NOTE

Determination of Insulin-like Growth Factor-I in Normal Subjects and in Patients with Growth Hormone Disorders by Radioimmunoassay Using Biosynthetic Homologous Peptide

TOSHIHIKO INOUE, NORIAKI WATANABE, SHIRO SAITO, MASAYUKI YAMASAKI, HARUHIKO SAITO AND NORIHISA SHIRAKAWA

The First Department of Internal Medicine and Department of Pediatrics
School of Medicine, The University of Tokushima
Kuramoto-cho, Tokushima 770, Japan

Abstract

A highly sensitive and specific RIA for IGF-I has been developed using recombinant DNA-derived IGF-I of very high purity and specific antiserum to it. This assay system could detect IGF-I at as low concentrations as 20-30 ng/ml. The intra-assay and interassay coefficients of variation at various concentrations of IGF-I were 4.9 to 6.5% and 5.4 to 8.0%, respectively. The recovery rate of pure IGF-I added to plasma was 77.0±3.7%. The antiserum did not cross-react with porcine insulin, biosynthetic human insulin, hGH, hEGF, the synthetic C-domain of IGF-I or that of IGF-II, but reacted equally with an analog, Thr59-IGF-I. Plasma IGF-I was extracted by the acid-ethanol method before assay to separate IGF-I from its binding protein. When plasma IGF-I was assayed without extraction, the inhibition curves of serial dilution of plasma samples from several individuals were not parallel to the standard curve of IGF-I. The plasma concentration of IGF-I was 147±49 ng/ml (mean±SD) in 156 normal adults aged from 20-59 years. As reported by others, the IGF-I levels were low in cord plasma (41.8±23.5 ng/ml) and plasma of patients with GH deficiency (64.6±42.0 ng/ml), while its levels were high in normal children of pubertal ages [(12-13 yr, 365±126 ng/ml) and in patients with active acromegaly (562±115 ng/ml)]. This RIA system is a simple and useful method for determining plasma IGF-I in normal and diseased states.

Furlanetto et al. (1977) described a radioimmunoassay (RIA) for somatomedin (Sm)-C, and Zapf et al. (1981) reported a RIA for insulin-like growth factor (IGF)-I. But despite the increasing necessity for determination of IGF-I/Sm-C concentrations in blood and other biological fluids in patients with growth hormone (GH) disorders and various other pathological conditions, the extremely limited supply of natural IGF-I has prevented the general use of IGF-I assay.

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Therefore, alternative methods of RIA for IGF-I using synthetic C-domain of that were developed by Hintz et al. (1980), by Kadowaki et al. (1983) and by us (Inoue and Watanabe, 1986). Moreover, recently, Schalch et al. (1984) reported the availability of an IGF-I analog produced by recombinant DNA technology, which has almost identical biological and immunochemical activities to those of natural IGF-I. In addition a homologous peptide of IGF-I with an identical primary structure to that of the natural one was synthesized by the recombinant DNA procedure, and a specific antibody to it was prepared in Central Research Laboratory of Fujisawa Pharmaceutical Co., Ltd., Japan (Niwa et al., 1986).

This paper describes the establishment of a highly sensitive and specific RIA for IGF-I using this biosynthetic IGF-I homolog and its antisera. Data obtained by this RIA on plasma IGF-I in normal subjects and patients with GH disorders are also reported.

Materials and Methods

Subjects

The concentration of IGF-I was determined in cord plasma of 10 normal term neonates, sera of 115 normal children 0 to 15 years old, plasma of 183 normal adults 20 to 89 years old, and plasma of 20 patients with pituitary dwarfism 8 to 21 years old (14.4±3.1 yr, M±SD) and 7 adult patients with active acromegaly. Blood samples were drawn from the ante-cubital vein into polypropylene tubes containing aprotinin (500 KIU/ml) with or without EDTA (1.2 mg/ml) between 0800 h and 1200 h, and centrifuged at 4°C for 15 min at 3,000 rpm. The sera or plasma were stored at −70°C until assayed.

