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HETEROGENEITY OF INSULIN-LIKE GROWTH FACTOR-I AFFINITY FOR THE INSULIN-LIKE GROWTH FACTOR-II RECEPTOR: COMPARISON OF NATURAL, SYNTHETIC AND RECOMBINANT DNA-DERIVED INSULIN-LIKE GROWTH FACTOR-I

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SUMMARY: Although insulin-like growth factors (IGF) I and II bind with high affinity to structurally discrete receptors, they bind with a lesser affinity to each other's receptor. We have evaluated the affinity of five different IGF-I preparations (three natural IGF-I preparations, one synthetic preparation, and one recombinant DNA-derived) for the IGF-II receptor in rat placental membranes, 18-54,SF cells and BRL-3A cells. In all tissues tested, the natural IGF-I preparations demonstrated an affinity for the IGF-II receptor which was 10-20% that of IGF-II. However, the recombinant and synthetic IGF-I preparations exhibited substantially lower affinities than natural IGF-I for this receptor, with only 10-25% reduction in (125-I)iodo IGF-II binding at peptide concentrations up to 400 ng/ml. Radioimmunoassay of the natural IGF-I preparations with an antibody directed against the unique C-peptide region of IGF-II demonstrated that contamination of IGF-I preparations with immunoreactive IGF-II could not exceed 5%. These results demonstrate that IGF-I purified from human plasma has a different affinity for the IGF-II receptor than does synthetic or recombinant IGF-I. Furthermore, these data are consistent wth the hypothesis that IGF-I, itself, may be heterogeneous, and that subforms may vary in their affinities for the IGF receptors. Alternatively, IGF-I preparations which have been considered to be pure may be contaminated with small amounts of IGF-II, resulting in overestimation of the affinity of IGF-I for the type II IGF receptor.

The somatomedins, a family of GH-dependent, insulin-like hormones, are represented in humans by two distinct peptides, insulin-like growth factors (IGF) I and II (1). First purified from human plasma by Rinderknecht and Humbel (2,3), IGF-I and -II consist of 70 and 67 amino acids, respectively. The primary structures of these two polypeptides are highly homologous, with 62% of the amino acid positions identical. While IGF-I and -II have been found to bind to structurally discrete receptors, their homology is reflected in an affinity for each other's receptor (4-8).

Because of the scarcity of natural IGF-I and -II purified from human plasma, attention has been directed towards the production of these polypeptides by solid-phase methods (9-11) or recombinant DNA technology (12). The identity of these laboratory-generated polypeptides with natural IGF-I and -II has been based not only upon amino acid composition and sequence analysis, but also upon their ability to bind to specific antibodies, carrier proteins and receptors. In this study, we have investigated the relative affinities of five IGF-I preparations (three natural, one synthetic, and one recombinant DNA-derived) for the IGF-II receptor in a variety of cells and tissues. Our results indicate striking differences in the affinities of these polypeptides, suggesting structural heterogeneity in these various IGF-I preparations.

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MATERIALS AND METHODS

Peptides: Five different preparations of IGF-I were employed in these investigations. Natural IGF-I was the generous gift of Dr. R. E. Humbel (Zurich) (batch 4), and had been purified to homogeneity (2). A second natural IGF-I preparation, also isolated from outdated human plasma, was the kind gift of Dr. K. Hall (Stockholm) (batch 5 I-1). The amino acid composition of this peptide was indistinguishable from the known composition of IGF-I, as was N-terminal sequence analysis (13). A third natural IGF-I preparation was purified at Stanford University from Cohn fraction IV of human plasma by acid-ethanol extraction, gel filtration and reverse phase liquid chromatography (14). N-terminal sequence analysis and amino acid analysis for tryptophan and histidine have verified that any potential contamination with IGF-II must be less than 1%. Recombinant DNA-derived IGF-I (Thr-59)-IGF-I) was purchased from Amgen Biologicals (Thousand Oaks, CA) (lot 403). This analog substitutes a threonine for a methionine at position 59. Purity, determined by HPLC, is greater than 97%. Finally, an IGF-I preparation was synthesized by the solid-phase method (sIGF-I), as described (9).

