Interaction of ovine somatomedin-C/IGF-I and IGF-I with specific IGF-I receptors on cultured muscle-derived fibroblasts

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Abstract. Binding of $^{125}$I-insulin-like growth factor-I and $^{125}$I-ovine somatomedin-C/IGF-I to monolayer cultures of muscle-derived ovine fibroblasts is described. Preliminary competitive binding experiments indicate that ovine fibroblasts possess independent cell surface receptors for IGF-I. Affinity of rIGF-II for IGF-I binding sites is minimal; rIGF-II binds to Type I IGF receptors at 1/1000 the strength of IGF-I. Insulin binds to the Type I IGF receptor at 1/100 the strength of IGF-I, whereas ovine somatomedin-C/IGF-I displays equivalent IGF-I binding as evidenced by overlapping competition of ovine somatomedin-C/IGF-I for $^{125}$I-IGF-I binding sites. Results from disuccinimidyl suberate cross-linking of $^{125}$I-IGF-I to muscle-derived ovine fibroblasts in the presence of related polypeptides verified the competitive binding data. Under reducing conditions, $^{125}$I-IGF-I: receptor complexes migrated to a relative molecular weight of approximately 135 000 daltons. Specific $^{125}$I-IGF-I binding was completely inhibited by $10^{-8}$ mol/l IGF-I, $7.2 	imes 10^{-8}$ mol/l ovine somatomedin-C/IGF-I, and $10^{-6}$ mol/l insulin and partially inhibited by $7.2 	imes 10^{-9}$ mol/l ovine somatomedin-C/IGF-I and $6.5 	imes 10^{-9}$ mol/l rIGF-II. $^{125}$I-ovine somatomedin-C/IGF-I: receptor complexes also migrated at a relative molecular weight of 135 000 daltons. No migratory band was observed at 250 000 to 260 000 daltons with either $^{125}$I-IGF-I or $^{125}$I-ovine somatomedin-C/IGF-I indicating that little labelled moiety bound to the Type II IGF receptor. Based on these preliminary competitive binding studies and cross-linking data, we conclude that ovine somatomedin-C/IGF-I is primarily interacting with the Type I IGF membrane receptor on ovine skeletal muscle fibroblasts.

Insulin-like growth factors somatomedins comprise a family of polypeptides (Perdue 1984; Froesch & Zapf 1985) with similar structure (Froesch & Zapf 1985) and biological activities (Myal et al. 1984; Dodson et al. 1985; Mattson et al. 1986; Heaton et al. 1986). However, IGF-I preferentially binds to the Type I receptor (Perdue 1984; Nissley et al. 1985), whereas IGF-II has greatest affinity for the Type II IGF receptor (Perdue 1984; Froesch & Zapf 1985; Nissley et al. 1985). The Type I receptor has a different biochemical structure than the Type II IGF receptor. These receptors can be distinguished from one another after dithiothreitol reduction and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) by their relative molecular weights. Under these conditions, the Type I IGF binding subunit (a subunit) migrates as a 130–135 k dalton monomer, whereas the Type II receptor migrates at 260 k daltons (Nissley et al. 1985). Because of these differences, competitive binding (Rechler et al. 1983) and bifunctional affinity labelling (Oppenheimer & Czech 1984) methods have been used to distinguish the two types of receptors.

Knowledge of IGF receptor binding properties has been used initially to characterize an IGF preparation isolated from ovine serum (Hossner et al. 1985). Indeed, biological characterization of ovine somatomedin-C/IGF-I revealed that this
moiety promoted cell proliferation in a manner consistent with IGF-I (Dodson et al. 1985). However, direct evidence that ovine somatomedin-C/IGF-I binds preferentially to the Type I IGF receptor remains unavailable. A preliminary report has suggested that such binding occurs (Dodson et al., unpublished data). However, these data were obtained through the use of a mixed culture system and no data are available to demonstrate that ovine somatomedin-C/IGF-I can compete for specific IGF receptor sites or that ovine somatomedin-C/IGF-I can be covalently cross-linked to the Type I IGF receptor. Consequently, the present study was designed to test the hypothesis that ovine somatomedin-C/IGF-I binds exclusively to the Type I IGF receptor population of homogenous cultures of ovine, muscle-derived fibroblasts.

