels were associated with a more favourable cardiovascular risk profile in our study (Paragraph 3.2) and this relation remained after adjustment for insulin levels. This suggests that IGFBP-1 levels might be an independent marker for metabolic disturbances, which are associated with an increased risk of cardiovascular diseases. An additional argument for this is the observation that in NIDDM subjects, low IGFBP-1 levels correlate with an increased cardiovascular risk (14). Studies on the biological effects of IGFBP-1 have shown conflicting results. IGFBP-1 is capable of both inhibition as well as augmentation of IGF-I bioactivity (15). These conflicting observations may be explained by the recent findings that differential phosphorylation of IGFBP-1 significantly alters its affinity to IGF-I, thereby differentially modulating IGF-I bioactivity (16). The IGFBP-1 assay used in our study cannot discriminate between phosphorylated and non-phosphorylated IGFBP-1, but under normal conditions IGFBP-1 circulates mainly in a highly phosphorylated form (17). This latter form of IGFBP-1 would favor sequestration of IGF-I by IGFBP-1 resulting in a decreased IGF-I release to IGF-I receptors (15). The observed lower IGFBP-1 levels in subjects with a disadvantageous cardiovascular risk profile, might thus be an adaptive mechanism to increase IGF-I bioactivity at the vascular endothelium.

In Paragraph 3.3 we observed that circulating (total and free) IGF-I levels were positively related to bone mineral density in men but not in women. This suggests a gender-specific anabolic effect of circulating IGF-I on trabecular bone in men. However, this statistically significant relationship was not very powerfull suggesting that circulating IGF-I levels are not a very important determinant for bone mineral density. However, one needs to consider that interindividual differences in circulating IGF-I concentrations in old age may have already existed at a younger age. It is therefore possible that any association between IGF-I and bone mass in elderly subjects may be due to events that occurred long before the actual

measurements were performed.

The assessment of serum free IGF-I levels did not give much additional information to that of total IGF-I in this latter study, since the relationships between free IGF-I and total IGF-I and bone mineral density were almost identical. The relationship between circulating IGF-I levels and bone mineral density became only manifest after adjustment for body mass index. Obesity is generally considered as a protective factor for bone mineral density (18). However, this relationship between body mass index and bone mineral density is not linear, and a lowered bone mineral density is especially observed in very thin individuals (18). We observed an inverse relationship between body mass index and free IGF-I levels in men only, supporting that this might be a possible explanation for the observed gender specific effect.

It has been suggested that the anabolic actions on bone are mediated both by the (endocrine) production of IGF-I from the liver and by local production of IGF-I (19). IGF-I has been found to stimulate osteoblast activity and collagen synthesis in bone (20). Apart from direct effects on osteoblasts, IGF-I enhances intestinal absorption of calcium and phosphate by increasing the production of 1,25 $(OH)_2$ vitamin D, and by increasing the intestinal sensitivity to vitamin D (21). However, the situation is even more complex since bone cells not only locally synthesize IGF-I but also at least six IGFBPs regulating its bioactivity in an auto- and paracrine manner (22). Therefore it is at present difficult to conclude whether circulating (endocrine) or locally produced (autocrine/paracrine) IGF-I is from a physiological point of view most important for the effects of IGF-I on bone.

Finally, as physical fitness, muscle strength, and bone integrity are related, it might be possible that the observed relations between serum free and total IGF-I and skeletal BMD in our study are related to alterations in physical fitness secondary to aging. However, we did not observe a relation between (total and free) IGF-I and disability. Differences in physical activity seem thus not responsible for the observed relation between (total and free) IGF-I and BMD in men.

In Paragraph 3.4 we observed lower serum free IGF-I levels in subjects, who felt less healthy in comparison to other individuals of their own age. No such

relationship was observed for total serum IGF-I levels. This study has many limitations in its design. The finding may be not specific, since it has been found that IGF-I levels are lowered in all catabolic conditions (23). Lowered IGF-I levels are related with indices of malnutrition, hepatic function, chronic renal failure and are predictive of future life-threatening complications such as severe infections (24). Low IGF-I levels might thus have indirectly contributed to the feelings of a lesser health. Lowered IGF-I levels also occur in GH deficiency. It has been found that subjects with GH deficiency have a decreased subjective feeling of well being (25). Replacement therapy with growth hormone in these GH deficient subjects normalizes free IGF-I levels (26) but also improves psychological well-being (27).

To summarize the results of Chapter 3 in this healthy elderly population (age range 55-80 years):

-We observed a decrease of total serum IGF-I and IGFBP-3 levels with aging. Free serum IGF-I levels did not decrease in this age range, and tended to be higher in subjects over 70 years. We did not observe a relationship between free IGF-I levels and free androgen and free oestrogen indices in this healthy aging population.

-Higher free IGF-I (but not total IGF-I) levels were associated with a lowered prevalence of cardiovascular disease, while IGFBP-1 levels were associated with a more favourable cardiovascular risk profile.

-Circulating (total and free) IGF-I levels were positively related to bone mineral density in men but not in women.

-Finally, lowered free IGF-I (but not total IGF-I) levels were observed in subjects who felt less healthy in comparison to other individuals of their own age.