Natural IGF-II, isolated from outdated human plasma, was provided by Dr. K. Hall (Stockholm) (batch 8) (13). The amino acid composition of this peptide was indistinguishable from the known composition of IGF-II, and sequence analysis up to position 39 was identical to that published by Rinderknecht and Humbel (3). In addition, an IGF-II preparation was synthesized by the solid-phase method (sIGF-II), and shown to be homogeneous by chromatofocusing, HPLC and amino acid analysis (11). All lyophilized peptides were reconstituted as 1 ug/10 ul in 0.1M HCl, and stored at -70C for up to 3 months. Crystalline porcine insulin was obtained from Elanco Products Company (Indianapolis, IN).

Iodination of IGF-I (Zurich) and IGF-II (Stockholm) was performed by the method of Hunter and Greenwood (15) to specific activities of 250 uCi/ug. Before use in receptor binding studies, the iodinated peptides were purified by gel filtration over a Sephadex G-50 column (1.0 x 120 cm) at 4C, and eluted with 100 mM HEPES buffer, pH 7.4, containing 0.5% BSA, 120 mM NaCl, 1.2 mM MgSO4, 5 mM KCl, 15 mM Na acetate and 10 mM dextrose.

Cells and Membranes: The human fibroblast cell line N3652, derived from a 24 year old male donor, passage 11, was obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). Membrane preparations were made from BRL-3A rat liver cells (American Type Culture Collection, Rockville, MD), and from 18,54-SF cells, kindly donated by Dr. J. Wyche (New York, NY) (16). Both cell lines were maintained in serum-free media prior to binding studies. For the BRL-3A cells, confluent monolayers were detached with 1 mM EDTA in saline, centrifuged at 1000 xg, washed, and resuspended in cold 50 mM Tris HCl, pH 7.4, containing 1mM EDTA, 0.25M sucrose and 4mM iodoacetic acid. Cells were disrupted by 20-30 strokes in a glass dounce and centrifuged at 600 xg for 10 minutes. The supernatant was recentrifuged at 30,000 xg for 40 minutes, and the resultant pellet resuspended in binding buffer, and either used immediately or stored frozen at -20C for up to two weeks. 18,54-SF cells were detached from monolayer cultures with PBS plus 1mM EDTA, centrifuged, washed and resuspended in 10mM NaPO4, pH 7.4, containing 1mM EDTA, 0.25% sucrose, 0.15M KCl, 1mM PMSF and 4mM iodoacetic acid. Cells were frozen at -20C until used for membrane preparation. At that time, cells were quick-thawed and disrupted by sonication. The lysate was centrifuged at 12,000 xg for 30 minutes, and the resultant supernatant recentrifuged at 36,000 xg for 60 minutes. The membrane pellet was resuspended in 50mM HEPES, pH 7.4, containing 0.15M NaCl, 1mM EDTA, 1mM PMSF and 4mM iodoacetic acid, and frozen at -20C until use. Rat placentas were obtained during the final three days of pregnancy, and membranes prepared as described above, but using 0.05M Tris-HCl, 0.5% BSA, pH 7.4 as buffer.

Receptor Studies: Binding studies were performed in intact fibroblast monolayers, by a minor modification of the method of Rosenfeld and Dollar (6). Specific binding of IGF-I to intact fibroblast monolayers was inhibited by both insulin and by monoclonal antibodies directed against the type I receptor (17). For BRL-3A and 18,54-SF cells, membranes were resuspended in 100mM HEPES buffer, containing 120mM NaCl, 5mM KCl, 1.2mM MgSO4, 10 mM dextrose, 15 mM Na acetate, 1mM EDTA and 0.5% BSA. Five-50 ug of membrane were incubated with 10 pM of iodinated peptide in the presence or absence of unlabeled peptide for 16 hours at 4C. Subsequently, membranes were washed in several volumes of cold buffer, centrifuged, and pellets counted in an automatic gamma counter for determination of receptor bound radioactivity. Non-specific binding was determined in the presence of a partially purified somatomedin preparation, containing 1 ug/ml IGF-II and 2 ug/ml IGF-I. Assays using rat placental membranes were performed by the method of Daughaday et al (18).