Materials and Methods

**Ovine, muscle-derived fibroblasts**

Animals used for this study were male, 2- to 3-month-old, western white-face-crossed sheep obtained from the University of Idaho, Cooperative Sheep Research Center, Moscow, ID. Fibroblasts were isolated from ovine semimembranosus muscles during a routine satellite cell harvesting protocol and preplating regimen (Dodson et al. 1987). Isolated fibroblasts were seeded into uncoated plastic, 35-mm diameter cell culture plates in Dulbecco's Modified Eagles Medium (GIBCO, Grand Island, NY) supplemented with 15% horse serum (GIBCO) plus antibiotics (Dodson et al. 1987), and allowed to proliferate to 90% confluency. First through eighth-passage fibroblasts were used for these studies. All cultures were maintained in a humidified environment of 95% air and 5% CO₂ at 37°C.

**Iodination, competitive binding and bifunctional affinity labelling protocols**

Native human IGF-I (kindly provided by Dr Rene Humbel, Biochemisches Institut der Universität, Zürich, Switzerland) and recombinant IGF-I (Thr-59 IGF-I; AMGEN, Thousand Oaks, CA) were iodinated using iodobeads (Miskimins & Shimizu 1984) to a specific activity of 70 to 100 µCi/µg. Highly purified ovine somatomedin-C/IGF-I (Hossner et al. 1985) was iodinated to a specific activity of 90 to 100 µCi/µg using the lacto-peroxidase technique (Thorrell & Johnson 1971). Binding of radiolabelled IGF-I, or ovine somatomedin-C/IGF-I, in the presence of competitive antagonists was conducted on whole fibroblasts as similarly described (Shimizu & Shimizu 1980). In brief, these assay procedures were as follows: 35-mm diameter tissue culture plates containing cells to be analysed were placed on ice and washed three times with ice-cold binding buffer consisting of 0.1 mol/l Hepes, 0.12 mol/l NaCl, 0.0012 mol/l MgSO₄, 0.005 mol/l KCl, 0.01 mol/l D-glucose, 0.001 mol/l EDTA, 0.015 mol/l Na-acetate, 0.002 mol/l CaCl₂, and 1% BSA adjusted to pH 7.9. Treatment polypeptides contained in either phosphate buffered saline at pH 7.2, or in 0.01 mol/l HCl, approximately 2.0 × 10⁶ cpm of ¹²⁵I-radiolabelled polypeptide and binding buffer were then added to a final volume of 1.0 ml. Excess non-radiolabelled polypeptide (assay specific) was added to nonspecific binding (NSB) plates. Treatment and NSB plates were then floated on 15°C water for a 90-min incubation period. Following incubation, all plates were washed three times with 2.0 ml of ice-cold binding buffer then solubilized with 1.0 ml (each) of 0.5 mol/l NaOH. The dissolved cells of each plate were then counted in a gamma counter. Data were adjusted for NSB and presented as the percent of control counts as described. Cross-linking of radiolabelled IGF-I or ovine somatomedin-C/IGF-I to IGF receptors on the fibroblasts was conducted using the disuccinimidyl suberate reagent exactly as described by Pilch & Czech (1979). Competitive antagonists used were bovine insulin (Collaborative Research, Waltham, MA), rat IGF-II (Sigma Chemical Co, St. Louis, MO), highly purified ovine somatomedin-C/IGF-I (Hossner et al. 1985), highly purified human IGF-I (Humbel, Zürich, Switzerland), and recombinantly-produced Thr-59 IGF-I (AMGEN). Densitometric analyses of the autoradiogram was conducted using a Beckman R115 densitometer.

