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Divergence of Binding Sites, *in Vitro* Action, and Secretory Regulation of the Somatomedin Peptides, IGF-I and IGF-II (41428)

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Three separate lines of research, initially perceived as unrelated, have converged to form the present field of the somatomedins and the insulin-like growth factors (see (1, 2) for early reviews). Because these separate areas of research evolved their own terminology, there is a confusing array of names for the different factors under study. The first of these areas of research was based on the observation that a key target tissue of growth hormone *in vivo* responsible for the skeletal growth, namely cartilage, is not responsive to growth hormone *in vitro*. This tissue, however, is highly responsive to a GH-dependent factor in serum originally given the operational designation of "sulfation factor," because it was convenient to monitor the response by measuring sulfate uptake into cartilage. Later when extracts containing this factor were shown to evoke pleiotypic responses in cartilage and certain other tissues, the less restrictive term, "somatomedin," was introduced. Subsequent fractionations yielded three preparations believed to have somatomedin activity: a neutral fraction designated somatomedin A, an acidic fraction, somatomedin B, and a basic fraction, somatomedin C.

The second contributing line of research was based on the observation that only a relatively small fraction of the insulin-like action of serum on fat and muscle tissue *in vitro* could be neutralized by anti-insulin serum. Much of this nonsuppressible insulin-like activity (NSILA) could be extracted in acid–ethanol and was designated NSILA_s (the subscript stands for soluble), and the residual activity was insoluble and was called NSILA_p. When purification progressed further to show two separate fractions with NSILA_s activity, they were called NSILA_s-I and NSILA_s-II.

The third line of research contributing to this field arose from attempts to determine the constituents of bovine serum which stimulated cell division and growth of fibroblasts in culture. A partially purified fraction enriched for this action was called multiplication stimulating activity or MSA. A fibroblast stimulating activity present in conditioned media of Buffalo rat hepatocyte culture was also called MSA and has been used extensively in tissue culture studies.

Major advances in our knowledge of the structure of the somatomedin peptides has led to a new understanding of the structural and functional relationships between these various partially purified materials. Particularly significant are the contributions of Rinderknecht and Humbel (3, 4) in establishing the primary sequence of the two peptides originally called NSILA_s-I and -II. These closely similar but not identical peptides have extensive sequence homology with proinsulin and more remote homology with nerve growth factor and relaxin. To emphasize the relationship with insulin these workers renamed NSILA_s I and II "insulin-like growth factors-I and -II" (IGF-I and IGF-II).

The relationship of somatomedin C (Sm-C), a basic peptide, under study by Van Wyk and associates, to IGF-I has been partially clarified by a recent paper by Svoboda *et al.* (5). Twenty-two amino acids of the N terminal portion of the Sm-C molecule had identical sequences with IGF-I. Two tryptic fragments, however, had amino acid sequences unrepresented in the IGF-I molecule indicating that Sm-C differed slightly in structure or that the preparation was not homogeneous. In addition to this structural similarity, IGF-I and somatomedin-C are identical in their immunologic determinants, receptor binding properties and certain *in vitro* biologic actions; it is

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clear that somatomedin-C is in fact identical to or very similar to IGF-I (6).

Rats also possess a basic somatomedin that has been isolated by Rubin *et al.* (7) from serum of rats bearing a GH-secreting transplanted pituitary tumor. The isolated peptide was homogeneous as judged by SDS–polyacrylamide gel electrophoresis and amino terminal end group analysis. Amino acid analysis indicated a molecular weight of about 8000. The amino acid sequence of the first 29 N terminal residues established that 26 of the residues were identical to those in IGF-I. The sequence data, cross-reactivity with Sm-C antisera, and the receptor properties of this basic rat somatomedin provide strong evidence that this peptide is the rat equivalent of human IGF-I.

Although a great deal of important research has been conducted with peptides released into the medium by Buffalo rat hepatocytes in culture, the characterization of this multiplication stimulating activity (MSA) has been difficult. Moses *et al.* (8) found that when crude MSA peptides were gel filtered through G-75 Sephadex, most of the biologic activity resided in two of three major regions of peptide elution. Peak II material consisted of at least four separate active peptides after acid urea electrophoresis. The most prominent peptide, Peak II_1, appeared to be homogeneous with an estimated molecular weight of 8700. Peak III contained a number of peptides of smaller molecular weight. One component Peak III_2 had an estimated molecular weight of 7000 and was more than three times more potent than II_1 in a receptor binding system.

Marquardt *et al.* (9) have succeeded in preparing a homogeneous peptide from MSA Peak III material by high-performance liquid chromatography. Like IGF-II, it contained 67 amino acids. Amino acid sequencing of this peptide established that, except for five conservative amino acid substitutions, the sequence was identical to that of human IGF-II. It is likely that the heterogeneity of the larger MSA peptides present in the crude MSA preparations represents incomplete enzymatic processing of a preprohormone precursor.

There has been clarification of the status of a serum peptide fraction reported from Stockholm with mitogenic properties on human glial cells referred to as somatomedin B. Heldin *et al.* (10) have now shown that the mitogenic activity of somatomedin B is attributable to EGF contamination of the preparations. Because of this finding the term somatomedin B should be abandoned.

Another somatomedin fraction, isolated by Stockholm workers, somatomedin A, has the charge and size properties of IGF-II but also cross-reacts to a limited extent with the IGF-I/Sm-C antibody (11). It may represent a mixture of these two peptides.

An acidic peptide, ILS, has been isolated in partial purity by Posner *et al.* (12). This peptide has an isoelectric point of 6.2 to 6.8 by isoelectric focusing which may be lower than that of IGF-II, otherwise this peptide fraction closely resembles IGF-II in its receptor binding properties. Structural information is not available.

We can conclude from the above brief review of recent contributions that simplification of the classification of the somatomedin and insulin-like growth factors is now possible. In both man and rat two major insulin-like growth factors exist. The first is IGF-I which may be identical to somatomedin C. In the rat the basic somatomedin is the counterpart of human IGF-I. The second growth factor in the human is IGF-II which is probably similar to or contained in somatomedin A and ILS preparations. The most potent peptide present in the MSA preparations of rat origin is structurally very similar to human IGF-II. The molecular heterogeneity which exists in MSA and other insulin-like growth factors may represent post-translational or postsecretory molecular modifications. Because at least two species possess the same two types of insulin-like growth factors, it is important to ask whether or not these closely related peptides represent simple redundancy without functional differences or whether functional divergence can be recognized.

The comparative biologic effects of IGF-I and IGF-II on several tissues have been studied by Zapf *et al.* (13). In incubations with rat epididymal fat pads both peptides equally stimulated glucose uptake and glucose oxidation to CO_2. IGF-II was more potent, however, in inhibiting lipolysis by the fat pad. Isolated rat fat cells behaved differently when incubated with [^{14}C]-glucose; IGF-II was two to three times more potent than IGF-I in stimulating $^{14}CO_2$ production and ^{14}C incorporation into lipid. In the case of chick embryo fibroblasts, IGF-I and IGF-II were equally potent in promoting incorporation of [6-^{3}H]thymidine into DNA. IFG-I was more potent than IGF-II in stimulating [^{35}S]sulfate uptake by costal cartilage of fasted immature female rats.

The somatomedins, like other peptide hormones, exert their biologic effects primarily by reacting with cell surface receptors. Information about the cell surface receptors for somatomedins has rapidly developed by studying the competitive binding of ^{125}I-labeled and unlabeled peptides to cells or cell membrane preparations. Zapf *et al.* (13) provided specific information concerning the binding of IGF-I and IGF-II by several tissues. Rat fat cells bound about 8 times as much IGF-II as IGF-I, and IGF-II was much more potent than IGF-I in displacing the two labeled IGFs from its receptors. Chick embryo fibroblasts and chondrocytes bound both labeled peptides equally, and displacement of the two peptides by IGF-I and IGF-II was comparable. Rechler *et al.* (14) have confirmed these findings with chick embryo fibroblasts and went on to study binding of ^{125}I-IGF-I and ^{125}I-IGF-II by human fibroblasts. This binding was preferentially displaced by the homologous peptide. Rat liver membranes bound tracer IGF-II about 10 times more than IGF-I and IGF-II was much more potent in displacing ^{125}I-IGF-II from this binding site than IGF-I. Both peptides were equally potent in displacing ^{125}I-IGF-I from binding sites from liver membranes. With these various binding systems, the binding of ^{125}I-MSA (Peak II_1) was about one-half that of ^{125}I-IGF-II. The displacement produced by unlabeled MSA (Peak II) was similar to that of IGF-II but only 1/10th as potent.

In interpretating the various tissue specificities of IGF-I and -II binding, Zapf *et al.* (13) were unable to answer the question whether or not there were one or two different types of binding sites. Rechler *et al.* (14) concluded from their binding studies that there were three patterns of binding in the various tissues and compared the multiplicity of somatomedin/IGF receptors with the constancy of binding properties of the insulin receptor.

Studies of human and rat placental membranes have provided evidence of two distinct binding sites for IGF-related peptides. Human placental membranes were first observed by Marshall *et al.* (15) to contain separate membrane binding sites for insulin and somatomedin C. When the binding of IGF-I and IGF-II by human placental membranes was investigated in my laboratory, there was evidence of two separate binding systems (16). One system of high affinity and low binding capacity preferentially bound IGF-I; IGF-II was only about a third as effective in competing for this binding site as IGF-I. The second set of binding sites had a lower binding affinity and higher binding capacity for IGF-II. IGF-I had very little ability to displace ^{125}I-IGF-II from this binding site.

The somatomedin/IGF binding characteristics of rat placental membranes proved of great interest. These membranes had little ability to bind ^{125}I-IGF-I but bound IGF-II avidly. IGF-I and basic rat somatomedin had less than 1% of the ability of IGF-II to displace ^{125}I-IGF-II from its binding sites. An MSA preparation was about one-half as potent as IGF-II in displacing IGF-II from rat placental membranes. It is clear from these observations that rat placental membranes are enriched in binding sites which are highly specific for IGF-II and virtually devoid of the IGF-I preferential sites.

Labeling the receptors for IGF/Sm peptides by photo affinity coupling agents has also provided direct structural evidence of the existence of two types of receptors. Bhaumick *et al.* (17) reported that a solubilized receptor in human placental membranes could be photo affinity coupled to a ^{125}I-IGF-I-like peptide. This IGF receptor complex after solubilization migrated in gel electrophoresis with an apparent MW of 240,000. Like the insulin receptor it was converted to a smaller component of 140,000 MW in the presence of 2-mercaptoethanol. The somatomedin binding site thus defined differed from the insulin binding site because insulin could only weakly inhibit ^{125}I-IGF binding. A 30-fold purification of the putative receptor was achieved by lectin–agarose affinity chromatography. This suggests that the receptor is a glycoprotein.

Independently, Kasuga *et al.* (18) described a similar IGF-I receptor on cultured rat hepatocytes. Photo affinity labeling followed by subsequent electrophoresis revealed an apparent molecular weight of 300,000 which decreased to 130,000 after disulfide reduction. Binding of ^{125}I-IGF-I to this site was inhibited by IGF-I and MSA but not by insulin.

Evidence of a second IGF binding site was obtained by Kasuga *et al.* (18) after crosslinking ^{125}I-MSA to cultured hepatocytes and rat liver membranes. These membranes contained sites that bound ^{125}I-MSA in a complex with an apparent molecular weight of 260,000 that was not dissociated into smaller components by dithiothreitol.

This type of somatomedin receptor was also found by Massague *et al.* (19). ^{125}I-MSA was linked to its binding sites on plasma membranes of rat adipocytes and liver and from human placental membranes with three different coupling agents. In every case the affinity-labeled binding site had an estimated molecular weight of 255,000 by polyacrylamide electrophoresis. The binding site was not dissociated by dithiothreitol reduction.

Massague *et al.* (20) have further characterized the IGF-I and IGF-II receptors by the photo affinity coupling technique in rat adipocytes, rat liver, human placenta, and human skin fibroblasts. Only the type II receptor was detected in rat liver membranes whereas the type I receptor was predominant in placental membranes. The IM-9 lymphocytes had only the type I receptors demonstrated by this technique. The authors compared the IGF-I and IGF-II receptor binding with the insulin receptor binding in these cell types and a number of other tissue culture cell types.

Massague and co-workers proposed that the IGF-I (type I) receptor possessed a tetrameric structure composed of two α units, $M_r = 130{,}000$, and two β units, $M_r = 98{,}000$, joined by interchain disulfide bridges. This is the same basic structure previously reported for the insulin receptor. They also showed that the Type I receptor underwent similar proteolytic transformations after exposure to lysosmal proteases and elastase as did the insulin receptor. They predicted that the type I receptor and the insulin receptor would prove to have peptide sequence homology and had evolved from a common ancestral molecule. The structure of the type II receptor appears to be quite different from that of IGF-I in not being subject to dissociation after reduction of S–S bonds. In addition, the binding properties of the type II receptor are heterogeneous in different tissues.

Radioimmunoassays have been described for somatomedin C (21) and IGF-I (22) that have little cross-reactivity with IGF-II. The Sm-C RIA has been performed on unextracted serum under conditions that the authors believed avoided interference from serum binding proteins, but this has been challenged by Daughaday *et al.* (23) who found that direct serum assays detected only about one-third of the total Sm-C/IGF-I demonstrable after simple acid–ethanol extraction. This procedure had an excellent recovery of added IGF-I and excluded binding protein. When applied to the IGF-I RIA described by Zapf *et al.* (22), the acid–ethanol extraction proved superior to acid Sephadex gel filtration. Despite these major methodologic differences the two assays gave qualitatively similar results in disorders of GH secretion. Extremely low levels of Sm-C/IGF-I exist in patients with growth hormone deficiency, and patients with acromegaly have markedly elevated levels of Sm-C/IGF-I.