Acid-ethanol extraction of IGF-I

For removal of the bulk of plasma proteins, especially specific binding protein for IGF-I, acid-ethanol extraction of plasma or serum was performed by a slight modification of the method of Daughaday et al. (1980). For this, a solution (0.8 ml) of 12.5% 2N-HCl and 87.5% ethanol (vol/vol) was added to plasma or serum (0.2 ml) in a polypropylene tube with thorough mixing. The mixture was stood at room temperature for 30 min, and then centrifuged at 4°C for 30 min at 3,000 rpm. The supernatant (0.5 ml) was transferred to another polypropylene tube, neutralized with 0.2 ml of 0.855 M Tris buffer, pH 11.2, and mixed with 1.3 ml of assay buffer [0.01 M phosphate buffer (pH 7.4) containing 0.15 M NaCl, 0.025 M EDTA, 0.01% sodium azide and 0.5% bovine serum albumin (BSA)]. The original plasma or serum sample was thus diluted 1:20. The IGF-I levels in untreated plasma of 10 normal adults were measured, and the inhibition curves with untreated and acid-treated plasma were obtained to examine their parallelism to the standard curve in IGF-I RIA.

Peptides and antisera to IGF-I

The biosynthetic homologous peptide of IGF-I and its specific antisera were kindly provided by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). This recombinant DNA-derived IGF-I was demonstrated to be more than 99% pure by high-performance liquid chromatography (HPLC). Antiserum was raised in two New Zealand white rabbits by immunization with ovalbumin conjugated IGF-I five times with intervals of 32, 41, 40 and 58 days between successive injections. The antiserum recognizes five portions of the IGF-I molecule: the N terminal portion (the first five amino acid residues), middle portions (residues 13 to 20 and 21 to 33) and C terminal portions (residues 47 to 53 and 60 to 70) (Niwa et al., 1986). For RIA, this specific antiserum was used at a final dilution of 1:35,000.

Human growth hormone (hGH) and its specific antibody were kindly provided by the National Hormone and Pituitary Program, NIAMDD, Bethesda, MD, U.S.A. Porcine insulin and biosynthetic human insulin were obtained from Eli Lilly and Co. (Indianapolis, IN, U.S.A.). Human epidermal growth factor (hEGF) was from Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan), and synthetic IGF-I (26-46), and IGF-II (27-45) were generous gifts from Prof. Noboru Yanaihara, Shizuoka College of Pharmacy, Shizuoka, Japan. Thr59-IGF-I was a product of Amgen Biologicals (Thousand Oaks, CA, U.S.A.) and was purchased through Amersham Japan in Tokyo.
Iodinations of IGF-I and hGH

A sample of 4 μg of IGF-I was labelled with 0.5 mCi of Na-125I (New England Nuclear Corp., Boston, MA, U.S.A.) by the chloramine T method (Hunter and Greenwood, 1962). The reaction mixture was applied to a column (1 × 25 cm) of Sephadex G-50 fine (Pharmacia Fine Chemicals AB, Uppsala, Sweden) and material was eluted with 0.1 M ammonium acetate buffer, pH 7.0, containing 0.2% BSA. Fractions of 1 ml of effluent were collected and the three successive fractions starting the first radioactive peak were used as tracers. The specific activity of 125I-IGF-I was 75–85 μCi/μg. 125I-Labelling of hGH was also done by the chloramine T method, and material was purified on a column (1 × 17 cm) of Sephadex G–75.

The specific activity of 125I-hGH was 100 μCi/μg.