Results

Binding of radiolabelled ¹²⁵I-IGF-I to muscle-derived ovine fibroblasts in the presence of related polypeptides is presented in Fig. 1. Highly purified IGF-I and a recombinantly-produced Thr-59 IGF-I competed for ¹²⁵I-IGF-I binding sites in a similar manner. Half-maximal inhibition of ¹²⁵I-IGF-I binding by these IGF-I moieties occurred at about 2 × 10⁻⁹ mol/l. Insulin also competed for ¹²⁵I-IGF-I binding sites. However, insulin was about 100-fold weaker in inhibiting ¹²⁵I-IGF-I binding than IGF-I. Half-maximal inhibition of ¹²⁵I-IGF-I binding occurred with insulin levels near 10⁻⁷ mol/l. At maximum insulin concentrations of 10⁻⁵ mol/l, insulin was capable of competing for 70% of ¹²⁵I-IGF-I binding. Interestingly, rIGF-II was the least effective in binding ¹²⁵I-IGF-I.
Inhibition of $^{125}$I-IGF-I or Thr-59 $^{125}$I-IGF-I binding by related polypeptides. Nonspecific binding was determined by the addition of $1.2 \times 10^{-7}$ to $2.5 \times 10^{-7}$ mol/l IGF-I per well. Specific binding was determined by subtracting nonspecific binding from total binding. Specific binding ranged from 1.4 to 2.7% of total input radioactivity. $^{125}$I-IGF-I and Thr-59 $^{125}$I-IGF-I mass added ranged from $8 \times 10^{-11}$ to $3 \times 10^{-10}$ mol/l per well. Each point represents the mean of at least four observations. Thr-59 $^{125}$I-IGF-I/Thr-59 IGF-I (○—○), $^{125}$I-IGF-I/IGF-I (●—●), $^{125}$I-IGF-I/Collaborative Research Inc insulin (▲—▲), $^{125}$I-IGF-I/Sigma Chemical Co rIGF-II (■—■).

Inhibition of $^{125}$I-ovine somatomedin and $^{125}$I-IGF-I binding by the alternative unlabelled moiety. Specific binding and $^{125}$I-mass added were similar to that described in Fig. 1. Each point represents the mean of five observations. $^{125}$I-IGF-I/ovine somatomedin-C/IGF-I (▲—▲), $^{125}$I-ovine somatomedin-C/IGF-I/human IGF-I (●—●).
inhibiting $^{125}$I-IGF-I binding to ovine fibroblasts, in vitro. Addition of as much as $2 \times 10^{-7}$ mol/l rIGF-II resulted in only a 30% inhibition of $^{125}$I-IGF-I binding. Affinity of IGF-I binding sites for rIGF-II was approximately 1000-fold less than that for IGF-I.

The relationship of ovine somatomedin-C/IGF-I to IGF-I is addressed in Fig. 2. Highly purified ovine somatomedin-C/IGF-I competed for $^{125}$I-IGF-I binding sites in a manner consistent with ovine somatomedin-C/IGF-I being more closely analogous to IGF-I than either insulin or rIGF-II. Greater than 80% inhibition of $^{125}$I-IGF-I binding occurred with ovine somatomedin-C/IGF-I at levels near $10^{-7}$. The reciprocal competitive binding curve is also presented in Fig. 2. Binding of $^{125}$I-ovine somatomedin-C/IGF-I to ovine fibroblasts was efficiently inhibited by increasing levels of IGF-I. The close proximity of the $^{125}$I-IGF-I/ovine somatomedin-C/IGF-I curve to the $^{125}$I-ovine somatomedin-C/IGF-I/human IGF-I curve suggests that ovine somatomedin-C/IGF-I and IGF-I are closely related polypeptides.