Specific methods of measurement of IGF-II have recently been described which permit comparison of IGF-I and IGF-II serum concentrations in endocrine and other conditions. Daughaday *et al.* (24) developed a radioreceptor assay which measures the displacement of ^{125}I-IGF-II by unlabeled IGF-II from its binding site on rat placental membranes. As mentioned previously, these membranes are virtually devoid of IGF-I binding sites, and IGF-I and insulin cross-react less than 1% with this assay. Before assay, IGF-II is extracted from small aliquots of serum, and binding proteins are excluded by the acid–ethanol extraction procedure used for the IGF-I RIA that recovers nearly all serum IGF-II. As compared to a normal human serum working standard, serum from normal adult individuals, 20–69 years of age, had 0.73 ± 0.3 unit/ml. This was estimated to be equivalent to 800 ng/ml of IGF-II. Slightly higher values were observed in endocrinologically normal short children and aged individuals more than 70 years of age. The mean concentration of serum of hypopituitary children was 56% of that of the endocrinologically normal short children. A small rise in mean IGF-II measured by RRA occurred after GH treatment. No increase in IGF-II was noted in acromegaly. Ten of fourteen sera from patients with tumor-induced hypoglycemia were higher than normal, but IGF-I measured by RIA was markedly reduced in the sera of these patients. This confirms earlier work of Megyesi *et al.* (25) with a radioreceptor assay with ^{125}I-NSILAs and rat liver membrane that is now known to preferentially detect IGF-II.

Uremia is another condition in which there is divergent regulation of serum levels of IGF-I and IGF-II. In adult hemodialyzed patients, serum levels of IGF-II by RRA are elevated, but IGF-I levels determined by RIA are lowered in the experience of Goldberg *et al.* (26). It seems unlikely that the elevation of IGF-II can be attributed to decreased renal catabolism when at the same time serum levels of the structurally closely related IGF-I are depressed.

IGF-II related peptides may be important in fetal and neonatal development in the rat. Daughaday *et al.* (27) found that at term mean maternal serum was 55% higher than nonpregnant female rat serum. Fetal IGF-II RRA was elevated at birth and rose to more than six times the level in nonpregnant female rats on Day 5 of life. IGF-I RIA levels in fetal and neonatal life are lower than those observed in adult female rats. These findings with the IGF-II RRA are qualitatively similar to the measurement in MSA by RIA by Moses *et al.* (28), but the degree of elevation of MSA by RIA was 20 to 200 times greater in late term fetal rat serum than in maternal serum. By this MSA RIA, the levels are extremely low in normal rat serum. These observations suggest that fetal rat serum contains peptides that are better recognized by the MSA RIA than by the IGF-II RRA.

Zapf *et al.* (22) have recently described a RIA for human serum IGF-II. IGF-I cross-reacts with their antiserum about 10% as potently as does IGF-II. An independent RIA for IGF-I permits correction for this interference. The authors found that the mean level of serum IGF-II was 647 ± 125 (SD) ng/ml whereas in hypopituitary dwarfism it was 252 ± 99. The levels in acromegaly were not elevated. No alteration of serum IGF-II RIA was found in patients with hypoglycemia associated with tumors. IGF-I levels in these patients were reduced.

Hintz *et al.* (29) have raised an antibody to the portion of the IGF-II molecule analogous to the C peptide portion of proinsulin. A RIA with this serum was highly specific for human IGF-II with negligible cross-reaction with IGF-I. The results with this assay confirmed the measurements reported by Zapf *et al.*

These various assays have established that normally IGF-II is present in three to five times the concentration of IGF-I and its serum concentration is less dependent on GH. No increase of IGF-II occurs in acromegaly and the lowering of IGF-II in hypopituitarism is less profound than that of IGF-I. The high levels of IGF-II found by RRA but not by IGF-II RIA in sera of patients with tumor-induced hypoglycemia suggest that the tumor peptide may be immunologically different from normal IGF-II.

In summary the structural information which has been reviewed supports the conclusion that there are two closely related peptides classes with insulin-like and somatomedin properties. Despite their similar structure and overlapping biologic effects, the affinity of these two peptide classes differ for different tissue binding sites. Structural information has confirmed the presence of an IGF-I preferential site composed of subunits analogous to the insulin receptor. This suggests that this receptor evolved from a primitive insulin receptor as the structure of the IGFs diverged from the insulin molecule. Sometime in evolution a separate receptor preferentially binding IGF-II appeared. Its evolutionary precursor is a mystery. The new measurements of serum levels of IGF-I and IGF-II indicate that divergence of regulation of the two peptide classes exist in respect to GH dependence, in fetal life (at least in the rat), in uremia, and in ectopic production by tumors. The analytic tools are now available to define the biologic significance of these peptides and their participation in normal and pathologic states.

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