Radioimmunoassay procedure

A mixture of 0.4 ml of assay buffer, 0.1 ml of diluted antiserum (working dilution, 1:5,000), 0.1 ml of standard IGF-I or sample, and 0.1 ml (10,000 cpm) of 125I-labelled IGF-I was incubated overnight at 4°C in a glass tube. Bound and free 125I-IGF-I were separated by the polyethylene glycol (PEG) method (Desbuquois and Aurbach, 1971): a mixture of 2% bovine gamma-globulin (0.1 ml) and 25% PEG (0.9 ml) was added to the tubes with thorough shaking. After further incubation at 4°C for 1 hr, the tubes were centrifuged at 4°C for 30 min at 3,000 rpm and the radioactivities of the precipitates were counted in a well-type gamma counter (Aloka, Tokyo, Japan). Plasma GH levels were measured by RIA with an hGH kit (AFP-4793B) from NIAMDD.

Effects of various peptides on binding of IGF-I to antiserum

The specificity of the antiserum was examined with porcine insulin, biosynthetic human insulin, hGH, hEGF, IGF-I (26–46), IGF-II (27–45) and Thr59-IGF-I.

Gel filtration of extracted and unextracted plasma from a normal adult female

The molecular heterogeneity of plasma IGF-I-like immunoreactivity was examined by gel filtration. Samples of 0.5 ml of plasma from a normal adult female (24 yr, plasma IGF-I level: 176 ng/ml) with and without extraction with acid-ethanol were applied to a column (1 × 24 cm) of Sephadex G-50 fine, the material was eluted with 0.1 M ammonium acetate buffer (pH 7.0) containing 0.2% BSA and fractions of 0.5 ml were collected. The calibration was made with OD (280 nm) for Vo, 125I-hGH (MW 22,000), 125I-IGF-I (MW 7,600), 125I-human insulin (MW 6,000) and Na-125I for Vt.

Statistical methods

Data are expressed as mean ± standard deviation. The significance of differences between groups was examined by Student’s t-test.

Results

Binding of labelled IGF-I to antiserum

Fig. 1 shows typical elution patterns of radioactivity and binding activity of
iodinated IGF-I on gel filtration on a Sephadex G-50 fine column (1×25 cm) at room temperature. There were 2 radioactive peaks. The first peak, consisting of fractions No. 14 through No. 19, showed IGF-I-like immunoreactivity with maximum binding (Bo/T) of approximately 65% to the diluted antiserum (1:35,000) in fraction No. 16, but the second peak showed no binding activity. Fractions No. 15, 16 and 17 were stored at −70°C for use as a tracer in the assay.

**Standard curve**

Fig. 2 shows standard curves for RIA for IGF-I obtained at different times with the same lot of tracer. The lower limit of detectability with 95% confidence was 1–1.5 ng/ml. The labelled IGF-I retained tracer activity for up to three months.

**Validity of the assay**

The intra- and interassay coefficients of variation were examined by replicate determinations (n=8 for intra-assay and n=5 for interassay) at 3 concentrations of standard IGF-I solution and a known IGF-I concentration (128 ng/ml) of pooled normal plasma. The intra-assay coefficients of variation were 4.9% at 2.5 ng/ml, 7.9% at 5 ng/ml, 6.5% at 10 ng/ml and 4.6% for pooled plasma. The interassay coefficients of variation were 5.4%, 6.3%, 6.5% and 8.0%, respectively. The recovery rate of recombinant IGF-I added to plasma, which was subsequently subjected to acid-ethanol extraction, was 77±3.7% (n=5).

**Specificity of anti-IGF-I antiserum**

None of the peptides tested, including porcine insulin, biosynthetic human insulin, hGH, hEGF, IGF-I (26–46) and IGF-II (27–45), cross-reacted with antiserum against the biosynthetic IGF-I. However, the inhibition curve obtained with Thr59-IGF-I completely overlapped that of IGF-I (Fig. 3).

**Difference between parallelisms to the standard curve of extracted and unextracted plasma**

A dose-response curve for plasma extracted by the acid-ethanol method was parallel to the standard curve, but that for unextracted plasma was not (Fig. 3). Therefore, the IGF-I levels of unextracted plasma from 10 normal adults were remarkably lower than those of their extracted plasma, being approximately 25% of the latter (Table 1). Even when 125I-IGF-I was added 72 h after the first incubation (non-equivalent).
Fig. 3. Inhibition curves of acid-ethanol extracted plasma, unextracted plasma and various peptides in IGF-I radioimmunoassay. Points and bars represent mean±SE (n=5).

