A highly sensitive and specific assay for determination of IGF-I bioactivity in human serum

Jian-Wen Chen,1 Thomas Ledet,2 Hans Ørskov,1 Niels Jessen,1 Sten Lund,1 Jonathan Whittaker,3 Pierre De Meyts,3 Maj Britt Larsen,1,4 Jens Sandahl Christiansen,1 and Jan Frystyk1

1Medical Research Laboratories and Medical Department M, and 2Laboratory of Biochemical Pathology, Aarhus University Hospital, DK-8000 Aarhus C; 3Receptor Biology Laboratory, Hagedorn Research Institute, and 4Department of Immunochemistry, Novo Nordisk, DK-2820 Gentofte, Denmark

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Chen, Jian-Wen, Thomas Ledet, Hans Ørskov, Niels Jessen, Sten Lund, Jonathan Whittaker, Pierre De Meyts, Maj Britt Larsen, Jens Sandahl Christiansen, and Jan Frystyk. A highly sensitive and specific assay for determination of IGF-I bioactivity in human serum. Am J Physiol Endocrinol Metab 284: E1149–E1155, 2003. First published February 25, 2003; 10.1152/ajpendo.00410.2002.—At present, the circulating bioactivity of insulin-like growth factor I (IGF-I) is estimated by immunological measurements of IGF-I levels. However, immunoassays ignore the modifying effects of the IGF-binding proteins (IGFBPs) on the interaction between IGF-I and the IGF-I receptor (IGF-IR). Therefore, we developed an IGF-I kinase receptor activation assay (KIRA) based on cells transfected with the human IGF-IR gene. The bioassay was sensitive (detection limit 0.08 μg/l), specific (cross-reactivity of insulin, insulin analogs, and proinsulin was <1%; IGF-II cross-reactivity was 12%), and accurate (within- and between-assay coefficients of variation <7 and <15%). The operational range of the assay (0.25–10.0 μg/l) allowed for determination of IGF-I bioactivity in serum from patients with, for example, growth hormone deficiency, type 1 diabetes, and acromegaly. Addition of IGFBPs dose dependently reduced the KIRA signal, whereas addition of IGF-II to preformed complexes (1:1 molar ratio) of IGF-I and IGFBP dose dependently increased IGF-I bioactivity by displacement of bound IGF-I. In conclusion, the KIRA will enable us to compare IGF-I bioactivity with existing immunological measurements of IGF-I in serum and, hopefully, to elucidate the factors that determine IGF-I bioactivity in vivo.

Peptides. Two assays directed against the human IGF-IR were developed. One assay detected total (i.e., activated as well as inactivated) IGF-IR levels in crude cell lysates. The coating antibody (MAB391), biotinylated detecting antibody (AF-305-NA), and recombinant human IGF-IR standard were developed. One assay detected tyrosine-phosphorylated (i.e., ligand-activated) IGF-IR levels only. We used a monoclonal antibody directed against the extracellular domain of the human IGF-IR (MAD1, Gropep, Adelaide, Australia) for coating and an europium-labeled monoclonal anti-phosphotyrosine antibody (PY20, PerkinElmer Life Sciences, Turku, Finland) for detection. Recombinant human IGF-I was from Austral Biologicals (San Ramon, CA), and recombinant human IGF-II, IGFBP-1, -2, and -3 were from R&D Systems. Streptavidin-europium was acquired from PerkinElmer Life Sciences and was used in a final dilution of 1:1,000. Human serum albumin (HSA) was obtained from ICN Biomedic (Aurora, OH). Human insulin, insulin aspart (NovoRapid), appears to be involved in common diseases, e.g., atherosclerosis and cancer (3, 14).

At the cellular level, IGF-I bioactivity is modulated by the presence of IGFBPs, which inhibit as well as stimulate IGF-I-mediated effects (12). In addition, several IGFBP proteases participate. Cleavage of the IGFBPs reduces the ligand affinity, and this is believed to represent a mechanism by which IGF-I bioactivity is increased (2).