The results of Chapter 3 suggest that measurement of free serum IGF-I levels provides additional and other information concerning the IGF-I/IGFBP system, which would not be obtained by measuring total serum IGF-I levels. We observed a discrepancy in the ratio of free and total IGF-I levels under several (patho)physiologic circumstances. Since the free serum IGF-I levels measured in our study probably reflect the majority of the biologically active fraction of IGF-I, the observed relationships for free IGF-I suggest that IGF-I has predominantly anabolic effects in this aging population.

In Chapter 4 (Paragraph 4.1) we discussed the problems encountered in the diagnosis of the growth deficiency syndrome. Besides discussing the parameters to measure when growth hormone deficiency is suspected, we have presented recommendations to answer the question which individuals should be tested for growth hormone deficiency in the first place.

In Paragraph 4.2 we demonstrate that measurement of circulating IGFBP-3 levels provides no (additional) advantages above serum total IGF-I levels in the diagnosis of acromegaly. This is not surprising since there is a close correlation between serum total IGF-I and IGFBP-3 levels in all clinical conditions (1) and as discussed in chapter 1, IGFBP-3 binds almost 75-95% of the IGF-I present in the circulation (28).

In Paragraph 4.3 we have studied the physiological and clinical relevance of the measurement of serum total and free IGF-I and IGFBP-3 levels in untreated patients with acromegaly. In acromegalics, serum free IGF-I levels did not change with age, while mean 24 h serum GH, total IGF-I and IGFBP-3 levels tend to decrease with age. We observed that although serum total and free IGF-I, but not IGFBP-3 levels are suitable biochemical parameters for screening for acromegaly, total and free serum IGF-I levels do not correlate with disease activity in acromegaly.

Diabetic retinopathy and nephropathy develop only after the age of 15 years (29). It therefore has been suggested that GH and IGF-I levels are involved in the pathogenesis of diabetic retinopathy and nephropathy (29). In healthy subjects there is an almost linear increase in serum IGF-I levels from early childhood into adolescence, while after puberty a significant decrease is apparent throughout adulthood (12, 13)(see Chapter 1, Figure 5). In diabetic patients total IGF-I levels during puberty are lower and peak later, but statistically significant differences in total IGF-I levels between diabetic patients and healthy controls are only manifest during the later stage of puberty (30).

In Chapter 5.1 we have studied the changes in the IGF-I/IGFBP system in IDDM type I, and whether these changes are related to the presence of diabetic microvascular complications. We observed lower free IGF-I and total IGF-I levels and IGFBP-3 levels and higher IGFBP-1 levels in insulin-treated adult IDDM

subjects than in age- and sex-matched controls. In addition, free IGF-I levels decreased significantly with age in IDDM subjects in comparison to age- and sex-matched healthy controls. Most previous studies in IDDM also observed low to lowered total IGF-I and IGFBP-3 levels and high fasting IGFBP-1 levels, despite hypersecretion of GH (31-33). Our study confirms these previous reports and extends these results by demonstrating lowered fasting free IGF-I levels in insulin-treated IDDM patients. It has always been assumed that the insulin-like effects of IGF-I were not of physiological relevance, because IGF-I circulates bound to IGF-binding proteins and the amounts of free IGF-I were too small as to be significant (34). This view has to be revised, since it is now known that IGFBPs play an important role in regulating bioavailability and thus bioactivity of IGF-I (35). It has been demonstrated in animals that infusion of IGFBP-1 leads to a prompt increase of blood glucose (36), which is reversed by administration of human recombinant IGF-I. In addition, administration of recombinant IGF-I normalizes expression of renal glucose transporters in diabetic rats (37). Thus, it can be argued that reduced circulating IGF-I levels and high IGFBP-I levels may have direct effect on glucose homeostasis. Generally poor glycemic control in type I diabetes, the most important risk factor for diabetic complications, is associated with reduced rather than elevated IGF-I levels (34).

In diabetic subjects " tissue wounding" is a more common phenomenon than in normal subjects because of alterations in tissues induced by glycosylation, cross-linking of proteins, shear stress and a variety of other changes (38). Moreover, it is now accepted that wound repair of many types requires an increase of IGF-I in the damaged area (39). In diabetic animals an impairment in tissue repair has been observed due to a deficiency in the production of IGF-I (40).

In our study circulating free IGF-I levels were decreased to a lesser extent (after adjustment for age) in IDDM patients with retinopathy than in those without. Hansson et al. have shown that immunoreactive IGF-I is greatly increased in areas of angiogenic activity (41). The difference in free IGF-I levels may thus be the expression of a different degree of (retinal) angiogenesis between IDDM patients with retinopathy and those without. Angiogenesis occurs as response to ischemia

caused by nonperfusion of the smaller retinal blood vessels and capillaries (42). This ischemia may initiate an increased release of factors (like IGF-I) which stimulate local vasoproliferation and vasodifferentiation in an latest and abortive attempt to provide nutrition for tissue deprived of the required amount of oxygen (42). Diabetic proliferative retinopathy is considered in this view as an insufficient adaptive growth response of the retinal microvessels. This hypothesis fits well with data which suggest that alterations of growth factors (like IGF-I) occur late in the sequence of events leading to angiogenesis, but do not play an early or initiating role ((43) , see Figure 1).

