Receptors for epidermal growth factor and insulin-like growth factor-I on preimplantation trophoderm of the pig

A. N. CORPS, D. R. BRIGSTOCK, C. J. LITTLEWOOD and K. D. BROWN
Department of Biochemistry, AFRC Institute of Animal Physiology and Genetics Research, Babraham, Cambridge CB2 4AT, UK

Summary

$125^I$-labelled epidermal growth factor ($125^I$-EGF) and $125^I$-labelled insulin-like growth factor-I ($125^I$-IGF-I) bound to trophoderm cells from pig blastocysts obtained on days 15–19 of pregnancy. Specific binding was detected on freshly isolated cell suspensions and on cells cultured for several days. The binding of $125^I$-EGF was inhibited by increasing concentrations of EGF, but not by various other growth factors and hormones. Chemical cross-linking of $125^I$-EGF to its receptors using disuccinimidyl suberate (DSS) revealed a radiolabelled band of relative molecular mass 160 000, similar to that identified as the EGF receptor in other cell types. The binding of $125^I$-IGF-I was inhibited by both IGF-I and insulin, indicating that the receptors were either type I IGF receptors or insulin receptors. Cross-linking of $125^I$-IGF-I to serum-free supernatants from trophoderm cultures showed that the cells secreted an IGF-binding protein, giving a complex of relative molecular mass about 45 000. The presence of receptors for EGF and IGF/insulin suggests that these factors could be involved in regulating the growth and development of the early blastocyst.

Key words: epidermal growth factor, insulin-like growth factor, growth factor receptors, trophoderm (pig).

Introduction

The pig conceptus undergoes rapid growth and development during the period before and after implantation (Anderson, 1978). Although the factors that control cell proliferation and remodelling in the developing blastocyst are not known, uterine secretions may play an important role in these processes (see Roberts and Bazer, 1988; Brigstock et al. 1989). Uterine luminal fluid has growth-promoting activity for cells in culture, and has been shown to contain insulin-like growth factor-I (IGF-I), heparin-binding growth factors (HBGFs), and a novel factor that has been termed uterine luminal fluid mitogen (Simmen et al. 1988, 1989; Brigstock et al. 1989). In addition, uterine tissues from several species have been shown to contain various polypeptide mitogens, including IGF-I, epidermal growth factor (EGF), colony-stimulating factor-I and HBGFs (reviewed in Brigstock et al. 1989).

The two most likely target tissues for the growth factors present in uterine secretions are the uterus itself, forming an auto- or paracrine system, and the blastocyst, notably the cells of the trophoderm, which are in direct contact with the uterine fluid. In this paper, we demonstrate that receptors for EGF and IGF-I are present on the trophoderm of porcine blastocysts at 15–19 days post coitum. We also show that cells cultured from trophoderm secrete an IGF-specific binding protein.

Materials and methods

EGF was prepared from mouse submaxillary gland (Savage and Cohen, 1972). Radioisotopes, and recombinant [thr$^{5}$]-IGF-I, platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) were obtained from Amersham International. EGF and IGF-I were iodinated by the soluble lactoperoxidase method (Thorell and Johansson, 1971), and the labelled products were purified by gel filtration as described previously (Brown and Blakeley, 1983; Corps and Brown, 1988). Bovine serum albumin (BSA), insulin and angiotensin were obtained from Sigma. Vasopressin and substance P were from Cambridge Research Biochemicals, bombesin was from Bachem and transforming growth factor $\beta$ (TGF$\beta$) was from R & D Systems.

The reproductive tracts of gilts (15–19 days pregnant) were removed about 20 min after stunning and exsanguination at a local slaughterhouse, and transported on ice to the laboratory within 10 min. