Assay of Somatomedin C by Cartridge Extraction Prior to Radioimmunoassay With Antiserum Developed Against Synthetic Somatomedin C

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ABSTRACT

The whole molecule of human somatomedin C (SM-C) prepared by the total synthesis method was used as an antigen to produce an antiserum for a radioimmunoassay. Since plasma proteins that bind SM-C interfere with the assay, a method was developed that uses acid dissociation followed by C-2 cartridge extraction to strip SM-C from its binding proteins before assay. This assay has no cross-reactivity with human proinsulin or insulin-like growth factor II (IGF-II). The SM-C values in 339 normal subjects showed age-dependence, increasing from childhood to a peak at age 14 to 16 years and decreasing sharply before adulthood. In adults, the SM-C values decreased gradually with age. All 13 patients with acromegaly who were tested had an increased SM-C value, with no overlap with the normal range. The 12 patients with prolactinoma but non-growth-hormone-producing pituitary tumor had no increase in SM-C. Two children with pituitary deficiency had low SM-C values; one of these children received growth hormone therapy, and his SM-C value increased from undetectable to normal. By three weeks after discontinuation of the therapy, his SM-C value was again undetectable. Of 20 children with short stature and...
constitutional delay of growth and development, SM-C was below normal in 70 percent and normal in 30 percent. Two patients with malnutrition had below-normal SM-C values.

Introduction

Somatomedin C (SM-C), also called insulin-like growth factor I (IGF-I), is a polypeptide chain of 70 amino acid residues with three disulfide bonds. Structurally, it is similar to proinsulin but has a shorter connecting peptide (C-peptide), 12 amino acid residues instead of 33 as in proinsulin. One major difference is that the biologically active SM-C molecule includes the 12-amino acid C-peptide, in contrast to proinsulin from which the 33-amino acid C-peptide is enzymatically removed to form the active hormone, insulin.

A radioimmunoassay for SM-C would be useful in the management of growth hormone-related pituitary disorders because the concentration of SM-C circulating in the blood is constant, whereas that of growth hormone fluctuates during the day. Because the biosynthesis of SM-C is under the control of growth hormone, circulating levels of SM-C reflect growth hormone secretion.

The first problem in developing a SM-C assay is the lack of a source of SM-C antigen. There is no depot organ for this hormone in the body, and it has to be isolated either from whole plasma or from a by-product of commercial blood protein fractionation, Cohn fraction IV, however, the yield is very low. Only about 20 mg of crude SM-C was isolated from 6,000 kg of Cohn fraction IV. An alternative method is to use the 12-amino acid C-peptide as antigen to produce antiserum in the hope that there will be cross-reactivity against the whole molecule of SM-C. However, few animals produced antisera having such cross-reactivity.

Binding of SM-C by plasma proteins interferes or competes with binding of SM-C to antibody and thus poses a second problem in a SM-C assay. Sample treatments required prior to assay range from the simple addition of protamine to the assay buffer to acid-ethanol extraction to separation with acid buffer on a Sephadex column followed by lyophilization.

An antiserum was produced to synthetic SM-C, which solves the problem of finding readily available antigen. Acid dissociation and reverse-phase chromatography on a C-2 cartridge to extract SM-C prior to assay solve the second problem of interference by plasma binding protein in the interaction between SM-C and the antibodies to SM-C used as an assay reagent. The assay system was validated by analytical methods and used to quantitate SM-C levels in a large group of normal subjects. The system was then shown to give abnormal results in analyses of samples from patients with abnormalities of growth and growth hormone secretion.

Materials and Methods

Immunization of Animals

Whole SM-C prepared by Li and associates (total chemical synthesis method) was conjugated via glutaraldehyde to keyhole limpet hemocyanin (KLH) at a ratio of 50 μg of SM-C to 90 μg of KLH. Conjugate equivalent to 50 μg of SM-C was homogenized with complete Freund's adjuvant and injected intradermally into two goats at multiple sites. Booster injections of the same preparation of antigen were given monthly at
the same dose. After six booster injections, one goat produced antibody with a titer of 1:1200.