IGF-I has also been found to prevent and/or to protect -in a dose dependent fashion against glucose-induced inhibition of neuronal growth and apoptosis in sensory neurons in vitro (44). Since the retina consists of neuronal tissue, while apoptosis is a characteristic feature of diabetic retinopathy (45), we hypothesize that the observed relative steeper decrease of serum free IGF-I levels in IDDM subjects during aging in comparison to healthy controls (see Paragraph 5.1, Figure 2), might be a mechanism contributing to the progression of diabetic retinopathy during aging. In other words, the progressive decline of serum free IGF-I levels and IGF-I bioactivity during aging in diabetics, may ultimately result in a progressive loss of anti-apoptotic effects by IGF-I on retinal cells, and in the progression of diabetic retinopathy. Unfortunately no data are available on free IGF-I levels in vitreous fluid of eyes from diabetic patients with proliferative retinopathy.

The hypothesis that IGF-I is involved in the control of GFR is supported by the observation that GH-deficient patients have lowered renal plasma flow (RPF) and GFR which both normalize during GH replacement therapy (46); also infusion of IGF-I increases GFR and RPF (47, 48). We observed lower values of serum (free and total) IGF-I levels in IDDM patients than in sex- and age-matched controls, which suggests at first glance no important role for IGF-I in the pathogenesis of renal hyperfiltration in IDDM.

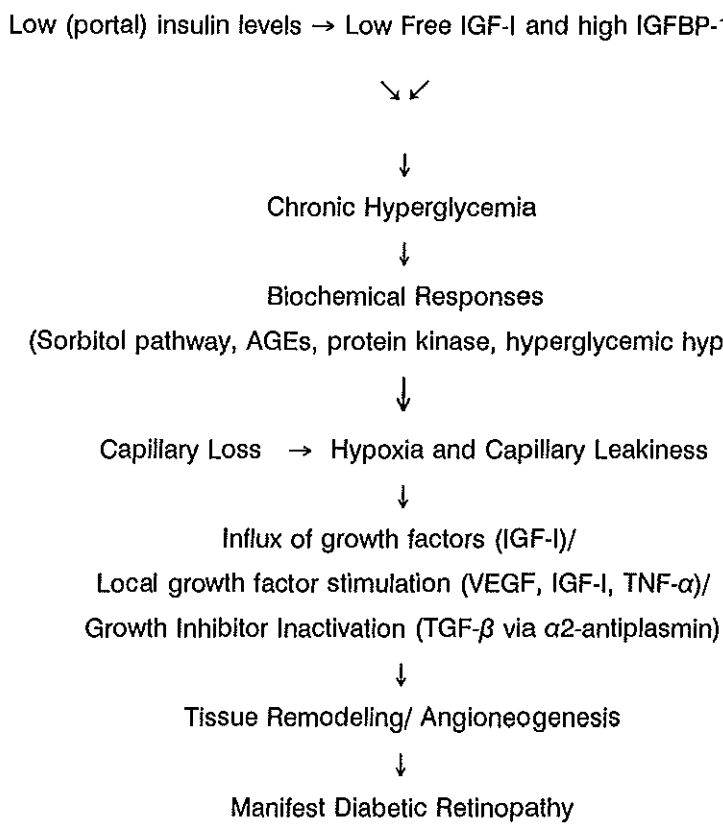

Figure 1. A tentative model of the pathogenesis of diabetic proliferative retinopathy (modified after (42)).

However, in an experimental animal model of diabetes, which was also characterized by low to normal serum IGF-I levels, an increased binding of IGF-I to the IGF-I receptors in the kidney was found in comparison to controls (49). In accordance with this observation, after infusion of recombinant human IGF-I, plasma free IGF-I and IGF-I bound IGFBP-3 levels were at any dosage lower in diabetic rats than in normal rats, due to the more rapid distribution and/or elimination of IGF-I from plasma in diabetic rats (50). The availability of circulating IGF-I may thus determine the degree of kidney IGF-I accumulation in diabetic animals, and, therefore, its contribution to the alterations that precede diabetic nephropathy (51).

It has been suggested that IGF-I contributes to the kidney hypertrophy and glomerular hyperfiltration found in diabetes (52). Kidney hypertrophy and glomerular hyperfiltration are considered in diabetes mellitus as early symptoms detrimental to the kidney (53). However, kidney hypertrophy and glomerular hyperfiltration per se may not be detrimental to the kidney. Glomerular hypertrophy and glomerulosclerosis are not tightly linked (54). Mice transgenic for IGF-I develop glomerular hypertrophy but do not develop glomerulosclerosis (55). Renal disease is not observed in acromegaly (56), and pregnancy-induced hyperfiltration does not predict renal disease in non-diabetic individuals (57). Also, normal individuals with one kidney and hyperfiltration in that kidney appear to have a good prognosis and will usually not develop renal disease (58). In ischemic acute renal failure, it has been suggested that IGF-I, by stimulating hyperfiltration and renal blood flow, provides oxygen and nutrients to ischemic kidney cells thereby protecting these cells from dying (59). We hypothesize that increased binding of IGF-I to the IGF-I receptor in IDDM subjects might also protect kidney cells from ischemia (hypoxia). The progressive decrease of serum IGF-I levels in diabetic patients starting in late puberty, might subsequently lead to a progressive loss of these IGF-I-dependent protective mechanisms during later life, thereby contributing to the development of manifest diabetic nephropathy.

