Cultured Fibroblast Monolayers Secrete a Protein That Alters the Cellular Binding of Somatomedin-C/Insulinlike Growth Factor I

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Abstract
We studied somatomedin-C/insulinlike growth factor (Sm-C/IGF-I) binding to human fibroblasts in both adherent monolayers and in suspension cultures. The addition of Sm-C/IGF-I in concentrations between 0.5 and 10 ng/ml to monolayer cultures resulted in a paradoxical increase in 125I-Sm-C/IGF-I binding and concentrations between 25 and 300 ng/ml were required to displace the labeled peptide. The addition of unlabeled insulin resulted in no displacement of labeled Sm-C/IGF-I from the adherent cells. When fibroblast suspensions were used Sm-C/IGF-I concentrations between 1 and 10 ng/ml caused displacement, the paradoxical increase in 125I-Sm-C/IGF-I binding was not detected, and insulin displaced 60% of the labeled peptide. Affinity cross-linking to fibroblast monolayers revealed a 43,000-mol wt 125I-Sm-C-binding-protein complex that was not detected after cross-linking to suspended cells.

The 43,000-mol wt complex was not detected after cross-linking to smooth muscle cell monolayers, and binding studies showed that 125I-Sm-C/IGF-I was displaced >90% by Sm-C/IGF-I using concentrations between 0.5 and 10 ng/ml. Because fibroblast-conditioned medium contains the 43,000-mol wt complex, smooth muscle cells were incubated with conditioned medium for 24 h prior to initiation of the binding studies. 125I-Sm-C/IGF-I-binding increased 1.6-fold compared to control cultures and after cross-linking the 43,000-mol wt complex could be detected on the smooth muscle cell surface. Human fibroblast monolayers secrete a protein that binds 125I-Sm-C/IGF-I which can be transferred to the smooth muscle cell surface and alters 125I-Sm-C/IGF-I binding.

Introduction
Cultured human skin fibroblasts have been studied extensively as a model to determine the effects of somatomedins on cell growth and DNA synthesis (1, 2). Several investigators have analyzed the characteristics of binding of 125I-somatomedin-C/insulinlike growth factor I (Sm-C/IGF-I) to cultured fibroblasts and compared its binding properties with its cell growth-stimulating properties (3, 4). To perform binding studies, suspended cell systems have been commonly used (3–5). Suspended fibroblasts have been shown to possess two types of somatomedin receptors (6, 7). The type I receptor has two subunits, Mr = 135,000 (alpha) and 95,000 (beta), and binds Sm-C/IGF-I with a higher affinity than IGF-II and both with a greater affinity than for insulin; whereas the type II receptor has an Mr = 260,000, binds IGF-II preferentially, and does not bind insulin. In order to determine the effects of somatomedins on DNA synthesis, however, adherent monolayer cultures must be used because fibroblasts in suspension do not undergo DNA synthesis and division. Exposure to trypsinization and removal of the cells to create suspended cell preparations has been shown to alter the binding of peptide growth factors to cell surface receptors (8) and various conditions such as treatment at 4°C and the use of chelating agents have been adopted in order to minimize these alterations. It has been reported that human fibroblast monolayers possess specific Sm-C/IGF-I receptors, that bound Sm-C/IGF-I is fully dissociable, that displacement of bound 125I-Sm-C/IGF-I occurs at concentrations of unlabeled Sm-C/IGF-I between 1 and 100 ng/ml (9, 10), and that insulin when added at a concentration of 10 μg/ml displaces >60% of 125I-Sm-C/IGF-I from the monolayers. In contrast other investigators have reported that although there are type I receptors present in fibroblast monolayers, binding to the type I receptor may represent only a small portion of the total 125I-Sm-C/IGF-I that is bound (11).

In attempting to perform similar binding studies we were not able to effectively displace labeled Sm-C/IGF-I from fibroblast monolayers using concentrations in the range of 1.0–10 ng/ml, and the addition of insulin resulted in no displacement. Because monolayer cultures are required to demonstrate Sm-C/IGF-I stimulation of replication, we undertook studies using monolayer cultures to define the factors responsible for our inability to displace the labeled peptide effectively and to test the hypothesis that an additional cell surface protein was present that could bind Sm-C/IGF-I but not insulin.