Table 1. Relation between IGF-I levels of acid-ethanol extracted and unextracted plasma of 10 normal subjects.

<table>
<thead>
<tr>
<th>Sample</th>
<th>EP (ng/ml)</th>
<th>UEP (ng/ml)</th>
<th>UEP/EP</th>
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<tbody>
<tr>
<td>1</td>
<td>104</td>
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</tr>
<tr>
<td>2</td>
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<td>20</td>
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</tr>
<tr>
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<tr>
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<td>0.25</td>
</tr>
<tr>
<td>SD</td>
<td>46.4</td>
<td>26.8</td>
<td>0.58</td>
</tr>
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</table>

EP: Acid-ethanol extracted plasma (ng/ml)
UEP: Unextracted plasma (ng/ml)

liberated method), the dose-response curve of unextracted plasma was not parallel to the standard curve (data not shown).

Gel filtration of extracted and unextracted plasma from a normal adult female

On gel filtration, the amount of IGF-I recovered from extracted plasma was 52 ng and the recovery rate was 64.5%. Most of the IGF-I-like immunoreactivity of the extracted plasma was eluted in the same fractions (No. 28–36) as $^{125}$I-IGF-I, but a small portion of IGF-I-like immunoreactivity was found in fractions No. 20–21 (Fig. 4). In contrast, the IGF-I-like immunoreactivity of unextracted plasma was eluted only in the position of larger molecular weight (fractions No. 18–23), and the total amount
Fig. 4. Gel filtration profile of acid-ethanol extracted (○--○) and unextracted (●--●) plasma from a normal adult female of 24 years of age. The IGF-I concentration of each fraction was determined by RIA as described in the Materials and Methods.

Fig. 5. Serum or plasma IGF-I levels in cord plasma, normal children and adults. Columns and bars represent mean±SD for each group. Numbers of individuals are shown in parentheses.
of IGF-I recovered was 5.4 ng. Therefore, IGF-I in unextracted plasma from this female constituted less than 10% of the IGF-I in extracted plasma.

Plasma IGF-I concentration in normal subjects

There was essentially no difference between the IGF-I values in serum and plasma samples from the same individuals (n=10, Y=0.76X+13.0, r=0.87, p<0.01). Although the GH concentration was high in cord plasma (12.5±2.0 ng/ml, M±SD), the IGF-I concentration in it was quite low (41.8±23.5 ng/ml, p<0.001) compared with that in sera of 82 normal children aged 7 to 15 years (266±118 ng/ml). The highest values, which showed overlap with values in acromegalic patients, were attained at the age of 12 to 13 years (206–660 ng/ml), and then values gradually decreased to those of adults. The plasma IGF-I concentration in normal adults of 20 to 59 years old was 147±49 ng/ml. The plasma IGF-I level fell gradually with age and became very low in subjects of over 70 years of age (Fig. 5). In general, females had slightly higher plasma IGF-I levels than males of the same age.

Plasma IGF-I concentrations in patients with pituitary dwarfism and active acromegaly

In 20 children with pituitary dwarfism, the mean plasma IGF-I concentration was 64.6±42.0 ng/ml, which was lower than that in normal children of corresponding ages. Seven patients with active acromegaly (plasma GH, 21.4±15.7 ng/ml) had high levels of plasma IGF-I (562±115 ng/ml).