At present, human studies use the circulating concentration of free or total IGF-I as an estimate of IGF-I bioactivity (10). However, these immunological measurements ignore the modifying effects of IGFBPs and IGFBP proteases on the interaction between IGF-I and the IGF-I receptor (IGF-IR). Therefore, we established a specific and sensitive IGF-I kinase receptor activation assay (KIRA) based on principles described by Sadick (20). This assay has enabled us to determine the serum concentration of IGF-I that can phosphorylate (i.e., activate) the IGF-IR in vitro at physiological conditions.

Materials and Methods

Address for reprint requests and other correspondence: J.-W. Chen, Medical Dept. M, Aarhus Kommune Hospital, Norrebrogade 44, DK-8000 Aarhus C, Denmark (E-mail: wen@iekf.au.dk).

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and porcine proinsulin were kindly provided by Novo Nordisk (Bagsværd, Denmark), and insulin lispro (Humalog) was from Eli Lilly Denmark (Copenhagen, Denmark).

**Cell line and media.** We used human embryonic renal cells (293 EBNA) transfected with cDNA encoding the full-length human IGF-IR gene, as previously described (17). Briefly, 293 EBNA cells constitutively expressing EBV nuclear antigen (Invitrogen, Germany) were maintained in continuous culture medium that contained 10% fetal calf serum (FCS), the antibiotics penicillin and streptomycin (50,000 U and 50 mg/l, respectively) and 500 mg/l of Geneticin (Invitrogen, Karlsruhe, Germany). The human IGF-IR cDNA was subcloned into the episomal expressing vector pCEP4 (Invitrogen), and the cells were transfected with the construct using Lipofectamine (Invitrogen) according to the manufacturers’ instructions. To select for stable transformant, 48-h-posttransfected cells were grown in medium supplemented with 250 mg/l hygromycin B (Invitrogen). Hygromycin-resistant colonies were expanded and assayed for IGF-IR expression using 125I-labeled IGF-I binding assay (17). Cultures with high-level expression of IGF-IR were recloned by limiting dilution.

The transfected cells were cultured in 175-mm2 tissue culture flasks (Nune, Roskilde, Denmark) using Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, Copenhagen, Denmark) prepared according to the manufacturer’s instructions, but still in the presence of hygromycin B (250 mg/l).

**Buffers.** Antibody coating buffer was made of 15 mM sodium carbonate and 35 mM sodium hydrogen carbonate (pH 9.6). Blocking solution contained 40 mM phosphate (pH 8.0), 0.05% (wt/vol) NaN3, 0.6% (wt/vol) NaCl, 0.2% (wt/vol) Tris, 8% (vol/vol) EDTA, and 1% (wt/vol) HSA. Lysis buffer was made of 50 mM HEPES (pH 7.4; Genentech Media prep, Sigma-Aldrich), and 1% (wt/vol) HSA. A serially diluted 125I-IGF-I standard was used as a standard. Standards or crude lysates diluted (1:10) were dissolved in assay buffer and analyzed in duplicate. Duplicate samples contained 100 µl of standard or diluted sample and 100 µl of biotinylated second antibody (AF-305-NA). Plates were incubated overnight at 5°C and then washed and kept for 3 h at room temperature with 200 µl of streptavidin-labeled europium dissolved in assay buffer. After six washes, enhancement solution was added to the plates, and contents were read in the fluorometer.

**Competitive IGF-IR-binding studies.** Competitive binding of IGF-I, IGF-II, and insulin to the IGF-IR of 293 EBNA cells was evaluated by the method described previously (7). Briefly, 293 EBNA cells (400,000/well) were incubated with moniodinated 125I-IGF-I (generously supplied by Novo Nordisk) together with increasing concentrations of unlabeled peptides for 30 min at 5°C. The total radioactivity of the IGF-I tracer was kept constant (10,000 counts·min−1·well−1) while the concentration of unlabeled peptide was increased up to 1,000 µg/l (0, 5, 10, 25, 50, 100, and 1,000 µg/l, respectively). The highest concentration of unlabeled IGF-I was used as an estimate of nonspecific binding. After incubation with tracer and unlabeled peptide, the cells were washed twice with cold KRB buffer containing 0.5% HSA and lysed, as mentioned before. The radioactivity of crude lysates was counted for 5 min.