Radioimmunoassay: The IGF-II content of the several different IGF preparations was determined by RIA, using a rabbit-generated antibody directed against the 8-amino acid C-peptide region of IGF-II (19). The radioligand for this assay was the synthetic, tyrosylated octapeptide, kindly provided by Dr. D. Chang (Peninsula Laboratories, San Carlos, CA). Because of the different primary structures of the C-peptide regions of IGF-I and IGF-II, IGF-I does not cross-react with this antibody, even at concentrations as high as 10 ug/ml.

(Methyl-3-H) Thymidine Incorporation: Stimulation of 3-H-thymidine incorporation in human fibroblasts was performed by the methods of Conover et al (20). (Thr-59)-IGF-I and natural IGF-I were tested by themselves, and together with dexamethasone (10^{-7} M), and dexamethasone plus 0.25% human hypopituitary serum.

RESULTS: The ability of sIGF-I and (Thr-59)-IGF-I to bind to IGF-I receptors, antiserum and binding proteins has been documented in prior studies (9, 10, 12). Figure 1A depicts competition for (125-I)iodo IGF-I binding sites in human fibroblasts by natural IGF-I isolated from human plasma and by (Thr-59)-IGF-I. Using fibroblast monolayers from a 24 year old donor, both IGF-I preparations exhibited high affinity, with 50% displacement at 3.5-5 ng/ml. Identical results were observed in human fetal fibroblasts. Figure 1B compares the ability of natural IGF-I and (Thr-59)-IGF-I to stimulate (methyl-3-H) thymidine incorporation in human fibroblast monolayers. In the presence of IGF-I alone, (methyl-3-H) thymidine incorporation was stimulated 6.8-fold by natural IGF-I and 6.9 fold by (Thr-59)-IGF-I. Both IGF-I preparations demonstrated equivalent synergism with dexamethasone and low concentrations of human hypopituitary serum.

However, when (125-I)iodo IGF-II was employed as radioligand, and tested in membranes derived from cells and tissues rich in IGF-II receptors, natural IGF-I was found to compete far more effectively than either (Thr-59)-IGF-I or sIGF-I. With rat placental membrane preparations, 50% competition was observed with 13 ng/ml of IGF-II (Figure 2). By comparison, two different preparations of natural IGF-I resulted in 50% competition at 66 and 250 ng/ml. On the other hand, only minor competition was observed with increasing concentrations of sIGF-I or (Thr-59)-IGF-I. At concentrations as high as 400 ng/ml, (Thr-59)-IGF-I reduced (125-I)iodo IGF-II binding by only 18%, while sIGF-I reduced binding by only 24%.