Methods
Cell culture techniques. Human fibroblasts were purchased from the Human Mutant Genetic Cell Repository (Camden, NJ) or obtained from foreskins of newborn donors. Fibroblasts that were purchased were dermal in origin and were obtained from donors of varying ages. These include GM-10, 12 wk fetal; GM-4392, 16 wk fetal; GM-4525, 17 wk fetal; GM-1437, newborn; GM-498, 3 yr; GM-1362, 14 yr; GM-6291, 90 yr; GM-731, 96 yr. All cultures were plated in plastic petri dishes (Falcon Labware, Oxnard, CA) using Eagle’s minimum essential medium (MEM) supplemented with penicillin 100 U/ml, streptomycin 100 μg/ml, purchased from Grand Island Biological Company (Gibco, Grand Island, NY) and 10% bovine serum (Colorado Serum Co. Denver, CO). Fetal calf serum (FCS), used in some experiments, was purchased from two sources; HyClone Laboratories (Denver, CO; lot 110454) and Gibco (lot 36K824). Cells were routinely cultured in 10-cm dishes (Falcon 3001) in MEM...
containing 10% calf serum and were incubated at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. The medium was changed every third day and the cells were harvested by trypsinization (trypsin 0.1%, EDTA 0.03%, Gibco) and replated approximately once every 10 d using the split ratio of 1:4. Both stock and test cultures were examined at monthly intervals for *Mycoplasma pneumoniae* and were found to be uncontaminated. For studies of ¹²⁵I-Sm/C-IGF-I binding stock cultures were subcultured into 24-well plates (Falcon 3004, 16-mm diam) using a plating density of 10,000 cells/cm² except where variations in plating density were required to achieve differences in final cell density. Cells were cultured for 3 d in MEM supplemented with 10% FCS then the medium was changed and the incubation continued for an additional 3 d. Cell number was determined in triplicate wells using a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL) on the day that the binding experiment was performed.

**Determination of ¹²⁵I-Sm/C-IGF-I binding to monolayer fibroblast cultures.** Quiescent monolayers were washed three times with phosphate-buffered saline (PBS) and then the cultures were exposed to binding medium containing MEM plus Hepes 20 mM and 0.1% bovine serum albumin (BSA) (pH 7.4). ¹²⁵I-Sm/C-IGF-I (prepared as described below) was added at a concentration of 80,000 cpm/well in a final incubation volume of 0.25 ml. After a 2-h incubation at 4°C (there was no further increase in binding during a 3- or 4-h incubation) the medium was aspirated, the cells were washed three times with cold PBS, and then solubilized using 0.3 N NaOH for 1 h at 22°C. Cell lysates were transferred to plastic tubes and ¹²⁵I-Sm/C-IGF-I that was cell associated was determined in a Beckman gamma spectrometer (Beckman Instruments, Inc., Palo Alto, CA). Non-specific binding was determined by subtracting the radioactivity bound in the presence of an excess (1.0 μg/ml) of unlabelled Sm-C-IGF-I that had been partially purified by a previously described method (12). Total radioactivity bound was consistently in the range of 5-9% and non-specific binding was consistently <15% of the total bound or the experiment was discarded. In all experiments in which competition curves were generated, unlabelled Sm-C/IGF-I or insulin was added at increasing concentrations varying between Sm-C/IGF-I 0.1 and 300 ng/ml or insulin between 1.0 and 10.0 μg/ml.

**Suspended fibroblast cultures.** Fibroblast cell suspensions to be used for binding studies were prepared as follows: confluent monolayers were treated with 0.1% trypsin in 0.03% EDTA for 1 min at 24°C and the reaction was stopped by addition of MEM plus 10% FCS. The cells were pelleted then resuspended in 0.25 ml of binding buffer and added to 12 × 75-mm polystyrene tubes (1–1.5 × 10⁶ cells/tube). ¹²⁵I-Sm/C-IGF-I (120,000 cpm) was added to each tube. Tubes were incubated for 2 h at 8°C with agitation every 15 min then washed once with 0.5 ml of PBS and once with 1.0 ml of PBS. After centrifugation the cell pellet was counted.

**Preparation of growth factors.** Pure preparations of Sm-C/IGF-I were used in all experiments except to determine nonspecific binding. The purification procedure and criteria for purity have been previously published (13). Purified Sm-C/IGF-I was iodinated by a modified chloramine-T method (14) to a specific activity of 300 μCi/μg. After iodination the peptide was further purified by affinity chromatography using a Sepharose-linked IgG Sm-C/IGF-I antibody. Immune Sm-C/IGF-I-I that was used to determine nonspecific binding was purified by acid extraction of acromegalic plasma followed by SP-Sephadex and G-50 Sephadex gel filtration chromatography as previously published (12). Porcine (Pit-5862) and human insulin were a gift from the Eli Lilly Co. (Indianapolis, IN). IGF-II was purified by a previously described method and purity was confirmed by amino acid sequencing (15).

**Preparation of conditioned medium.** Quiescent fibroblasts or smooth muscle cell monolayers that had been grown to confluence on 10-cm plates were washed three times with PBS and exposed to serum-free MEM for 2 h at 37°C. The conditioned medium was removed, centrifuged at 2,000 × g for 15 min to remove cellular debris, and stored at −20°C until use (usually within 2 wk).