**KIRA assay validation.** The validation of the KIRA assay included several steps. 1) A serial dilution of 293 EBNA cells (6,250 to 800,000 cells/ml) was used to investigate the effect of cell number on the specific KIRA signal after incubation with 0, 1, 10, and 100 µg/l IGF-I. Furthermore, we examined the effect of increasing cell number on the IGF-IR level in crude cell lysates. 2) Assay specificity was examined by incubating 293 EBNA cells with serial dilutions of ≤10,000 µg/l of human insulin, insulin aspart, insulin lispro, or porcine proinsulin. The cross-reactivity with IGF-II was also tested, and the concentrations of IGF-II were 0.5, 2.5, 10, 50, and 100 µg/l, respectively. 3) The impact of IGFBPs on the IGF-I KIRA signal was assessed by coincubating increasing concentrations of IGFBP-1, -2, or -3 with IGF-I. The tested molar ratios between IGF-I and the IGFBPs ranged from a hundredfold molar excess of IGF-I to a hundredfold molar excess of IGFBP. Moreover, by addition of different concentrations of IGF-II (0.5, 2.5, 10, 50, and 100 µg/l, respectively) to preformed complexes of IGF-I (2.5 µg/l) and...
IGFBP-1, 2, or 3 (10 μg/l), the effect of IGF-II on the IGF-I KIRA signal was investigated. 4) Enzymatic degradation of IGFBP-3 at the cellular level is believed to increase IGF-I bioavailability by lowering IGFBP-3 ligand affinity (2). To study whether the cells contained surface-associated IGFBP-3 proteases, we incubated the cells for 0, 10, and 20 min with KRB buffer containing 0.5% HSA and 40 μg/l of IGFBP-3. The presence of IGFBP-3 proteolysis was investigated by Western ligand binding, as previously described (9). 5) The effect of serum dilution (from 1:5 to 1:40) on the KIRA signal was also tested. Sera were obtained from two normal subjects, one patient with acromegaly and one patient with type 1 diabetes. 6) Assay reproducibility was estimated by calculation of within-plate and between-plate coefficients of variation (CVs), the latter by repetitive measurements of a control sample.

Clinical evaluation. To study the relationship between IGF-I bioactivity and serum levels of free and total IGF-I, we studied 11 normal male subjects (41.6 ± 3.0 (SE) yr), 11 male subjects with type 1 diabetes (43.0 ± 2.5 yr; diabetes duration 24.8 ± 2.7 yr; Hb A1c 8.3 ± 0.2%), 11 patients (5 males and 6 females) with acromegaly (44.9 ± 3.6 yr), and 8 patients (5 males and 3 females) with growth hormone deficiency (GHD; 39.4 ± 5.6 yr). These four groups were included to assess whether the KIRA was able to determine IGF-I bioactivity in samples with low as well as high concentrations of free and total IGF-I. It was not the purpose to test whether the KIRA was superior to free and total IGF-I in discriminating between groups with different GH status, and it should be stressed that among the four groups, healthy controls were matched with type 1 diabetic patients, whereas patients with acromegaly were matched with patients with GHD. All samples were collected in accordance with the Declaration of Helsinki.

Other assays. IGF-I was determined by an in-house non-competitive monoclonal antibody-based time-resolved immunofluorometric assay, as previously described (8). This assay is characterized by high sensitivity (detection limit <0.005 μg/l) and specificity (IGF-II cross-reactivity <0.02%). Serum total (extractable) IGF-I was determined in acid ethanol serum extracts with within- and between-assay CVs averaging <5 and 10%, respectively (8). Serum free IGF-I was determined using ultrafiltration by centrifugation at conditions approaching those in vivo (11). Amicon YMT 30 membranes and MPS-1 supporting devices were used (Amicon, Beverly, MA). Before centrifugation, serum samples were diluted (1:11) in KRB buffer (pH 7.4) containing 50 g/l HSA (Behring, Marburg, Germany). From each dilution, triplicates of 600 μl were applied to the membranes and incubated (30 min at 37°C) and centrifuged (1,500 rpm at 37°C). The lower detection limit of free IGF-I in the ultrafiltrates was 0.020 μg/l. Within- and between-assay CVs of free IGF-I averaged 15 and 20%, respectively.

Statistics. For data with normal distribution, Student’s t-test was used to compare differences between groups; otherwise, the nonparametric Mann-Whitney test was employed. A two-tailed P value of <0.05 was considered statistically significant. All data are means ± SE.

RESULTS

Assay characteristics. We developed an assay for total IGF-IR to study the relationship between cell number, IGF-I KIRA signal, and IGF-IR density. The standard curve was linear from 0.1 to 100 μg/l, and within this range the specific signal increased from 10,000 ± 1,600 to 580,000 ± 55,000 counts/s (CPS).

Furthermore, crude cell lysates diluted in parallel with the standard curve (data not shown). The estimated detection limit was 0.2 μg/l [nonspecific binding (NSB) + 3 SD]. Within- and between-assay CVs averaged <3 and 15%, respectively.

Total IGF-IR levels in crude cell lysates increased with increasing cell number up to 400,000 cells/ml,
after which the total IGF-IR concentration leveled off (Fig. 1A). Accordingly, the amplification of the specific KIRA signal was less pronounced when the number of cells exceeded 400,000/ml (Fig. 1B). However, when IGF-IR activation was expressed as phosphorylated IGF-IR (specific signals of IGF-I KIRA) per total IGF-IR, the IGF-I KIRA was virtually unaffected by the number of cells (Fig. 1C). Furthermore, the present experiment showed that the concentration of phosphorylated IGF-IR was not increased as the level of IGF-I exceeded 10 μg/l. On the basis of these findings, we decided to use 400,000 cells/ml for further experiments and 10 μg/l of IGF-I as the highest standard.

A representative IGF-I KIRA standard curve is shown in Fig. 2. The CVs of the standards averaged <7%. The detection limit (NSB + 3 SD) was found to be 0.08 μg/l. Plate-to-plate variation was estimated by repetitive measurements of a control sample assayed 34 times on 6 different days and averaged 13%.

Analysis of serially diluted serum samples showed that the signal was relatively independent of the dilution, in particular when dilutions ranging from 1:5 to 1:20 were compared (Fig. 2). At higher dilutions the signal decreased (P < 0.02 when sera diluted 1:5 and 1:40 were compared). On the basis of this finding, we decided to dilute all serum samples 1:10 for further experiments.

The specificity of the IGF-I KIRA was studied by incubating 293 EBNA cells with serial dilutions of IGF-II, human insulin, insulin aspart, insulin lispro, and porcine proinsulin, respectively. The maximum cross-reactivity averaged 0.7% for both insulin analogs, 0.8% for human insulin, 0.06% for proinsulin, and 12% for IGF-II.

Coincubation of IGF-I with increasing concentrations of IGFBP-1, -2, or -3 dose dependently reduced the IGF-I KIRA signal (Fig. 3A). At low concentrations (a 10- to 100-fold molar excess of IGF-I), the IGFBPs had almost no impact on IGF-I bioactivity. However, the IGF-I KIRA signal decreased by >80% when the IGFBPs were added in a 10- to 100-fold molar excess of IGF-I. Addition of IGF-II to preformed complexes of IGF-I and IGFBP (in 1:1 molar ratio) displaced IGF-I...
from the IGFBPs and enhanced IGF-I bioactivity dose dependently (Fig. 3B).