Discussion

The new biosynthetic IGF-I used in the present study has the same primary structure as that of the natural peptide extracted from human blood. The purity of this synthetic peptide was confirmed to be more than 99% by HPLC (Niwa et al., 1986). The antibody developed to this pure IGF-I has high affinity and specificity for the peptide. This antibody was able to be used at a final dilution of 1:35,000 and showed no cross-reactivity with porcine insulin, biosynthetic human insulin, hEGF or synthetic C-domains of IGF-I and II even at high concentrations of 10-100 μg/ml. On the other hand, a biosynthetic analog of IGF-I, Thr59-IGF-I, reacted as well as IGF-I with the antibody. In addition to having high affinity and specificity, our RIA for IGF-I has excellent sensitivity and can detect as little as 1–1.5 ng/ml of the peptide. Therefore, plasma or serum samples diluted 1:20 to 1:40 could be used for the assay. Moreover, the validity of the RIA was satisfactory as indicated by the low intra- and interassay coefficients of variation. Another merit of this assay is that ¹²⁵I-labelled IGF-I maintains tracer activity for up to 3 months when kept frozen at −70°C.

For the assay, plasma or serum samples must be extracted with acid to separate IGF-I from its binding protein. We examined the molecular heterogeneity of the IGF-I carrier protein complex in plasma by gel filtration. When untreated plasma was chromatographed on a Sephadex G-50 fine column, IGF-I-like immunoreactivity was eluted only in the position of void volume, a molecular weight more than 30,000 (30K) daltons. On the other hand, on the same column acid-treated plasma gave a major peak of IGF-I-like immunoreactivity in the position of 7,000 M. W. and a minor peak in the position of void volume. The mean ratio of the IGF-I values of acid-treated and acid-untreated plasma was 4:1, but there was wide variation from one individual to another. These results indicate that without acid treatment, the IGF-I carrier protein complex of 150 K M. W., which is the major source of total IGF-I activity in the circulating blood (Hintz, 1984), cannot be detected by our RIA and only the
50 K complex is likely to be measured. However, the 50 K IGF-I carrier protein complex seems to show individual variation, and so does not always reflect the IGF-I concentration in the blood. Furthermore, the inhibition curve of acid-untreated plasma was not parallel to that of the standard curve, suggesting the existence of some interfering substances in native plasma. Two methods for acid treatment of plasma or serum for separation of IGF-I from its binding protein have been described: column chromatography with acidified elution buffer (Hintz et al., 1980; Zapf et al., 1981), and extraction with acid-ethanol (Daughaday et al., 1980). The latter method, by which a number of samples can be processed at the same time and fairly rapidly, seemed to be more useful, and so was employed in our assay system.

The clinical data obtained by our RIA are consistent with those reported by others (Bala et al., 1981; Clemmons et al., 1984; Furlanetto et al., 1977; Underwood et al., 1980; Zapf et al., 1981). A very low IGF-I concentration in cord plasma may be due to a negative feedback mechanism due to an elevated plasma GH level, as proposed by Hintz et al. (1977). In children, especially those of under 2 years old, the blood IGF-I level is low, despite rapid physical development. From 3 years of age, the blood IGF-I level gradually rises, reaching the adult level at 7 or 8 years of age. Thereafter, the level continues to increase as children grow and reaches the maximum in early puberty.

The plasma IGF-I levels in normal adults decreased gradually with age and became very low in subjects of over 70 years old, possibly due to either reduced sensitivity of hepatic cells to GH stimulation or reduced production of IGF-I in the liver. In general, the plasma (or serum) IGF-I level tends to be slightly higher in females than in males of the same age, suggesting a role of sex hormones in regulating the IGF-I concentration. These data indicate that it is important to take age and sex into consideration when individual plasma IGF-I concentrations are compared with normal values. Results obtained in this study showed that the plasma IGF-I levels in patients with pituitary dwarfism were only 20% of the normal level, while those in patients with active acromegaly were approximately four times as high as normal, as reported by others (Clemmons and Van Wyk, 1984; Furlanetto et al., 1977; Hintz et al., 1980; Zapf et al., 1981).

These results indicate that this modified RIA system should be useful for IGF-I determination in basic and clinical studies.

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References