Our findings may have important therapeutic consequences for the treatment of IDDM. It has been suggested that under normal physiological conditions insulin

and IGF-I, and the insulin receptors and IGF-I receptors keep each others effects in check (60). Insulin is necessary for a normal GH response with a subsequent response of IGF-I (see Chapter 1, Figure 5). In IDDM there seems to be a derangement of the growth hormone(GH)-IGF-I axis (61). The mechanism behind the imbalance in the GH-IGF-I axis in IDDM has been suggested to be due to low portal insulin levels leading to decreased production of IGF-I with subsequent increased GH levels (62). The elevation in GH, due to loss of feedback inhibition of IGF-I as a result of low insulin, may worsen hyperglycaemia by counteracting insulin's action. The use of recombinant IGF-I has therefore proposed as an adjunct to insulin therapy and several recent studies suggest indeed beneficial effects of recombinant IGF-I in IDDM (34, 63-66).

However, the initial enthusiasm concerning possible beneficial effects of recombinant IGF-I therapy in IDDM has been tempered by reported side effects of IGF-I in several studies. The main reasons for the occurrence of these side effects may be the use of supraphysiologic doses of recombinant IGF-I (uncomplexed to IGFBPs) and/or the use of total serum IGF-I levels to monitor IGF-I therapy. However, we propose that free IGF-I levels, supposedly the major biologically active hormonal form of IGF-I, should be primarily used to monitor recombinant IGF-I therapy. Measurement of serum free IGF-I levels may be necessary to ensure that only safe IGF-I doses are used which restore the normal physiological function of the GH-IGF-I axis. To illustrate this latter problem with an example: when normal human subjects were infused with 10 μg IGF-I/kg per h for several days (67), fasting plasma glucose levels statistically significant fell from 4.7 to 4.3 mmol/L, total IGF-I levels rose from 31 to 133 nmol/L (about 4 fold increase) and free IGF-I levels rose from 0.08 nmol/L to 1.5 nmol/L (almost 20 times increase). (For comparison: we observed mean free IGF-I levels of 0.15 nmol/L with a range of 0.04-0.58 nmol/L in healthy controls in Paragraph 5.1).

In Paragraph 5.2 we have studied the role of the IGF-I/IGFBP system in four patients with congenital partial lipodystrophy. In the existing literature the acromegaloid features, acanthosis nigricans, and polycystic ovaries frequently observed in subjects with congenital partial lipodystrophy usually have been

attributed to hyperinsulinemia and insulin interaction with the IGF-I receptor. Our study suggests that the acromegaloid features, acanthosis nigricans, and polycystic ovaries in this syndrome, may also be the consequence of a direct interaction of IGF-I to its receptor through an increased free IGF-I/IGFBP-1 ratio. We suggest that the circulating increased free IGF-I/IGFBP-1 ratio in congenital partial lipodystrophy may reflect increased IGF-I bioactivity at the target cell and is caused by the hyperinsulinemia.

In conclusion, our studies suggest that determination of free IGF-I and IGFBPs is of help to study normal physiology and pathologic changes of the IGF-I/IGFBP system during aging. Determination of free IGF-I and IGFBPs seems important in understanding situations in which there is an apparent discrepancy between GH secretion and total IGF-I levels. It is of diagnostic value in acromegaly and might help to assess clinical disease activity in diseases like GH deficiency.

Determination of free IGF-I and IGFBPs give new insights in the pathogenesis of diabetic microvascular complications and might be an important tool to assess the effect of IGF-I therapy in diabetes. Also it helps to unravel the contribution of the endocrine vs the local IGF-I/IGFBP system in the complex regulation of metabolism and growth in health, aging and disease. The studies presented in this thesis suggest that both the determination of free IGF-I as well as of IGFBPs 1 and 3 in serum are valuable methodological contributions to a better understanding of the IGF/BP system.

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Summary

The aim of this thesis was to investigate the (patho)physiologic role of the insulin-like growth factor I/insulin-like growth factor binding proteins (IGF-I/IGFBP) system in health and disease under several conditions like aging, GH deficiency, acromegaly, diabetes mellitus and congenital partial lipodystrophy.

In **Chapter 1** the homologies in molecular structure and functions between IGF-I and insulin and their respective receptors is discussed. IGF-I, like insulin, consists of an A- and B-chain, a conserved connecting C-peptide, which is shorter than the C-peptide of insulin, and a D-domain. IGF-I, initially also termed non-suppressible insulin-like activity, stimulates glucose uptake, amino acid uptake, and inhibits lipolysis. IGF-I also has insulin-like effects on cellular proliferation and differentiation. Since IGF-I at high concentrations crossreacts with insulin receptors and insulin at high concentrations crossreacts with the IGF-I receptor, it is difficult to distinguish the components activated in the IGF-I signal transduction pathway through the IGF-I receptor from those activated via the insulin receptor. IGF-I and insulin are complementary in their anabolic actions in vivo and although there is also some overlap in functions: IGF-I, be it as an endocrine hormone or as an autocrine/paracrine cytokine, is considered to be more primarily involved in cell and tissue differentiation, whereas insulin mediates more primarily the metabolic functions such as lowering of blood glucose and glucose homeostasis.
Most circulating IGF-I is bound to the insulin-like growth factor binding proteins (IGFBPs), which titrate the supply of IGF-I to its receptors in the target tissues, while a very small component circulates as the free form. The IGFBPs are a family of proteins that non-covalently bind IGF-I with high affinity. To date at least six IGFBPS have been identified. They have a high degree of sequence homology. The IGFBPs have been proposed to have four major functions that are essential to coordinate and regulate the biological activities of the IGF-I: 1] to act as transport proteins in plasma and to control the efflux of IGF-I from the vascular space; 2] to prolong the half-life of IGF-I and thereby regulating its metabolic clearance; 3] to provide a means of tissue- and cell-type specific localization; and 4] to directly modulate interaction between IGF-I and the IGF-I receptor, thereby indirectly