**Determination of Sm-C/IGF-I binding to smooth muscle cultures.** Smooth muscle cells were isolated from the aortas of 3-wk-old pigs by a previously published method (16). Stock cultures were maintained in 10-cm dishes (Falcon 3001) in Dulbecco's modifed Eagle's medium (DME) supplemented with 10% FCS (Gibco), glatmine 10 mM (Gibco), penicillin 100 U/ml, and streptomycin 100 μg/ml (Gibco). Media was changed every third day until growth was confluent (11 d), then the cells were removed by exposure to 0.1% trypsin, 0.03% EDTA (Gibco), and replated between cell densities of 10,000–12,000 cells/cm². To determine Sm-C/IGF-I binding to porcine aortic smooth muscle cells these cells were plated in 24-well 16-mm dishes (Falcon 3004) at a density of 6,000 cells/cm² in DME containing 10% FCS. After 6 d of incubation cells were washed extensively with PBS and then exposed to serum-free medium containing a 50% concentration of conditioned medium from either human fibroblast cultures or porcine aortic smooth muscle cells. After a 14–48-h incubation in DME containing the conditioned medium, the confluent monolayers were again washed extensively and ¹²⁵I-Sm-C/IGF-I (80,000 cpm/well) was added to 0.25 ml of DME supplemented with 20 mM Hepes, 0.1% BSA, pH 7.4, and the incubation continued for 2 h at 8°C. Specific binding of cell-associated ¹²⁵I-Sm-C/IGF-I was determined as described for the fibroblast cultures.

**Affinity cross-linking studies.** Affinity labeling was performed according to a modification of methods described by Adams et al. (17) and Wilkins and D'Ercole (18). Briefly, the conditioned media was incubated with specific antigen (pre-C-IGF-I at 10 μg/ml) for 10 min in Hepes binding buffer with BSA 1%, pH 7.4, with or without varying concentrations of unlabeled Sm-C/IGF-I and insulin. The cross-linking agent, disuccinimidyl suberate (DSS; Pierce Chemical Co., Rockford, IL) was then added in a final concentration of 0.5 mM and the samples incubated for 10 min at 22°C. The reaction was stopped by the addition of 50 mM Tris and the samples hydrolyzed by boiling for 5 min. The proteins were then separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

For studies on the monolayer cultures the fibroblasts or smooth muscle cells were grown to confluence in 35-mm dishes (Falcon), washed three times with Hepes binding buffer containing BSA 1%, pH 8.0, and incubated with ¹²⁵I-Sm-C/IGF-I in the same buffer at 4°C for 2 h with or without varying concentrations of unlabeled Sm-C/IGF-I, insulin or the anti-type I-receptor monoclonal antibody. The hybridoma cell line producing the monoclonal antibody to the type I receptor, designated alpha-IR3, was a gift of Dr. Steven Jacobs (Burroughs Wellcome, Research Institute Park, NC). This antibody, which is directed at the type I Sm-C/IGF-I receptor (19), was amplified in ascites fluid and purified by sequential precipitation in 18% and 15% sodium sulfate. In a preliminary study of the time of incubation (2–18 h), the longer incubations resulted in a slight increase in intensity of labeled bands seen on autoradiography, but in no differences in the protein species that bound to ¹²⁵I-Sm/C-IGF-I. The monolayers were then washed twice with cold Hepes buffer without BSA, pH 7.4, and DSS was added in a final concentration of 0.1 mM in 1.0 ml of the same buffer. After a 10-min incubation at room temperature, the reaction was quenched with 3 vol 10 mM Tris, pH 7.0, and 1 mM EDTA. After 5 min, the Tris-EDTA was aspirated and the cells were solubilized in 0.1 ml of 50 mM Tris, 2% SDS with or without 100 mM diethiothreitol (DTT). The cell lysate was transferred to a 1.5 ml microfuge tube and boiled for 5 min, and the proteins were separated on a 3–14% gradient SDS-PAGE (20). The gels were fixed with 10% acetic acid and 30% methanol, washed, dried, and then exposed to Kodak X-4 mat film (Eastman Kodak Co., Rochester, NY). The autoradiograms were then developed by standard techniques. The molecular weights of the standards used were: myosin 200,000; phosphorylase B 97,400, BSA 68,000; ovalbumin 43,000; alpha-chymotrypsinogen 25,700; beta-lactoglobulin 18,400; lysozyme 14,300 (Bethesda Research Laboratories, Gaithersburg, MD).

**Results**

Between 5% and 9% of the radiolabeled Sm-C/IGF-I bound to the fibroblast monolayers. When increasing concentrations of unlabelled Sm-C/IGF-I (1–20 ng/ml) were added, however, ¹²⁵I-Sm/C-IGF-I binding increased 40–65% over basal and decreased only when concentrations were increased to between 25 and 300

**Somatomedin-C Binding Alteration by 35,000-mol wt Protein 1549**
ng/ml (Fig. 1). In contrast, when 125I-Sm-C/IGF-I binding was determined using suspension cultures, low concentrations of Sm-C/IGF-I (0.5–10 ng/ml) displaced significant amounts of the labeled peptide (Fig. 1). To determine whether this phenomenon was limited to one fibroblast strain, monolayer cultures prepared from eight other fibroblast strains were analyzed for the capacity of Sm-C/IGF-I to displace labeled Sm-C/IGF-I. In all the cell lines tested, there was a significant increase in 125I-Sm-C/IGF-I binding at low concentrations (1–10 ng/ml) and no lines showed >29% displacement at the 50 ng/ml concentration (Table I).