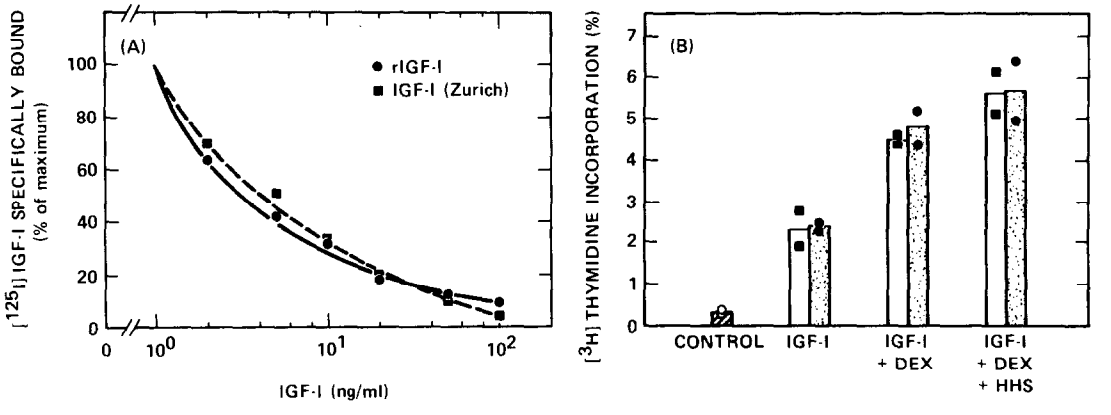

Figure 1. IGF-I binding and action in human fibroblast monolayers. (A) Competition with (125-I)iodo IGF-I for binding to human fibroblast monolayers. Points represent the means of triplicate determinations, and is representative of four separate experiments. In this experiment, specific binding averaged 3.2%. (B) Stimulation of (methyl-3-H) thymidine incorporation into human fibroblasts by natural IGF-I (Zurich) and (Thr-59)-IGF-I. Data are shown for stimulation by IGF-I alone, IGF-I + 10^{-7}M dexamethasone, and IGF-I + dexamethasone + 0.5% human hypopituitary serum (HHS). Bars indicate the mean of duplicate determinations, and individual data are depicted by the two symbols with each bar. Open bars: natural IGF-I; stippled bars: (Thr-59)-IGF-I.

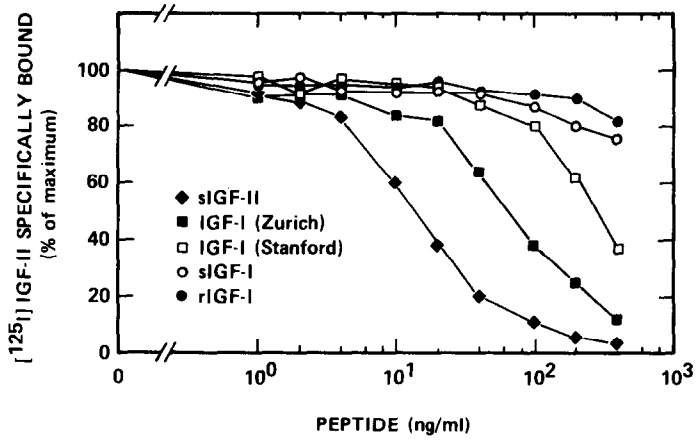

Figure 2. Competition with (125-I)iodo IGF-II for binding to rat placental membranes. Specific binding was 29.9%. (Thr-59)-IGF-I is designated rIGF-I.

Similar results were observed with other cell lines (Figure 3). With BRL cells (Figure 3A), 50% competition with (125-I)iodo IGF-II was observed with 4 ng/ml of IGF-II. Equivalent competition required 34 ng/ml of natural IGF-I. However, (Thr-59)-IGF-I concentrations as high as 500 ng/ml resulted in only 10% reduction in (125-I)iodo IGF-II binding. In membrane preparations derived from the 18,54-SF cell line, 50% competition was observed at IGF-II concentrations of 3 ng/ml and natural IGF-I concentrations of 20 ng/ml (Figure 3B). However, (Thr-59)-IGF-I, at concentrations as high as 400 ng/ml, resulted in only a 25% decrease in (125-I)iodo IGF-II binding. As is typical of IGF-II receptors, insulin showed little, if any, affinity for this receptor, even at concentrations as high as 1-10 ug/ml.

To exclude the possibility that the discrepancies in recognition of the IGF-II receptor were due to contamination of the natural IGF-I preparations with IGF-II, three IGF-I preparations were radioimmunoassayed