controlling its biological actions. Free IGF-I, by analogy with sex and adrenal steroids and thyroid hormones, may be the major biological active hormonal form of IGF-I. The levels of free IGF-I at the tissue level are modulated by the rate of IGF-I production, the clearance, and the degree of binding to IGFBPs. Growth hormone (GH) and insulin both stimulate hepatic IGF-I production, and IGF-I feeds back to suppress GH and insulin release. Age is also a major determinant of plasma IGF-I concentrations. Part of the age-related decrease of plasma IGF-I levels in adults is believed to be due to the decrease in GH secretion after puberty. The aim of this thesis is presented in the final part of chapter I.

In **Chapter 2** we have investigated the relationship between the IGF-I/IGFBP system and blood cells in healthy subjects. Is it possible to measure IGF-I receptors on blood cells in vitro and what is the (clinical) importance of this assay. We studied especially the following questions: do IGF-I receptors on peripheral blood cells reflect what happens with the IGF-I receptors on other IGF-I target cells elsewhere in the body and what are the relationships and effects of (free and total) IGF-I and the IGFBPs on IGF-I receptor characteristics and what happens with the IGF-I receptor characteristics after an oral glucose load? We have adapted and further developed a previously described method to measure the IGF-I receptor characteristics on blood cells in vitro. The observed affinity constants for the IGF-I receptor on blood cells using this method showed a concentration range that was the same as that measured for serum free IGF-I levels. The number and affinity of IGF-I binding sites on PBMCs did not change 120 minutes after an oral glucose load. Our observations suggest that studies of the IGF-I receptor characteristics on blood cells are cell-specific, and cannot be extrapolated to other cell types in the body without restrictions. We therefore concluded that measurement of IGF-I receptors on blood cells does not give a good impression of the action of IGF-I on other (relevant) target tissues in the body and do not acutely change after a oral glucose load.

In **Chapter 3** we have investigated the relationships between the IGF-I/IGFBP system and age, sex steroids, cardiovascular diseases, bone mineral density and quality of health in a healthy elderly population. Other questions studied were: what is the potential significance of measuring serum free IGF-I levelsand does the measurement of serum free IGF-I levels give additional and/or other information than the total IGF-I assay?

We observed a decrease of total serum IGF-I and IGFBP-3 levels with aging in a healthy elderly population (age range 55-88 years). Free serum IGF-I levels did not decrease in this age range, and tended to be higher in subjects over 70 years. We did not observe a relationship between free serum IGF-I levels and free androgen and free oestrogen indices in this healthy aging population.

Higher serum free IGF-I (but not total IGF-I) levels were associated with a lowered prevalence of cardiovascular disease, while higher IGFBP-1 levels were associated with a more favourable cardiovascular risk profile.

Circulating (total and free) IGF-I levels were positively related to bone mineral density in the lumbar spine in men but not in women.

Finally, lowered free IGF-I (but not total IGF-I) levels were observed in subjects who felt less healthy in comparison to other individuals of their own age.

The results of Chapter 3 suggest that measurement of free serum IGF-I levels provides additional and other information concerning the IGF-I/IGFBP system, which would not be obtained by measuring total serum IGF-I levels. We observed a discrepancy in the ratio of free and total IGF-I levels under several (patho)physiologic circumstances. Since the free serum IGF-I levels measured in our study probably reflect the majority of the biologically active fraction of IGF-I, the observed relationships for free IGF-I suggest that IGF-I has predominantly anabolic effects in this aging population.

In **Chapter 4** the potential role of the IGF-I/IGFBP system in disorders of GH secretion (GH deficiency, acromegaly) were investigated. We have studied the following questions: Whom is to be tested and how to diagnose growth hormone deficiency in adults? Which components of the circulating IGF-I/IGFBP system can

be used for this purpose? Do serum IGFBP-3 and/or free IGF-I levels reflect the clinical activity in acromegaly better than serum total IGF-I levels?
Besides discussing the parameters to measure when growth hormone deficiency is suspected, we have presented recommendations to answer the question which individuals should be tested for growth hormone deficiency in the first place.
Measurement of circulating IGFBP-3 levels provides no (additional) advantages above serum total IGF-I levels in the diagnosis of acromegaly and we observed that both serum total and free IGF-I levels are suitable biochemical parameters for screening for acromegaly, although total and free serum IGF-I levels do not correlate with disease activity in acromegaly.

In **Chapter 5** we have focused on the relationship between the IGF-I/IGFBP system, age and the presence of diabetic microvascular complications in insulin-dependent diabetes mellitus (IDDM) type I. We observed lower fasting free IGF-I and total IGF-I levels and IGFBP-3 levels and higher IGFBP-1 levels in insulin-treated adult IDDM subjects than in age- and sex-matched controls. In addition, free IGF-I decreased significantly with age in IDDM subjects, but not in the healthy controls. Circulating free IGF-I levels were decreased to a lesser extent (after adjustment for age) in IDDM patients with retinopathy than in those without.
In chapter 5 we have also studied the relationships between the IGF-I/IGFBP system and the IGF-I receptors on peripheral blood cells in patients with congenital partial lipodystrophy in order to study the role of the IGF-I/IGFBP system in the development of this syndrome which is characterized among other things by severe insulin resistance, hyperinsulinemia and acromegaloid features. Our study suggests that the acromegaloid features, acanthosis nigricans, and polycystic ovaries in this syndrome, may be the consequence of a direct interaction of IGF-I to its receptor through an increased free IGF-I/IGFBP-1 ratio. We suggest that the circulating increased free IGF-I/IGFBP-1 ratio in congenital partial lipodystrophy may reflect increased IGF-I bioactivity at the target cell and is caused by the hyperinsulinemia.