Results of covalently cross-linking $^{125}$I-IGF-I to ovine fibroblasts in the presence of putative antagonists is shown in Fig. 3. Under reducing conditions, $^{125}$I-IGF-I: receptor complexes migrated to a relative molecular weight of approximately 135 k daltons. $^{125}$I-IGF-I binding to the 135 k dalton receptor subunit was completely inhibited by $9 \times 10^{-8}$ mol/l IGF-I, $2 \times 10^{-7}$ mol/l Thr-59 IGF-I, and $7.2 \times 10^{-8}$ mol/l ovine somatomedin-C/IGF-I. On the basis of densiometric readings, $7.2 \times 10^{-9}$ mol/l ovine somatomedin-C/IGF-I and $10^{-6}$ mol/l insulin inhibited $^{125}$I-IGF-I binding by 57% and 77%, respectively. Inhibition by ovine somatomedin-C/IGF-I was dose-dependent. At $6 \times 10^{-8}$ mol/l, rIGF-II showed some cross-reactivity with Type I IGF receptors by inhibiting 49.3% of $^{125}$I-IGF-I binding. $^{125}$I-ovine somatomedin-C/IGF-I: receptor complexes, alone (lane H), also migrated at a relative molecular weight of approximately 135 000 daltons. No band was observed with either $^{125}$I-IGF-I or $^{125}$I-ovine somatomedin-C/IGF-I alone at approximately 260 000 daltons which would have been consistent with either of these moieties binding to the reduced Type II, IGF receptor. A minor radiolabelled band was, however, detected at 235 k daltons (Lanes A, D, F, G, H).

**Discussion**

The presence of somatomedin-like molecules in domestic animals has been well established. Bovine serum possesses two somatomedin-like moieties: one that is structurally identical to human IGF-I and one that is similar to IGF-II in function (Honegger & Humbel 1986). Radioimmunoassay
and radioreceptor data have suggested that ovine serum contains both an IGF-I like polypeptide (Wilson & Hintz 1982) and an IGF-II like moiety (Zangger et al. 1987). Ovine somatomedin-C/IGF-I, however, represents the only reported ovine somatomedin-like moiety that has been isolated and initially characterized in vitro. This polypeptide has a molecular size of 6900 daltons (Hossner et al. 1985) and promotes proliferation of myogenic satellite cells in a manner more similar to IGF-I than IGF-II (Dodson et al. 1985).

Preliminary data indicate that ovine somatomedin-C/IGF-I binds to (Dodson et al., unpublished data) and acts through (Dodson et al. 1985) IGF-I receptors on satellite cells. Concrete data to support this conclusion is obscured, however, as the previous data were obtained through the use of heterogenous cell cultures (e.g., nonmyogenic cells also existed in these primary cell cultures). In addition, the reported studies did not include an IGF-I component either as a competitive antagonist or as a labelled species for competition. In the present study we attempted to circumvent these problems by using a homogeneous cell system and by including IGF-I for all binding studies performed.

The preliminary competitive binding data (Fig. 1 and 2) demonstrate that independent receptors for IGF-I exist on muscle-derived ovine fibroblasts. Rat IGF-II displays only limited affinity for the Type I IGF receptor, however. At rIGF-II levels of 10^{-7} mol/l only 20% to 30% inhibition of ^{125}I-IGF-I binding occurred. Rechler & Nissley (1986) noted that IGF receptors characteristically bind 10% to 100% of the alternative IGF moiety. In ovine-derived fibroblasts, the minimum range of rIGF-II cross-reactivity appears to be the norm. In this respect these data are in agreement to those previously reported for rIGF-II and basic somatomedin inhibition of ^{125}I-basic somatomedin binding to T47D human breast cancer cells in vitro (Myal et al. 1984).