IODINATION

The purchased* SM-C was used to prepare radiolabeled tracer. A modified lactoperoxidase method15,19 was used to iodinate 2.5 µg of SM-C with 1,500 µCi of 125I. The labeled mixture was purified by gel filtration on two columns, first on a Bio-Rad P-6 column (0.7 × 15 cm) for desalting and then on a Sephadex G-50 column (1 × 45 cm) for final purification. The elution buffer used with both columns was 0.2 M ammonium acetate at pH 4.8 containing 0.1 percent Tween 20 and 0.1 percent gelatin.

The purified labeled SM-C was tested for homogeneity by high pressure liquid chromatography (HPLC), in a Varian model 5000† with reverse-phase C18 column 300 × 4 mm‡. The mobile phase was a linear gradient of 25 percent to 45 percent acetonitrile in one percent aqueous trifluoroacetic acid. The total elution time was 20 minutes; one-minute fractions were collected and the radioactivity in each fraction was counted.

RADIOIMMUNOASSAY

The SM-C purchased from AmGen also was used to prepare a standard curve as follows. A solution of 10 ng of SM-C in 200 µl of assay buffer (0.04 M sodium phosphate containing 0.1 percent Tween 20 and 0.1 percent gelatin, pH 7.4) was serially diluted 1:2 with assay buffer to 0.08 ng per 200 µl. A mixture of 50 µl of tracer containing 10,000 cpm, 50 µl of diluted antiserum that would bind 45 percent of the tracer, and 200 µl of standard or 200 µl of extracted sample was incubated at 4°C for three days. The free tracer and bound tracer were separated with a second antiserum, burro anti-goat IgG. All standards and samples were assayed in duplicate.

CROSS-REACTIVITIES

Human proinsulin§ was dissolved in assay buffer at 2,000, 500, 200, and 125 ng per ml, and 200-µl samples of each solution (containing 400, 100, 50, and 25 ng of proinsulin, respectively) were assayed in duplicate to check the cross-reactivity of human proinsulin with the antiserum. The cross-reactivity of insulin-like growth factor II11 was determined with the same procedure at 400, 200, 100, and 50 ng per 200 µl.

DISSOCIATION OF SM-C FROM BINDING PROTEINS

Elution With Neutral Buffer. A 0.3-ml sample of normal plasma was incubated with 125I-labeled SM-C (8 × 106 cpm) at 4°C for two hours. A 0.2-ml sample of this mixture was applied to a Sephadex G-100 column (1 × 50 cm) which was eluted with assay buffer at pH 7.4. The radioactivity of one-ml fractions was counted in a gamma counter. The column was precalibrated with blue dextran, hemoglobin, cytochrome c, and potassium ferrocyanide (molecular weights: 2 × 106, 64,500, 13,000, and 368, respectively).

Acid Dissociation and Elution With Acidic Buffer. From the same plasma, a 0.2-ml sample was incubated with 125I-labeled SM-C (20 × 106 cpm) in the same manner as that described previously. After the incubation, the sample

* AmGen Biologicals, Thousand Oaks, CA.
† Varian, Sunnyvale, CA.
‡ Alltech Associates, Inc., Deerfield, IL.
§ Gift from Eli Lilly Co., Indianapolis, IN.
1 IGF-II, gift from Dr. Juergen Zapf, University of Zurich, Switzerland.
was mixed with 1.5 ml of 1 M acetic acid and incubated again for two hours at 4°C. Then 0.2 ml of the mixture was applied to the same Sephadex G-100 column which was equilibrated and eluted with an acidic buffer (0.2 M ammonium acetate containing 0.1 percent gelatin and 0.1 percent Tween 20, pH 4.8). The one-ml fractions were assayed for radioactivity.

Acid Dissociation, Cartridge Extraction, and Then Elution With Acidic Buffer. Another 0.2-ml sample from the same plasma was incubated with $^{125}$I-labeled SM-C (3.58 × 10$^6$ cpm) and then mixed with 1.5 ml of 1 M acetic acid as described in previous paragraph. This mixture was applied to a 0.5-g C-2 cartridge that was pretreated with three ml of methanol followed by six ml of 0.5 M acetic acid containing 0.1 percent trifluoroacetic acid. Then, the cartridge was washed three times with 2-ml portions of 0.5 M acetic acid containing 0.1 percent trifluoroacetic acid and eluted with three ml of acetonitrile per one percent aqueous trifluoroacetic acid mixture (75:25). The eluant was collected and dried at room temperature under reduced pressure in a running centrifuge.** The dried material was dissolved in 0.3 ml of the acidic buffer; radioactivity was counted; and 0.2 ml of this solution was applied to the same Sephadex column and eluted with the acidic buffer; one-ml fractions were collected and assayed for radioactivity.