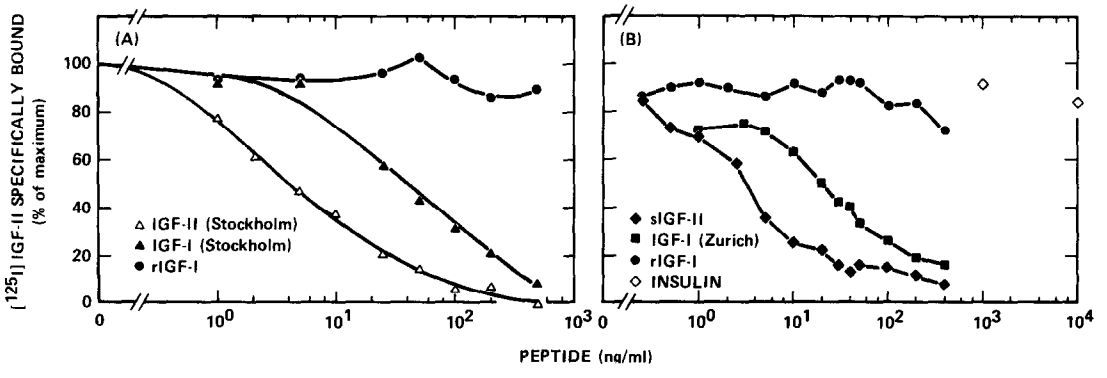

Figure 3. Competition with (125-I)iodo IGF-II for binding to (A) BRL-3A rat liver cell membranes and (B) 18,54-SF cell membranes. Specific binding in BRL-3A membranes was 8.6%. Specific binding in 18,54-SF membranes was 19.6%. (Thr-59)-IGF-I is designated rIGF-I.

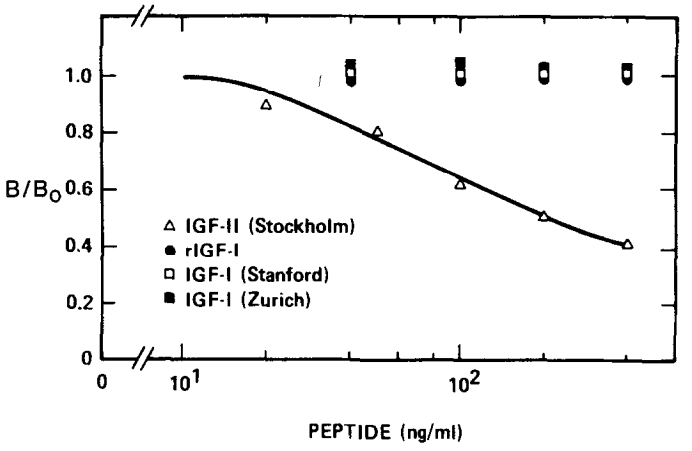

Figure 4. Radioimmunoassay for IGF-II C-peptide. (Thr-59)-IGF-I is designated rIGF-I.

for IGF-II by the method of Hintz and Liu (19). This assays employs an antibody generated against the synthetic 8-amino acid C-peptide region of IGF-II. Since this segment has no apparent homology with the C-peptide region of IGF-I, this RIA is highly specific for human IGF-II. As can be seen in Figure 4, neither (Thr-59)-IGF-I, natural IGF-I (Zurich) or natural IGF-I (Stanford) were recognized by this antibody, even at peptide concentrations of 400 ng/ml. In this assay, natural IGF-II (Stockholm) competed with (125-I)iodo IGF-II-C-peptide at concentrations of 20 ng/ml. Thus, we estimate that any potential contamination of IGF-I preparations with immunoreactive IGF-II must be less than 5%.

DISCUSSION: Prior studies of IGF-I preparations isolated from human plasma have demonstrated a consistent affinity of IGF-I for the IGF-II receptor (1, 4-8). In general, IGF-I has been found to compete for this receptor with 5-20% of the potency of IGF-II. Thus, initial observations that synthetic or recombinant DNA-derived IGF-I preparations recognized the IGF-II receptor with a substantially lower affinity than natural IGF-I preparations were surprising. Nevertheless, these findings have been demonstrated in a variety of cells and tissues rich in IGF-II receptors, including rat placental membranes, 18,54-SF membranes, BRL-3A rat liver cell membranes, K562 human erythroleukemia cells (data not shown) and human fibroblasts (data not shown). In all of these studies, both synthetic and recombinant DNA-derived IGF-I demonstrated an affinity for IGF-II receptors which was only 1-10% of that exhibited by natural IGF-I preparations. Similar observations have been described by Daughaday et al (18) and recently by Ewton and coworkers (21) in L6 myoblasts. In these cells, competitive binding and affinity cross-linking studies have demonstrated that recombinant DNA-derived (Thr-59) IGF-I has a low affinity for type II receptors.