Knowledge of distinct IGF binding characteristics inherent to ovine fibroblasts should help to determine if ovine somatomedin-C/IGF-I acts similarly to IGF-I in its binding ability to IGF receptors. Indeed, if ovine somatomedin-C/IGF-I were like rIGF-II, then little inhibition of ^{125}I-IGF-I binding should be observed with increasing levels of unlabelled ovine somatomedin-C/IGF-I. Alternatively, if ovine somatomedin-C/IGF-I were, indeed, the ovine analog to IGF-I, then ovine somatomedin-C/IGF-I competition for ^{125}I-IGF-I binding should be quite efficient. The position of the ^{125}I-IGF-I/ovine somatomedin-C/IGF-I competition curve relative to the ^{125}I-ovine somatomedin-C/IGF-I/human IGF-I curve suggests that ovine somatomedin-C/IGF-I is very closely related, if not identical in binding ability, to IGF-I.

We had previously shown that ovine somatomedin-C/IGF-I cannot promote cell proliferation when the Type I IGF receptors were saturated with insulin (Dodson et al. 1985). Furthermore, we recently suggested that because insulin could compete for ^{125}I-ovine somatomedin-C/IGF-I binding to myogenic satellite cells that ovine somatomedin-C/IGF-I must be IGF-I like (Dodson et al., unpublished data). The results of the initial competitive binding studies, described herein, provide supporting evidence for this hypothesis.

Additional evidence that ovine somatomedin-C/IGF is IGF-I like is shown by the cross-linking data of Fig. 3. Type I IGF receptors display greater affinity for IGF-I than for insulin or IGF-II (Rechler et al. 1983; Nissley et al. 1985). If ^{125}I-IGF-I is covalently cross-linked (Pilch & Czech 1979) to the Type I IGF receptor and the complex is reduced by dithiothreitol and separated by SDS PAGE, a labelled band composed of ^{125}I-IGF-I: α-binding subunits should be observed at a relative molecular weight of 130 000 to 135 000 daltons (Nissley et al. 1985). The cross-linking results presented in Fig. 3 are consistent with ^{125}I-IGF-I binding to the Type I IGF receptor, and as ovine somatomedin-C/IGF-I effectively competes for ^{125}I-IGF-I binding, we propose that ovine somatomedin-C/IGF-I acts as the ovine analog to IGF-I.

This preliminary hypothesis is further supported by the cross-linking data in which high levels of highly purified rIGF-II failed to inhibit completely ^{125}I-IGF-I binding, whereas lesser amounts of ovine somatomedin-C/IGF-I were quite effective in this regard. Furthermore, ^{125}I-ovine somatomedin-C/IGF-I cross-linked to muscle-derived ovine fibroblasts and separated by SDS PAGE under reducing conditions resulted in a 130 000 to 135 000 dalton band. No band at 260 000 daltons was observed suggesting that ovine somatomedin-C/IGF-I is interacting primarily with the Type I IGF receptor.

The additional radiolabelled band detected at
235 k daltons may represent unreduced Type II IGF receptor complexes (Rechler et al. 1983), incomplete reduction of the Type I IGF receptor complexes (Chernausek et al. 1981; Massague & Czech 1982) or nonspecific cross-linking of alternative receptor subunits by disuccinimidyl suberate (Massague & Czech 1982). Strong competitive antagonists of 125I-IGF-I binding (IGF-I, Thr-59 IGF-I, ovine somatomedin-C/IGF-I, and insulin) eliminated 98%, 99%, 99% and 89% of this band, respectively, whereas rIGF-II at 6 × 10⁻⁸ mol/l inhibited only 53% of the 125I-IGF-I bound. If this band represented labelled Type II receptors then rIGF-II at this high of a level would have completely eliminated its detection and insulin would not have shown any cross-reactivity at all. Inhibition by ovine somatomedin-C/IGF-I was dose-dependent. Consequently, we conclude that the ovine somatomedin-C/IGF-I preparation isolated by the Hossner et al. (1985) method binds to primary cultures of ovine, muscle-derived fibroblasts in a manner similar to IGF-I and not insulin or rIGF-II. These qualitative observations, along with previously reported biological data (Dodson et al. 1985) strongly suggest that ovine somatomedin-C/IGF-I is, indeed, the ovine analog to IGF-I.

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