Because high concentrations of unlabeled insulin have been shown to compete effectively for binding of 125I-Sm-C/IGF-I to type I receptors, increasing concentrations of unlabeled insulin were incubated with 125I-Sm-C/IGF-I to determine the capacity of this peptide to displace bound 125I-Sm-C/IGF-I from the adherent monolayers and from cells in suspension. Unlabeled insulin displaced 60% of the labeled material from suspended cells (Fig. 2) but had no effect on binding of 125I-Sm-C/IGF-I to the adherent monolayers. Again when eight other cell lines were tested unlabeled insulin was incapable of displacing 125I-Sm-C/IGF-I from adherent cultures (Table I). It appears, therefore, that in monolayer culture most of the 125I-Sm-C/IGF-I is binding to a site that does not bind insulin.

Alterations in the experimental conditions prior to performing the binding experiments on monolayers did not change the sensitivity of fibroblast 125I-Sm-C/IGF-I binding to competition by unlabeled Sm-C/IGF-I. Specifically, when monolayer cultures were grown to densities between 20,000 and 150,000 cells/cm² or when the interval since the last media change prior to performing the binding experiment was varied from 1 to 5 d, Sm-C/IGF-I binding was not altered. If the fibroblast monolayers were exposed to serum-free media for 48 h with two media changes prior to initiation of the experiment, however, the paradoxical increase in Sm-C/IGF-I binding was eliminated but the cultures were still relatively insensitive to competition by unlabeled Sm-C/IGF-I.

To investigate the possibility that in monolayer cultures Sm-C/IGF-I was binding to a site other than the type I receptor, a specific type I receptor monoclonal antibody (alpha-IR3) was added at increasing concentrations to both suspended and monolayer cultures and coincubated with 125I-Sm-C/IGF-I. This antibody caused a no displacement of radiolabeled Sm-C/IGF-I from the monolayers cultures, even when concentrations as high as 10⁻¹ M were used, but competed effectively with 125I-Sm-C/IGF-I.

Table I. Binding of Sm-C/IGF by Several Fibroblast Strains

<table>
<thead>
<tr>
<th>Cell designation</th>
<th>Donor age</th>
<th>% of control bound*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3-6 ng/ml</td>
<td>50 ng/ml</td>
</tr>
<tr>
<td>GM-10</td>
<td>12 wk</td>
<td>133±7</td>
</tr>
<tr>
<td>4392</td>
<td>12 wk</td>
<td>140±12</td>
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<td>498</td>
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<td>112±11</td>
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<tr>
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<td>90 yr</td>
<td>114±10</td>
</tr>
<tr>
<td>731</td>
<td>96 yr</td>
<td>115±8</td>
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</tbody>
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* Control binding is defined as the amount of 125I-Sm-C/IGF-I bound in the absence of any unlabeled SM-C/IGF-I.

Figure 1. Binding of Sm-C/IGF-I to human fibroblast monolayers and cell suspensions. Human fibroblast monolayers (○) were grown to confluence (85,000 cells/well) as described in methods. After extensive washing with PBS, 0.25 ml of DME containing 2.5 mM Hepes, 0.1% BSA, pH 7.4 was added. 125I-Sm-C/IGF-I (80,000 cpm/well) was added and the incubation was continued for 2 h at 8°C. The monolayers were then washed three times with PBS, solubilized in 0.3 N NaOH, and the cell-associated radioactivity was determined. Cell suspensions (■) were prepared by exposing monolayer cultures to trypsin 0.1%, EDTA 0.03% for 1 min, and after transfer of 1.5 × 10⁶ cells/tube in 0.25 ml of buffer, were incubated with 120,000 cpm 125I-Sm-C/IGF-I for 2 h at 8°C. After centrifugation, the cell associated 125I-Sm-C/IGF-I was quantitated. Increasing concentrations of unlabeled Sm-C/IGF-I (0–300 ng/ml) were added simultaneously with 125I-Sm-C/IGF-I to triplicate cultures and each point is the mean value.

Figure 2. Competition between 125I-Sm-C/IGF-I and insulin for binding to fibroblast monolayers and suspension cultures. Both fibroblast monolayers (○; density = 88,000 cells/well) and suspension cultures (■) were prepared as described in Fig. 1. After addition of increasing concentrations of insulin (0–10 μg/ml) the incubations were carried out and cell-associated 125I-Sm-C/IGF-I was determined as described in Fig. 1.
IGF-I in suspension cultures (Fig. 3). This finding suggests that the $^{125}$I-Sm-C/IGF-I is bound to a site other than type I receptor (i.e., a receptor that does not bind insulin such as the type II receptor), and that the type I receptor binding represents only a very low percentage of the total binding observed.