Elution Curve of $^{125}$I-Labeled SM-C in Absence of Plasma. $^{125}$I-Labeled SM-C in 0.2 ml of acidic buffer was applied to the same Sephadex column and eluted with the acidic buffer; one-ml fractions were collected and assayed for radioactivity.

Subjects

Blood samples were collected from 339 normal subjects (both sexes and ages less than one year to 64 years); proper informed consent was given by the subjects or by their parents. Blood also was drawn from patients with growth-hormone-related disorders or other diseases (after informed consent was obtained).

Results

Purity of Labeled SM-C

The Bio-Rad P-6 column separated the iodination mixture into two peaks—protein-bound iodine and free iodide. The fractions of protein peak were pooled and separated on the Sephadex G-50 column into three peaks (figure 1 Upper). The second peak showed the greatest binding, and the first peak (void volume) and third peak (free salt) showed little binding with the antiserum. The fractions of the second peak were pooled and tested for homogeneity by HPLC on a C$_{18}$ column with a linear gradient (25 percent to 45 percent) of acetonitrile in one percent aqueous trifluoroacetic acid. This yielded a single peak (figure 1 Lower) that had the same binding characteristics with the antiserum as the second peak from the Sephadex G-50 column. This indicates that further purification by HPLC is not necessary.

Dissociation of SM-C

After incubation of labeled SM-C with plasma, under neutral conditions (pH 7.4), the bound labeled SM-C was eluted in three major peaks representing molecular weights of approximately 150,000, 56,000, and 8,000 (figure 2 A). The first two peaks were protein-bound material; the last one presumably was unbound SM-C (molecular weight, 7,469).

¶ Analytichem International, Harbor City, CA.
** Speed Vac concentrator, Savant Instruments, Farmingdale, NY.
After elution under acidic condition (pH 4.8) from the same column, only the labeled SM-C bound to molecular weight 150,000 protein disappeared (figure 2 B). When the acidified mixture was further extracted with a C-2 cartridge, a single peak was eluted (figure 2 C); it coincided with the peak of labeled SM-C alone in the absence of plasma protein (figure 2 D). The recoveries of radioactivity were 86 percent from the C-2 cartridge extraction and 96 percent from the Sephadex G-100 column. Thus, acidification with acetic acid and C-2 cartridge extraction became the method used to extract all our plasma samples.

**SAMPLE COLLECTION AND SM-C EXTRACTION**

Blood (five ml from adults; two ml from children) was collected into EDTA tubes and centrifuged in a refrigerated centrifuge. The plasma was transferred to a glass tube, frozen in dry ice, and stored at −20°C in the laboratory until assayed. For assay, 0.2 ml of plasma was thawed, mixed with 1.5 ml of 1 M acetic acid, and applied to a C-2 cartridge that had been pretreated with three ml of methanol and six ml of 0.5 M acetic acid containing one percent trifluoroacetic acid. The cartridge was washed three
Figure 2. Dissociation of somatomedin C (SM-C) from plasma protein. A, 125I-Labeled SM-C was incubated with human plasma, applied to Sephadex G-100 column (1 x 50 cm), and eluted with pH 7.4 phosphate-buffered saline; one-ml fractions were assayed. Molecular weight markers: BD, blue dextran \(2 \times 10^5\); Hb, hemoglobin; Cyt c, cytochrome c; salt, potassium ferrocyanide. B, Dissociation with 1 M acetic acid and eluted from Sephadex G-100 with ammonium acetate, pH 4.8. C, Dissociated with 1 M acetic acid and extracted with C-2 cartridge. The cartridge was washed, and the SM-C was eluted from the cartridge, dried, dissolved, and applied to Sephadex G-100 column and eluted with ammonium acetate, pH 4.8. D, Labeled SM-C not bound to plasma protein eluted from Sephadex G-100 column with ammonium acetate, pH 4.8.