These differences cannot be ascribed to errors in amino acid composition of the synthetic and recombinant preparations. Both peptides have had careful documentation of amino acid sequence (9, 12). Furthermore, by

radioimmunoassay, competitive binding protein assays, binding to IGF-I receptors, and biological activity, these preparations behave in a manner identical to natural IGF-I (9, 10, 12). Similarly, our data indicate that natural IGF-I and (Thr-59)-IGF-I compete equally for the IGF-I receptor in human fibroblasts, and have equivalent potency in the stimulation of (methyl-3-H) thymidine incorporation. These investigations also demonstrate that the low affinity of (Thr-59)-IGF-I for the type II receptor cannot be ascribed to the substitution of threonine for methionine at position 59, since (Thr-59)-IGF-I and sIGF-I behave virtually identically in receptor binding studies employing (125-I)iodo IGF-II as radioligand.

The discrepant affinities of natural IGF-I, when compared to (Thr-59)-IGF-I and sIGF-I, suggest several possibilities. It is certainly conceivable that the natural IGF-I preparations, which are purified from human plasma, are contaminated by sufficient quantities of IGF-II to account for the apparent high affinity for the IGF-II receptor. While this possibility must be considered, it is of note that all three natural preparations have had determination of amino acid composition and, at the very least, N-terminal amino acid analysis. Additionally, a RIA employing an antibody directed against the unique C-peptide region of IGF-II failed to detect IGF-II activity in any of the natural IGF-I preparations. Although the lower limit of sensitivity of this assay is only 20 ng/ml, no IGF-II activity was observed at peptide concentrations as high as 400 ng/ml. Thus, the natural IGF-I preparations could, at the very most, be only 5% contaminated with immunoreactive IGF-II. It is uncertain whether this level of contamination could account for the relatively high affinity of these preparations for the IGF-II receptor.

A second possible explanation for the relative inability of (Thr-59)-IGF-I and sIGF-I to recognize the IGF-II receptor is that the tertiary structures of these peptides might be altered in a manner that would permit binding to the type I, but not the type II receptor. While our data are consistent with this possibility, we would have to conclude that the tertiary structures of both (Thr-59)-IGF-I and sIGF-I are sufficiently different from that of natural IGF-I to permit these discrepant binding patterns.

Finally, it is conceivable that IGF-I purified from pooled Cohn fraction IV of human plasma represents a heterogeneous collection of peptides that exist naturally in plasma, or are created during the process of peptide isolation and purification. Zumstein and Humbel (22) have reported that IGF-I from outdated human plasma contains a predominant form with a pI of 8.25. However, subforms with pIs of 8.7-9.0, 8.5, 7.9 and 7.5 are found in varying proportion in different Cohn fraction IV preparations. Heterogeneity of IGF-I on electrofocusing has also been reported by Svoboda and Van Wyk (23). It may well be that these forms may be equivalent by IGF-I radioimmunoassay or radioreceptorassay, and still have varying affinity for the IGF-II receptor. Recently, Blum and co-workers (24) have purified six somatomedin-like peptides from human plasma

Cohn fraction IV by a combination of chromatofocusing and reversed-phase high pressure liquid chromatography. Three of these peptides have basic isoelectric points, while three were neutral or mildly acidic. By rat liver cell membrane radioreceptorassay, the three basic peptides were found to have varying affinities for the IGF-II receptor, but in all cases, their affinities were <1% of the affinities exhibited by the acidic preparations. Whether these variants actually circulate in plasma, or represent products of the extraction and purification procedures remains to be established.

While the explanation for the binding differences among natural, recombinant and synthetic IGF-I remain unknown, these observations have important implications for investigations of IGF receptors. Data obtained with one IGF-I preparation cannot be automatically extrapolated to other forms of IGF-I. Rather, we may find that each IGF-I preparation provides us with a different perspective on the complex interactions of the IGFs and their receptors. Indeed, the different affinities of these IGF-I preparations for type I and II receptors may permit their use as specific probes of somatomedin action. Finally, potential contamination of natural IGF-I preparations with small quantities of IGF-II may have resulted in overestimation of the affinity of pure IGF-I for the type II IGF receptor.

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