To determine the characteristics of the $^{125}$I-Sm-C/IGF-I binding moiety, affinity cross-linking studies were performed. After cross-linking $^{125}$I-Sm-C/IGF-I-binding complexes from monolayer cultures were subjected to SDS-PAGE under reducing conditions and autoradiographed. Two major bands (Fig. 4) were observed: a 135,000-mol wt band, characteristic of the alpha-subunit of the type I receptor, and a more intensely labeled band at 43,000. A less intense band of ~260,000 mol wt was also observed. The 260,000-, 135,000-, and 43,000-mol wt bands were either reduced in intensity or obliterated in the presence of 50 ng/ml unlabeled Sm-C/IGF-I. High concentrations of insulin (20 μg/ml) partially displaced labeled Sm-C/IGF-I from the 135,000-mol wt band and the 260,000-mol wt band, but not from the 43,000-mol wt band (Fig. 4). Similarly alpha-IR3 inhibited labeling of the 135,000-mol wt subunit and the 260,000-mol wt band. In the presence of low concentrations of unlabeled Sm-C/IGF-I, however, the intensity of labeling of both the 135,000- and 43,000-mol wt bands was apparently increased (Fig. 5). To exclude the possibility that the binding protein in the 43,000-mol wt complex was associated with the culture dish or cellular matrix and not the cell surface, the matrix was extracted after cell removal with Triton X-100 and the counts per minute associated with the plastic surface also quantitated. Less than 8% of the total counts bound were detected in either the matrix or the dish.

To confirm that Sm-C/IGF-I binding to a protein in the 43,000-mol wt complex accounted for the lack of insulin displacement, quiescent fibroblast monolayers were suspended by trypsinization and incubated with $^{125}$I-Sm-C/IGF-I for 4 h at 8°C in the presence and absence of unlabeled Sm-C/IGF-I or insulin and cross-linked and the proteins were separated by SDS-PAGE. In contrast to the monolayers no binding to the 43,000-mol wt protein was present and the $^{125}$I-Sm-C/IGF-I was bound only to the 135,000-mol wt type I receptor (Fig. 6). The bound $^{125}$I-Sm-C/IGF-I could be displaced with either unlabeled Sm-C/IGF-I, insulin, or alpha-IR3. Similar results were obtained if the cells were mechanically removed from the plate and not exposed to trypsin.

To determine whether this binding protein in the 43,000-mol wt complex could be secreted by the fibroblast monolayers into the culture medium, conditioned medium from the fibroblast monolayers was incubated with $^{125}$I-Sm-C/IGF-I and reacted with DSS. After separation by PAGE, the $^{125}$I-Sm-C/IGF-I was cross-linked to three fibroblast media proteins. The molecular weights of the complexes were 43,000, 36,000, and 25,000 (Fig. 7). The 43,000- and 36,000-mol wt bands were less intensely labeled after addition of unlabeled Sm-C/IGF-I and IGF-II, but not after addition of insulin. In contrast, when conditioned medium was collected for 72 h from suspension cultures and cross-linked to $^{125}$I Sm-C/IGF-I, no 43,000-mol wt binding moiety was detectable (data not shown).

To determine the functional significance of this 43,000-mol wt protein complex, conditioned medium from quiescent fibroblast monolayers was incubated with $^{125}$I-Sm-C/IGF-I and the presence or absence of unlabeled Sm-C/IGF-I was confirmed.

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**Figure 3.** Competition between type I receptor antibody (alpha-IR3) and $^{125}$I-Sm-C/IGF-I for binding to fibroblast monolayers (77,000 cells/well) and suspension cultures. Fibroblast monolayers (o) and suspension cultures (●) were prepared as described previously and were exposed to $^{125}$I-Sm-C/IGF-I and increasing concentrations of antibody, (0.1–100 nM) then incubated as in Fig. 1. Cell-associated radioactivity after the incubation was determined as in Fig. 1. Each point represents the mean of triplicate determinations.

**Figure 4.** Autoradiogram of $^{125}$I-Sm-C/IGF-I cross-linked to fibroblast monolayers and separated on 3–14% gradient SDS-PAGE after reduction with 100 mM DTT. Lane A: without competing unlabeled peptide, showing predominant binding of $^{125}$I-Sm-C/IGF-I to the 135,000-mol wt alpha-subunit of type I Sm-C/IGF-I receptor and the 43,000-mol wt cell-associated, $^{125}$I-Sm-C/IGF-I binding protein complex. Lane B: competition with unlabeled Sm-C/IGF-I 50 ng/ml. Lanes C and D: competition with insulin, 0.5 and 20 μg/ml, respectively, showing a significant reduction in $^{125}$I-Sm-C/IGF-I binding to the 135,000-mol wt alpha-subunit of type I receptor without reduction in binding in the 43,000-mol wt complex. Lane E: competition with 10−7 M alpha-IR3, showing selective competition of $^{125}$I-Sm-C/IGF-I binding to the alpha-subunit of type I receptor and apparent increase in $^{125}$I-Sm-C/IGF-I binding in the 43,000-mol wt complex.
blast cultures obtained after 72 h of incubation was tested for its effects on the binding of $^{125}$I-Sm-C/IGF-I to porcine aortic smooth muscle cells (a cell type that does not secrete a binding moiety capable of associating with the cell surface). We have shown that quiescent porcine aortic smooth muscle cell monolayers possess type I receptors and that the $^{125}$I-Sm-C/IGF-I that binds to this receptor is displaceable by unlabeled Sm-C/IGF-I and insulin. After 24 h of exposure to either fibroblast or smooth muscle cell-conditioned medium, $^{125}$I-Sm-C/IGF-I binding was determined. Control monolayers exposed to smooth muscle cell-conditioned medium showed displacement of $^{125}$I-Sm-C/IGF-I at low concentrations of unlabeled Sm-C/IGF-I (1–5 ng/ml), and concentrations of unlabeled insulin between 1 and 10 μg/ml also caused significant displacement (Fig. 8). In contrast, cultures that were preincubated with fibroblast-conditioned medium showed a 60% increase in basal $^{125}$I-Sm-C/IGF-I binding that further increased when increasing concentrations of unlabeled Sm-C/IGF-I were added. Addition of unlabeled Sm-C/IGF-I in concentrations as high as 25 ng/ml were required to obtain any displacement, and insulin in concentrations as high as 1.0 mg/ml caused no displacement. To determine that this was not a property of one fibroblast line, conditioned media were obtained from eight different fibroblast strains and preincubated with smooth muscle cell monolayers. Media from all eight strains were shown to increase basal $^{125}$I-Sm-C/IGF-I binding and to change the relative sensitivity to competition by unlabeled Sm-C/IGF-I (data not shown). Exposure of smooth muscle cell cultures to conditioned media obtained from fibroblast suspension cultures for 24 h did not result in changes in the binding of radiolabeled Sm-C. To determine whether a factor in fibroblast monolayer-conditioned media could alter Sm-C binding to suspended fibroblasts, 120,000 cells/tube were exposed to a 20% (vol/vol) concentration of fibroblast monolayer-conditioned medium (that had been concentrated fivefold) for 14 h at 37°C. After addition of increasing concentrations of unlabeled Sm-C (1–10 ng/ml) $^{125}$I-Sm-C binding increased ~56% above basal at 4 ng/ml then decreased toward control levels.