Finally, conclusions and the general discussion are presented in **Chapter 6.**

Our studies suggest that determination of free IGF-I and IGFBPs is of help to study normal physiology and pathologic changes of the IGF-I/IGFBP system during aging. Determination of free IGF-I and IGFBPs seems important in understanding conditions in which there is an apparent discrepancy between GH secretion and total IGF-I levels. It is of diagnostic value in acromegaly and might help to assess clinical disease activity in diseases like GH deficiency.

Determination of free IGF-I and IGFBPs give new insights in the pathogenesis of diabetic microvascular complications and might be an important tool in the future to assess the effect of IGF-I therapy in diabetes. Also it helps to unravel the contribution of the endocrine vs the local IGF-I/IGFBP system in the complex regulation of metabolism and growth in health, aging and disease. The studies presented in this thesis suggest that both the determination of free IGF-I as well as of IGFBPs 1 and 3 in serum are valuable methodological contributions to a better understanding of the IGF/BP system.

Samenvatting

In dit proefschrift werd bij volwassenen de rol van de insuline-achtige groeifactor I (in het Engels afgekort als: IGF-I: Insulin-like Growth Factor I) en de eiwitten, waaraan het IGF-I gebonden is (in het Engels afgekort als IGFBPs: Insulin-like Growth Factor Binding Proteins) onderzocht. Zo werd gekeken naar veranderingen in het IGF-I en de IGFBPs bij het ouder worden, bij mensen met een tekort aan groeihormoon op de volwassen leeftijd (groeihormoondeficiëntie), bij mensen, die continu teveel groeihormoon aanmaken (acromegalie), bij mensen met suikerziekte, die helemaal geen insuline meer kunnen maken (ook wel type 1 insuline-afhankelijke diabetes genoemd) en een zeer zeldzame ziekte, de congenitale partiële lipodystrofie, een aandoening, die onder andere gekenmerkt wordt door insuline resistentie (van insulineresistentie is sprake wanneer normale insuline spiegels de bloedsuikers onvoldoende kunnen doen dalen. Als gevolg hiervan hebben personen met congenitale partiële lipodystrofie hogere insuline-spiegels (nodig) om een normale bloedsuikerspiegel te bereiken).

In **Hoofdstuk 1** worden de overenkomsten tussen de moleculaire structuur en de functies van respektievelijk het IGF-I en het insuline, en de IGF-I- en insuline-receptoren besproken. Het IGF-I molecuul is (vergelijkbaar met het insuline) opgebouwd uit een zogenaamde A en B keten, een C(connecting)-peptide, en een D-keten. Het IGF-I werd aanvankelijk in de literatuur aangeduid als de non-supressible insulin-like factor, dat wil zeggen een factor, die weliswaar de werking van insuline vertoonde, maar waarvan de werking niet onderdrukt kon worden met antilichamen, die specifiek gericht waren tegen het insuline molecuul.
Het IGF-I stimuleert evenals insuline de opname van glucose en aminozuren in de weefsels en onderdrukt de vetafbraak. Het IGF-I heeft daarnaast evenals insuline een stimulerende werking op celgroei en -differentiatie.
Aangezien IGF-I zich bij relatief hoge concentraties ook nog kan binden aan de insuline receptor, en omgekeerd het insuline zich bij relatief hoge concentraties kan binden aan de IGF-I receptor, is het moeilijk om de effecten van IGF-I en van insuline van elkaar te onderscheiden. Bovendien vullen IGF-I en insuline elkaar aan (zijn complementair) in hun werking in het menselijk lichaam. De functies van

beide moleculen en hun receptoren overlappen elkaar in zekere mate. Toch is er een zekere mate van specialisatie: IGF-I lijkt vooral betrokken bij (de vaak chronische) effecten op celgroei en -differentiatie, terwijl het insuline de belangrijkste rol speelt bij de (meer acute) metabole effecten op de suiker- en vetstofwisseling.

Ongeveer 99% van het IGF-I is in het bloed in een niet-vrije vorm aanwezig en gebonden aan eiwitten (de reeds bovengenoemde IGFBPs). Deze IGFBPs beïnvloeden de mate waarin het IGF-I zich kan binden aan de IGF-I receptor. Op dit moment zijn er in ieder geval zes verschillende IGFBPs bekend. De belangrijkste vier functies van de IGFBPs zijn: 1]het transporteren van het IGF-I in het bloed en het transport van het IGF-I vanuit de bloedbaan naar de weefsels, 2] het reguleren van het metabolisme en daardoor het beïnvloeden van de halfwaardetijd van het IGF-I in het bloed en in de weefsels, 3] het afgeven van IGF-I op een cel- en weefselspecifieke manier, en 4] het direct beïnvloeden van de interactie tussen IGF-I en haar receptor. Op deze manier worden door de IGFBPs langs een indirecte weg de biologische effecten van het IGF-I gecontroleerd.