Validation of Analytical Procedure

Recovery Study. A pooled sample was divided into two portions. To one portion was added SM-C standard at 62.5 ng per ml; the other portion without added SM-C standard was the baseline. Each portion was further divided into 20 aliquots, and then all 40 aliquots were put through the extraction and assay procedures. The results (mean ± SD) were 84 ± 9 ng per ml for the baseline aliquots and 129 ± 16 ng per ml for the aliquots with added standard, for a recovery of 72 percent of the added SM-C.

Linearity. Plasma samples from three normal subjects were extracted, diluted 1:5, 1:10, and 1:20 with assay buffer, and then assayed for SM-C. The results were proportional to the dilutions (table I).

Sensitivity and Cross-Reactivity

The sensitivity of this assay is at least 0.078 ng per tube because 0.078 ng of SM-C gave a 10 percent suppression on the standard curve (figure 3). There was no cross-reactivity with human proinsulin up to 2,000 ng per ml of plasma. The cross-reactivity with IGF-II was 1.1 percent. Because it was suspected that the cross-reactivity with IGF-II would be lower after extraction, 100 ng of IGF-II were mixed with 1.5 ml of 1 M acetic acid, extracted by C-2 cartridge, and radioimmunoassayed. There was no
measurable immunoreactive activity of the added IGF-II after extraction. That means that, after extraction, IGF-II up to 500 ng per ml will not show any measurable cross-reactivity.

**NORMAL SUBJECTS**

The concentration of SM-C in plasma samples from 339 normal subjects plotted against the age (figure 4) indicates age-dependence. The SM-C concentration increased sharply with age to a peak at age 14 to 16 years and then began to decline (table II).

**TABLE I**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Dilution</th>
<th>Somatomedin C, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Observed</td>
</tr>
<tr>
<td>Subject 1</td>
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<td>111</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>58.6</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>32.1</td>
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<tr>
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<td>88</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
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<tr>
<td></td>
<td>1:20</td>
<td>22.9</td>
</tr>
<tr>
<td>Subject 3</td>
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<td>136</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>1:20</td>
<td>34</td>
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</table>

**Patients**

Among the adult patients, all 13 acromegalic patients with high growth hormone values had high SM-C plasma values with no overlapping with normal (figure 5). Of the 12 patients with prolactin-producing pituitary adenoma but not growth-hormone-producing tumor (their growth hormone values were within the normal range), nine had normal SM-C and three had lower than normal levels. Both patients with nonfunctioning pituitary tumor had below-normal SM-C values and undetectable plasma growth hormone levels.

Among pediatric patients, of the 20 patients with short stature owing to constitutional delay of growth and development, SM-C concentration was lower than normal in 14 and normal in six. Two patients with growth hormone deficiency had lower than normal values; neither showed an increase in growth hormone concentration after insulin-induced hypoglycemia and arginine stimulation. One patient (both weight and height below the 5th percentile for his chronologic age of nine years) received human growth hormone (produced by recombi-
nant deoxyribonucleic acid [DNA] technology) subcutaneously, at two IU three times a week. His SM-C value increased from undetectable to 42 ng per ml, a level within the normal range for his age. Three weeks after treatment with growth hormone was discontinued, his SM-C value was undetectable.

Two patients with malnutrition or insufficient calorie intake had lower than normal SM-C values. One patient's body weight was well below the 5th percentile for her age, and the other was at the 10th percentile for his age. The boy improved his food intake to some extent; six months later his body weight was at the 25th percentile, and his SM-C was increased about 2-fold but was still slightly below the normal range for his age (15 years).

Two children who had excessive growth, but otherwise were healthy, had normal SM-C levels.

**Discussion**

The usefulness of a reliable SM-C assay in the evaluation of patients with acromegaly or with disorders of growth has been amply demonstrated by