To confirm that the effect of fibroblast-conditioned medium on $^{125}$I-Sm-C binding to smooth muscle cells was associated with attachment of a protein represented in the 43,000-mol wt $^{125}$I-Sm-C/IGF-I-protein complex, cross-linking studies were performed using smooth muscle cells that had been exposed to fibroblast-conditioned medium. After exposure to this medium and cross-linking, $^{125}$I-Sm-C/IGF-I was associated with three proteins in the smooth muscle cell monolayers (Fig. 9): a 135,000-mol wt band that was lessened in intensity in the presence of insulin and obliterated by unlabeled Sm-C/IGF-I, a 43,000-mol wt band that could not be displaced with unlabeled insulin, and a 260,000-mol wt band. In contrast, control cultures that had been exposed to smooth muscle cell-conditioned medium showed intense $^{125}$I-Sm-C/IGF-I labeling in the 135,000-mol wt band, as well as less intense labeling at 260,000. Both bands were displaced with increasing unlabeled Sm-C/IGF-I or insulin. Of interest was the observation that cross-link of radiolabeled Sm-C/IGF-I to smooth muscle cell-conditioned media showed that a 35,000-mol wt protein that bound radiolabeled Sm-C was present.
other Sm-C/IGF-I-binding bind insulin. It is possible that these binding proteins are not unique species it appears to associate with cellular membranes of both fibroblasts and smooth muscle cells. The latter characteristic is not common to all fibroblast-secreted Sm-C/IGF-I-binding proteins, because we found no evidence of an association of the fibroblast media 36,000- and 25,000-mol wt 125I-Sm-C/IGF-I-binding protein complexes with either fibroblast or smooth muscle cell lysates.

Discussion

These findings demonstrate that cultured human fibroblast monolayers secrete a 35,000-mol wt protein (43,000 minus 7,600, the molecular weight of 125I-Sm-C/IGF-I) into culture medium. This protein is capable of binding Sm-C/IGF-I and alters Sm-C/IGF-I binding to fibroblast monolayers. Furthermore, our affinity-labeling and competition studies suggest that it is the major binding species detectable after cross-linking to confluent monolayers. The protein has the characteristics of a Sm-C/IGF-I-binding protein because it is secreted by cultured cells into the media, binds Sm-C/IGF-I avidly, and does not bind insulin. The property that appears to distinguish it from other Sm-C/IGF-I-binding proteins secreted by fibroblasts is that

Figure 8. Binding of 125I-Sm-C/IGF-I to smooth muscle cell monolayers after exposure to fibroblast conditioned medium. Smooth muscle cell monolayers (92,000 cells/well) were exposed to fibroblast (○) or smooth muscle cell (●)-conditioned medium for 24 h at 37°C. After incubation the cultures were washed extensively, and 125I-Sm-C/IGF-I (80,000 cpm/well) was added. Increasing concentrations of unlabeled Sm-C/IGF-I (left) and insulin (right) were added to triplicate cultures. Cell-associated radioactivity was determined as in Fig. 1.

Figure 9. Autoradiogram of 125I-Sm-C/IGF-I cross-linked to porcine smooth muscle cell monolayers and separated on 3–14% gradient SDS-PAGE after reduction with 100 mM DTT. Lanes A–C: incubation was performed in conditioned medium from pig smooth muscle cells. Lanes D–F: incubation was performed in conditioned medium from fibroblasts. Lanes A and D: without competing unlabeled peptides. Lanes B and E: competition with 50 ng/ml Sm-C/IGF-I. Lanes C and F: competition with unlabeled insulin, 1 μg/ml.