Slechts een klein gedeelte van het IGF-I is in het bloed in een vrije (niet aan de IGFBPs gebonden) vorm aanwezig (ongeveer 1%). De vrije (niet gebonden) vorm van het IGF-I is te beschouwen als de belangrijkste biologische werkzame vorm van IGF-I in het lichaam en het vrije IGF-I is in dit opzicht vergelijkbaar met de vrije schildklierhormoon- en geslachtshormoonspiegels. De mate waarin het IGF-I in de vrije (niet gebonden) vorm in het serum aanwezig is, is afhankelijk van de mate van produktie en klaring van het IGF-I, en van de binding van het IGF-I aan de IGFBPs.

Het groeihormoon en het insuline hebben ook invloed op de IGF-I spiegels in het bloed en stimuleren beiden de aanmaak van het IGF-I in de lever. Het IGF-I heeft op zijn beurt weer een negatieve feedback op de groeihormoon- en insuline-afgifte. Leeftijd is een ander belangrijke factor, die effect heeft op de spiegels van het IGF-I in het bloed. De daling van de IGF-I spiegels in het bloed, die doorgaans met het ouder worden optreedt, is volgens de huidige inzichten ten dele toe te schrijven aan een afname van de groeihormoonsecretie met de leeftijd.

De vraagstelling van dit proefschrift wordt in het laatste deel van hoofdstuk 1 gepresenteerd.

In **Hoofdstuk 2** werden bij gezonde vrijwilligers de relaties tussen IGF-I en de IGFBPs enerzijds en bloedcellen anderzijds onderzocht. Is het mogelijk om IGF-I receptoren op bloedcellen te meten onder laboratorium omstandigheden en wat heb je daaraan in de kliniek? Specifiek werden de volgende vragen bestudeerd: weerspiegelen de IGF-I receptoren op bloedcellen de situatie van de IGF-I receptoren in andere weefsels van het menselijk lichaam en wat zijn daarbij de relaties en effecten van het (vrije en totale) IGF-I en de IGFBPs in het bloed op de affiniteit en het aantal bindingsplaatsen van de IGF-I receptor aanwezig op bloedcellen. Zijn er acute veranderingen van de IGF-I receptorkarakteristieken zichtbaar na een acute glucose gift? Om de IGF-I receptor op bloedcellen te meten hebben we een eerder beschreven methode aangepast en verder ontwikkeld waarmee in het laboratorium een aantal karakteristieken van de IGF-I receptor (aantal bindingsplaatsen en affiniteit) op bloedcellen gemeten kunnen worden. De zo door ons gevonden waarden voor de affiniteits-constanten van de IGF-I receptor op (rode en witte) bloedcellen, vertoonde dezelfde concentratie range als die van de vrije IGF-I spiegels in het serum. Er werden geen veranderingen gezien in de IGF-I receptor karakteristieken na een acute glucose gift. Onze experimenten suggereren dat de bestudering van de IGF-I receptor cel- en weefsel-specifieke resultaten oplevert en dat de IGF-I receptor in het ene weefsel niet zonder meer, die in andere weefsels weerspiegelt. Hieruit hebben we de conclusie getrokken dat de meting van IGF-I receptoren op bloedcellen niet gebruikt kan worden om een goede indruk te krijgen over de werking van het IGF-I en de IGF-I receptor op andere (relevante) doelwitweefsels in het lichaam. Bovendien lijkt het erop dat de IGF-I receptor geen acute veranderingen vertoont na een orale glucose belasting.

In **Hoofdstuk 3** hebben we gekeken naar de relaties van het IGF-I/IGFBP systeem met , respektievelijk, de leeftijd, geslachtshormonen, het optreden van cardiovasculaire ziekten, botdichtheid en de kwaliteit van leven in een populatie

gezonde ouderen. Andere vragen, die hierbij door ons aan de orde zijn gesteld, waren: wat is de betekenis van het meten van vrije IGF-I spiegels in het serum en geeft bepaling van de vrije IGF-I spiegel in het serum additionele en/of andere informatie dan bepaling van de totale IGF-I spiegels in het serum?
In een gezonde populatie ouderen in de leeftijd van 55 tot 80 jaar oud, vonden we met toenemende leeftijd een afname van de totale IGF-I en IGFBP-3 spiegels in het serum. De vrije serum IGF-I spiegels namen in deze groep ouderen echter niet af met de leeftijd. We vonden zelfs relatief de hoogste spiegels in personen van zeventig jaar en ouder. Er waren bovendien geen relaties tussen de vrije serum IGF-I spiegels en de vrije androgeen- en vrije oestrogeen-indices in deze gezonde oudere populatie.
Hogere vrije IGF-I spiegels in het serum waren geassocieerd met een verminderde prevalentie van cardiovasculaire ziekten, terwijl een dergelijk verband niet voor de totale IGF-I spiegels in het serum werd gevonden. Hogere IGFBP-1 spiegels in het serum waren geassocieerd met een gunstiger cardiovasculair profiel.
Circulerende (totale en vrije) IGF-I spiegels waren positief geassocieerd met een hogere botdichtheid in de lumbale wervelkolom bij oudere mannen maar niet bij oudere vrouwen. Tenslotte, werden verlaagde vrije (maar niet totale) IGF-I spiegels in het serum gevonden bij die ouderen, die zich minder gezond voelden dan hun leeftijdsgenoten.
De resultaten beschreven in Hoofdstuk 3 suggereren dat de meting van vrije IGF-I spiegels in het serum in een aantal gevallen additionele en andere informatie oplevert over het IGF-I/IGFBP systeem dan die verkregen wordt na de meting van de totale IGF-I spiegels in het serum. Aangezien de vrije IGF-I spiegel in het serum waarschijnlijk de belangrijkste biologische fractie van het IGF-I in het lichaam weerspiegelt, suggeren onze resultaten dat IGF-I vooral anabole effecten heeft in bij ouderen.