On the basis of competitive IGF-IR-binding studies of IGF-I, IGF-II, and insulin, it was possible to estimate the IC50 (the concentration of unlabeled peptide able to replace 50% of IGF-I tracer from the receptor). IC50 was estimated to be 9 μg/l for IGF-I, 100 μg/l for IGF-II, and 2,000 μg/l for insulin (Fig. 4).

To determine whether cell membrane-associated IGFBP proteases would interfere in the IGF-I KIRA, 293 EBNA cells were incubated with KRB buffer containing 0.5% HSA and 40 μg/l of IGFBP-3. After incubation for 0, 10, and 20 min, the IGFBP-3-containing buffer was aspirated, and IGFBP-3 proteolysis was estimated by Western blot. With this method we were unable to detect any IGFBP-3 proteolysis (data not shown).

Clinical evaluation. To examine the clinical applicability of the IGF-I KIRA, we compared the IGF-I KIRA signal with determinations of free and total IGF-I in serum samples from healthy overnight-fasted control subjects and patients with type I diabetes, acromegaly, and GHD (Table 1). The level of bioactive IGF-I was elevated in acromegalic patients and reduced in patients with type I diabetes, and the same pattern was seen for free and total IGF-I. In patients with GHD, levels of free as well as bioactive IGF-I appeared to be within the normal range, in contrast to total IGF-I. The CVs of the IGF-I KIRA averaged <6%.

DISCUSSION

Ligand binding induces conformational changes of the IGF-IR. This stimulates the intracellular kinase domain of the receptor, resulting in autophosphorylation of tyrosine residues (5, 13, 16). Because receptor autophosphorylation represents the first step in the intracellular signal cascade, phosphorylated IGF-IR levels may be used to assess ligand bioactivity.

Sadick (20) was the first to describe an IGF-I KIRA using MCF-7 cells, and this author showed that the IGF-I KIRA correlated well with classical end point bioassays such as the [3H]thymidine incorporation assay (20). The present assay resembles the KIRA described by Sadick; thus both assays use a short (15-min) sample incubation time, and this minimizes the possible time for the cells to produce IGFBPs that may interfere with the ligand activity during sample incubation. Furthermore, both methods are specific and accurate. However, our assay appears to be even more sensitive than that of Sadick, most likely because our technique is based on IGF-IR transfected cells.

We observed a small but clear bimodal effect of IGF-I on IGF-IR levels (Fig. 1A). Thus, for each of the tested cell densities, the concentration of the IGF-IR increased from 0 to 1 μg/l of IGF-I, whereas IGF-IR levels became downregulated when the concentration of IGF-I was increased to 10 and 100 μg/l. However, this bimodal effect did not involve IGF-IR phosphorylation, which increased with increasing IGF-I concentrations. Changes in the cellular IGF-IR density seem to play an important role in cell growth regulation (21), and in this context, the combination of the assay for total IGF-I levels and the KIRA may be useful to study whether factors that affect the cellular IGF-IR density also influence IGF-IR phosphorylation.

Our receptor-binding experiments with iodinated IGF-I showed that IGF-IR-binding affinity for IGF-II and insulin was more than 10 and 200 times lower, respectively, than that of IGF-I. Similar results have been obtained by others (18, 19), although different cell lines were used. Importantly, these data were in good accordance with our observation of an IGF-II cross-reactivity of 12% and an insulin cross-reactivity of <1%. Moreover, insulin analogs bound to the IGF-IR with comparable or lower binding affinity than that of insulin, as previously observed by others (15).