Preliminary physicochemical properties of the factor in the 43,000-mol wt 125I-Sm-C/IGF-I protein complex are: (a) it is retained by dialysis using 3,500-mol wt exclusion membrane, destroyed by proteases, (b) it is heat stable at 56°C for 2 h but partially inactivated by heating to 80°C for 1 h, and (c) it is stable at pH 2.5 and 10.6 (Table II).

Somatomedin-C Binding Alteration by 35,000-mol wt Protein
but represent proteolytic cleavage products of the binding moiety in the 43,000-mol wt complex that have retained the capacity to bind Sm-C/IGF-I but not to the cell surface. Whether or not this is the case the binding protein in the 43,000-mol wt moiety appears to be unique because it can associate with the cell surface. Furthermore, after exposure to this protein competition of unlabeled Sm-C/IGF-I or insulin for 125I-Sm-C/IGF-I binding to smooth muscle cells is greatly altered, suggesting that the 35,000-mol wt protein may directly alter binding to the type I receptor.

The conclusion that the 35,000-mol wt protein accounts for the differences observed in radiolabeled Sm-C/IGF-I binding between fibroblast monolayers and suspensions is supported by several observations. First, it is associated with the cell lysates of fibroblast monolayers but not the same cells in suspension, suggesting that it is responsible for the differences in displacement of 125I-Sm-C/IGF-I between these conditions. Second, because the 35,000-mol wt protein does not bind insulin its presence on monolayers may account for the inability of insulin to displace labeled Sm-C/IGF-I from monolayers (but not from suspension cultures). Similarly, because antibody to the type I receptor (alpha-IR3) does not recognize the 35,000-mol wt protein, this explains the lack of capacity of alpha-IR3 to displace labeled Sm-C/IGF-I from the monolayers. Third, the suspended fibroblasts do not appear to secrete this protein. Taken together with our finding that media containing the 35,000-mol wt protein can alter the binding characteristics of smooth muscle cultures in a similar fashion, our data support the concept that the 35,000-mol wt protein directly alters Sm-C/IGF-I cellular binding.

Our data do not provide a definitive explanation of the paradoxical increase in 125I-Sm-C/IGF-I binding that occurs when low concentrations of unlabeled Sm-C/IGF-I are added to these cultures. Gel electrophoresis data confirm that significant displacement of labeled Sm-C/IGF-I from either the 35,000-mol wt protein or the 135,000-mol wt type I receptor does not occur at concentrations of unlabeled Sm-C/IGF-I below 20 ng/ml and suggest that binding to these proteins is increasing at concentrations between 1 and 10 ng/ml. Two explanations for these results seem possible. First, when unlabeled Sm-C/IGF-I is added there could be a direct increase in the affinity (positive cooperativity) of either the 35,000-mol wt protein or the type I receptor or both that could result in increasing the binding of 125I-Sm-C/IGF-I. Second, it is possible that when low concentrations of unlabeled peptide are added it displaces radiolabeled Sm-C/IGF-I from binding proteins in the media and makes 125I-Sm-C/IGF-I more available to bind the cell surface receptor. This hypothesis is unlikely because radiolabeled peptide is present in excess under the experimental conditions. Furthermore, the smooth muscle cell media contains a protein that forms a 43,000-mol wt complex with 125I-Sm-C after cross-linking yet no paradoxical increase in 125I-Sm-C binding to the type I receptor appears to occur in these cells.

Affinity cross-linking studies indicate that the 35,000-mol wt protein is an important binding moiety associated with human fibroblast membranes obtained from monolayer cultures. Because the 35,000-mol wt protein appears to be a specific binding component of monolayer cultures and is not present in suspension cultures, it is possible that this protein is associated with the basement membrane of the monolayers. However, direct binding studies to basement membranes after cell removal showed very low levels of binding and after cross-linking to this material no 43,000-mol wt complex was detected. Furthermore, the 43,000-mol wt complex can be detected on the suspended cell surfaces if these cells are exposed to concentrated monolayer-conditioned media prior to the addition of cross-linking reagents. These data support the conclusion that the 43,000-mol wt complex is cell surface-associated.

The presence of this protein adds a third complex variable to understanding the binding of Sm-C/IGF-I to this cell type. Knaur and Cunningham (21) have reported that human fibroblasts secrete a protein that binds covalently to epidermal growth factor (EGF) binding protein (termed a protease nexin) and functions to enhance EGF binding to cultured fibroblast monolayers. Although the 35,000-mol wt protein reported here does not bind Sm-C/IGF-I covalently, it may function in a similar manner. This or a very similar protein may have previously been observed during preparation of membrane fractions from tissues. Grizzard et al. (22) noted a 45,000-mol wt 125I-Sm-C/IGF-I binding protein complex in preparations of placental membrane preparations from preterm placentas. Likewise, Armstrong et al. (23) used placental membranes and noted a band between 40,000 and 45,000 that cross-linked with labeled basic somatomedin. Although in the former study amniotic fluid was thought to be the source of the protein, it is notable that it was not completely removed by washing. Because fibroblasts are present in these tissues it is possible that these authors were cross-linking to a protein related to the one described herein. Finally, multiple studies report somatomedin-binding proteins of similar size in amniotic fluid, serum, and culture media (11, 18, 23).