In **Hoofdstuk 4** werd bekeken welke personen in eerste instantie getest dienen te worden op groeihormoondeficiëntie en welke parameters hiervoor gebruikt dienen te worden. We stelden ons in het bijzonder de vraag welke componenten van het

circulerend IGF-I/IGFBP systeem voor dit doel gebruikt kunnen worden. Bovendien stelden we ons de vraag of door meting van IGFBP-3 en/of vrije IGF-I spiegels in het serum betere informatie kan worden gekregen over de klinische activiteit van acromegalie dan door de meting van de totale IGF-I spiegels in het serum.
Behalve het bespreken van de parameters, die ons inziens gebruikt dienen te worden om een groeihormoon-tekort te objectiveren, presenteren we in dit hoofdstuk een praktische beslisboom, die gebruikt kan worden om uit te maken of iemand op het bestaan van groeihormoondeficiëntie getest dient te worden.
We stelden vast dat meting van IGFBP-3 spiegels in het serum geen extra voordelen heeft boven het bepalen van de totale IGF-I spiegels in het serum bij het stellen van de diagnose acromegalie. We vonden dat zowel de totale als de vrije IGF-I spiegels in het serum gebruikt kunnen worden voor de screening op acromegalie, maar dat beide parameters niet goed correleren met de klinische ziekteaktiviteit van de acromegalie.

In **hoofdstuk 5** hebben we gekeken naar de relatie tussen het IGF-I/IGFBP systeem, leeftijd, en het optreden van diabetische microvasculaire complicaties bij type I insuline-afhankelijke diabetes mellitus (IDDM). In de nuchtere toestand vonden we lagere vrije en totale IGF-I spiegels, lagere IGFBP-3 spiegels en hogere IGFBP-1 spiegels in het serum bij IDDM patiënten dan in een voor de leeftijd- en geslacht gematchte gezonde controle groep. Bovendien namen de vrije IGF-I spiegels in het serum bij IDDM diabetes patiënten in tegenstelling tot de gezonde controle groep significant met de leeftijd af. De vrije IGF-I spiegels in het serum waren (na correctie voor de leeftijd) relatief minder verlaagd bij IDDM patiënten met diabetische retinopathie dan bij patiënten zonder diabetische retinopathie.
In hoofdstuk 5 hebben we ook de relatie van het IGF-I/IGFBP systeem met de IGF-I receptoren op perifere bloedcellen bestudeerd bij patiënten met congenitale partiële lipodystrophie. We wilden nader bekijken of er veranderingen zijn in het IGF-I/IGFBP systeem bij deze patiënten en wat de rol van het IGF-I/IGFBP systeem is bij het onstaan van dit syndroom. Een congenitale partiële lipodystrophie wordt onder andere gekenmerkt door een ernstige insuline resistentie, hyperinsulinemie

en door acromegale kenmerken. Onze studie suggereert dat de acromegale kenmerken, maar ook het bij dit syndroom vaak optredende polycysteuze ovarium syndroom en acanthosis nigricans het gevolg zou kunnen zijn van een verhoogde directe interactie van IGF-I met de IGF-I receptor secundair aan het verhoogde vrije IGF-I/IGFBP-1 ratio in het serum bij deze patiënten.

Tenslotte worden in **Hoofdstuk 6** conclusies en de generale discussie gepresenteerd. De resultaten van onze studies suggereren dat het meten van het vrije serum IGF-I en de IGFBPs zinvol is om de normale fysiologie maar ook pathologische veranderingen in het IGF-I/IGFBP systeem bij het ouder worden te bestuderen. Bepaling van de vrije serum IGF-I spiegel en de IGFBPs lijkt belangrijk om die (pathologische) situaties te begrijpen waarbij er ogenschijnlijk een discrepantie bestaat tussen de mate van groeihormoonsecretie en de circulerende totale IGF-I spiegels. Ook kunnen ze een hulp zijn bij het stellen van de diagnose acromegalie en het meten van ziekteaktiviteit bij aandoeningen als groeihormoon-deficiëntie. Bepaling van de vrije IGF-I spiegels en de IGFBPs kan nieuwe inzichten in de pathogenese van de diabetische microvasculaire complicaties geven en kan in de toekomst een een belangrijk hulpmiddel worden om het effect van IGF-I therapie bij diabetes te monitoren. Het kan helpen om een beter inzicht te krijgen wat de bijdrage is van het endocriene IGF-I/IGFBP systeem en wat de bijdrage van het autocriene/paracriene IGF-I/IGFBP systeem in gezonde situaties, bij het ouder worden en bij het optreden van ziekten.
De gepresenteerde studies in dit proefschrift suggereren dat zowel de bepaling van het serum vrije IGF-I als van IGFBP-1 en IGFBP-3 waardevolle bijdragen leveren om de betekenis van het IGF-I/IGFBP systeem beter te kunnen begrijpen.

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Dankwoord

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