Table 1. Fasting serum levels of IGF-I bioactivity, free IGF-I, and total IGF-I in four groups

<table>
<thead>
<tr>
<th></th>
<th>Healthy Subjects</th>
<th>Patients With Type 1 Diabetes</th>
<th>Patients With Acromegaly</th>
<th>Patients With GHD</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I KIRA, μg/l</td>
<td>1.02 ± 0.11</td>
<td>0.49 ± 0.07*</td>
<td>4.15 ± 0.29*</td>
<td>0.82 ± 0.10†</td>
</tr>
<tr>
<td>Free IGF-I, μg/l</td>
<td>0.51 ± 0.11</td>
<td>0.12 ± 0.04*</td>
<td>4.04 ± 0.46*</td>
<td>0.45 ± 0.11†</td>
</tr>
<tr>
<td>Total IGF-I, μg/l</td>
<td>227 ± 19</td>
<td>177 ± 10*</td>
<td>674 ± 68*</td>
<td>88 ± 11*†</td>
</tr>
</tbody>
</table>

Results are means ± SE. IGF-I, insulin-like growth factor I; KIRA, kinase receptor activation assay; GHD, growth hormone deficiency. Free and total IGF-I levels were assessed with methods described in Refs. 8 and 11. *P < 0.05 vs. control; †P < 0.0001 vs. acromegaly.
One of the major challenges in the field of IGF research is to clarify how IGF-I bioactivity is regulated in vivo. At present, IGF-I bioactivity is almost exclusively estimated by immunological determinations of free and total IGF-I, but the results may not necessarily correspond to the fraction of IGF-I being accessible to the IGF-IR. The IGF-I KIRA methodology may be advantageous, because it allows for direct comparisons between serum levels of IGF-IR-accessible IGF-I, free IGF-I, and total IGF-I. Furthermore, the KIRA may be used to assess factors controlling the interaction between IGF-I and its receptor. However, to the best of our knowledge, such studies have never been performed previously, and we decided to establish a KIRA for these purposes. Because the equilibrium between IGF-I and the IGFBPs is highly dependent on temperature, pH, and buffer constitution (11), we preincubated samples at 37°C before assay and diluted samples in KRB adjusted to pH 7.4 with CO₂.

Sample dilution is necessary, because the cells become repressed at serum concentrations exceeding ~25% (data not shown). However, in contrast to what is seen in an immunoassay, where it is mandatory that the antigen dilutes in parallel with the standards, our data show that serial dilution of serum has almost no impact on the IGF-I KIRA, especially in the dilution range from 1:5 to 1:20. At first glance this finding may appear surprising, but the phenomenon is well recognized, and it has been described previously for free IGF-I as well as free thyroid hormones (11, 22). As predicted from the law of mass action, the IGFBPs liberate IGF-I during dilution to maintain equilibrium, and therefore the concentration of bioavailable IGF-I should not be multiplied with the dilution factor. Obviously, the buffering capacity of the IGFBPs is limited, and if the dilution exceeds 1:40, then the concentration of free IGF-I starts to decline. The effect of dilution on free IGF-I has recently been reviewed by Bang et al. (1), as well as by Ekins (6) some years ago.

To validate the clinical applicability of our KIRA, we compared IGF-I KIRA levels in serum samples from three overnight-fasted patient groups with low as well as high serum levels of free and total IGF-I. Of note, the KIRA was able to determine IGF-I bioactivity in samples with low as well as high levels of immunoreactive IGF-I (free IGF-I ranged from 0.012 to 6.50 μg/l; total IGF-I ranged from 49 to 1,134 μg/l). On the other hand, our KIRA did not yield a better discrimination between the groups than total and free IGF-I. However, in this context it is important to stress that the four groups were not strictly matched; therefore, further studies are needed to assess whether the KIRA may be useful in the diagnosis of GH disorders. As expected, the level of IGF-I bioactivity was somewhat higher than that of free IGF-I, but much lower than total IGF-I. On the other hand, in acromegalic patients, the differences between free and KIRA IGF-I were less pronounced than in healthy controls and patients with type 1 diabetes and GHD. This finding is likely a result of the different IGFBP profiles in the four study groups. The dissociation of lightly bound IGF-I from its binding protein may also have a role.

In conclusion, we have developed a highly sensitive, specific, and accurate IGF-I KIRA. We recognize that our assay may not necessarily reflect IGF-I bioactivity at the local tissue level (with the notable exception of vascular endothelial cells), but we believe that the assay will be able to provide us with new information on the factors that control IGF-I bioactivity in the circulation.

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