Other investigators have prepared human fibroblast membranes from suspension cultures for cross-linking studies and have not reported this protein (7). Because they detached the

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Table II. Effect of Heat, pH, and Proteolysis on the Activity of Fibroblast-conditioned Media

<table>
<thead>
<tr>
<th>Condition</th>
<th>125I-Sm-C/IGF-I binding % of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>205</td>
</tr>
<tr>
<td>pH 2.5</td>
<td>191</td>
</tr>
<tr>
<td>pH 10.6</td>
<td>213</td>
</tr>
<tr>
<td>56°C 2 h</td>
<td>183</td>
</tr>
<tr>
<td>80°C 1 h</td>
<td>99</td>
</tr>
<tr>
<td>−20°C 1 wk</td>
<td>186</td>
</tr>
<tr>
<td>−70°C 1 wk</td>
<td>177</td>
</tr>
<tr>
<td>Protease treatment 2 h 37°C</td>
<td>94</td>
</tr>
</tbody>
</table>

Conditioned media was collected from fibroblast monolayers after a 72-h incubation. It was concentrated 33-fold by lyophilization then exposed to the perturbation listed. Acidification and base exposure were conducted for 30 min at room temperature. The protease preparation was a mixture of proteolytic enzymes obtained from Streptomyces griseus linked to agarose purchased from Sigma Chemical Co. It was used at a final concentration of 0.04 U/ml.

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2. It is also possible that some 125I-IGF-I/Sm-C is bound to type II receptors and, therefore, cannot be displaced with insulin. A 260,000-mol wt band was detected after cross-linking but insulin and alpha-IR3 could compete with 125I-IGF-I/Sm-C for binding to this protein, suggesting that it is a dimer of the alpha-subunit of the type I receptor and not the type II receptor. Nevertheless, it is possible that the type II receptor was not efficiently extracted with SDS and is present in greater concentration than our extraction method would indicate.
cells from the monolayer it is possible that the 35,000 protein was removed, or alternately, as shown by Grizzard et al. (22), it might not have been detected because 5% polyacrylamide gels were used. Our data support the hypothesis that removal of the cells from the plate results in a lower cellular affinity for the 35,000-mol wt protein because exposure of suspended cells to concentrated conditioned media prior to binding studies results in reestablishment of the paradoxical increase in 125I-Sm-C/IGF-I binding. Inasmuch as the suspended cells do not secrete the 35,000-mol wt binding protein its continued synthesis and secretion during the binding experiment may be required for it to be detected. In contrast, when affinity cross-linking studies have been performed by other investigators directly on fibroblast monolayers not only is the type I receptor present but a lower molecular weight IGF-I complex (43,000) is also present (5).

Taken together, these findings suggest that this binding moiety may be important in modifying the binding, and possibly the biologic action of Sm-C/IGF-I in many types of proliferating cells.

These findings are difficult to reconcile with published reports of Rosenfeld and Dollar (9). They have reported that unlabeled Sm-C/IGF-I displaces significant quantities of labeled peptide from newborn foreskin fibroblast monolayers when added in concentrations of 1–10 ng/ml. In their hands addition of insulin at 8.0 µg/ml results in a 60% displacement of bound 125I-Sm-C/IGF-I from monolayer cultures, and their results are consistent with the existence of only one class of receptors. Differences between our studies and theirs cannot be attributable to tissue source, donor age, feeding schedule, or preincubation periods, as each were similar or identical. Because these authors did not detail the plating densities utilized nor the purity of the peptides employed, it remains possible that those differences account for the discrepancies noted for Sm-C/IGF-I displacement. The discrepancies in the findings of insulin displacement remain unresolved.

These findings indicate that fibroblasts secrete a protein that functions not only to alter Sm-C/IGF-I binding to human fibroblast receptors but also has the potential for altering cellular response to this peptide. Our laboratory has previously described the secretion of a somatomedinlike factor by fibroblasts that augments the cellular response to exogenously added Sm-C/IGF-I (24). Therefore, it is possible that the 35,000-mol wt protein represents a closely related factor. Fibroblast DNA synthesis can be stimulated by very low concentrations of Sm-C/IGF-I (e.g., 1–10 ng/ml). This finding indicates that the 35,000-mol wt protein may not be blocking the response to Sm-C/IGF-I in that the relatively high concentrations required for competition in these studies (e.g., 25–300 ng/ml) are not required for replication. It is possible that this factor actually enhances the cellular responsiveness to exogenously added Sm-C/IGF-I. Secretion of this factor could represent an important signal molecule in cell-cell interaction between fibroblasts or between fibroblasts and smooth muscle cells. In this regard there is evidence that after injury to vessel walls fibroblasts may suppress smooth muscle cell division and proliferate in areas where smooth muscle cells previously have dominated (25). Further studies will be required to analyze the effect of this peptide on smooth muscle cell division.

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References