**TABLE II**

<table>
<thead>
<tr>
<th>Age, year</th>
<th>n</th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
<th>90 Percent Confidence Limits</th>
<th>P*</th>
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<tbody>
<tr>
<td>0-5</td>
<td>32</td>
<td>42</td>
<td>21</td>
<td>5-82</td>
<td>12-73</td>
<td>--</td>
</tr>
<tr>
<td>6-10</td>
<td>69</td>
<td>69</td>
<td>34</td>
<td>4-186</td>
<td>26-111</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>11-13</td>
<td>32</td>
<td>138</td>
<td>52</td>
<td>44-215</td>
<td>68-207</td>
<td>&lt; 0.001</td>
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<tr>
<td>14-16</td>
<td>43</td>
<td>169</td>
<td>43</td>
<td>48-256</td>
<td>104-249</td>
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<td>78-210</td>
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<tr>
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<td>52-132</td>
<td>&lt; 0.001</td>
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<td>42-116</td>
<td>52-100</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>&gt; 45</td>
<td>29</td>
<td>63</td>
<td>17</td>
<td>31-104</td>
<td>42-97</td>
<td>&lt; 0.01</td>
</tr>
</tbody>
</table>

*For difference from next younger age group
others. Nonetheless, the availability of reliable SM-C assays has been restricted to only a few laboratories because of difficulties in making antibodies specific for SM-C in titers and amounts adequate to support a reliable assay for clinical use over long periods of time. Because of the number of patients seen by the present authors for whom SM-C assays would be applicable, the development of a dependable assay for SM-C was undertaken. However, the facilities were lacking to isolate SM-C from large amounts of human plasma and it was not possible to obtain an antiserum with useful cross-reactivity despite repeatedly using the 12-amino acid C-peptide in different species of animals. Hence, the synthetic whole molecule of SM-C as antigen was tried when it became available and an alternative method was developed to make a reliable, clinically useful assay for SM-C.

The interference of binding proteins in the radioimmunoassay for SM-C is well known. A number of methods have been used to minimize this problem, such as exposure of the plasma sample to heparin or protamine, acid-ethanol extraction, acid Sephadex column separation, or treatment with acid and then lyophilization. When plasma interference is high—or when hormone concentration is low—assay strategies, including an extraction procedure, have become increasingly popular. In the case of SM-C assay, a concentration procedure is unnecessary because plasma SM-C concentrations are high and the assay is adequately sensitive. However, the interference of binding proteins is a major problem. Extraction was used by the present authors to remove the interfering SM-C binding proteins prior to assay.

Various cartridges became commercially available for extraction, including silica, amino, and C18 cartridges. These have been applied in the extraction of a number of hormones prior to assay—

![Figure 5. Somatomedin C (SM-C) concentrations in patients with various diseases. Because normal SM-C concentration is age dependent, symbols are used to indicate whether or not a value is normal. Particularly in pediatric patients, the normal value of SM-C is lower in a young child than in an older child; thus, the far right column shows both normal and below-normal at the same SM-C level. Arrow, Change in malnutrition patient at six months after improving food intake.](image-url)
e.g., assays of adrenocorticotropin, atrial natriuretic peptide, and calcitonin.\textsuperscript{2,3,13} Although use of such cartridges changes assays from direct to indirect assays, it greatly improves their reliability and sensitivity. One windfall advantage is that extraction assays may decrease the risk of blood-transmitted diseases, which at present is a concern of all laboratory personnel. The extraction procedure requires mixing the patient’s plasma with a high concentration of strong acid as a first step, before applying the sample to the cartridge, and this will inactivate most of the microorganisms present.

The molecular weights of the major SM-C binding proteins were 150,000 and 56,000, as estimated on a Sephadex G-100 column. Studies of acid dissociation showed that the binding of SM-C with the larger protein was weaker than that with the smaller protein. The SM-C bound to the larger protein could be released with 1 M acetic acid, but that bound to the smaller protein could not be released by acid alone (it required the additional help of a C-2 cartridge). The molecular weights of these two binding proteins are similar to those found by other investigators using Sephadex G-200 columns.\textsuperscript{7,20} The 150,000-dalton binding protein has been shown to be growth hormone-dependent; in patients with growth hormone deficiency, this binding protein disappeared and then reappeared when the patients received growth hormone therapy.

By using synthetic SM-C as an antigen and acid dissociation and cartridge extraction to remove interfering binding proteins, a clinically useful radioimmunoassay for SM-C was developed by us. In addition to establishing a normal range for various age groups, it has been shown that patients with acromegaly have high levels of SM-C. Preliminary data suggest that children with growth hormone deficiency and many with constitutional delay of growth and development have low SM-C levels. Finally, by using this assay, it has been shown by us that patients with undernutrition have low circulating levels of SM-C.

References


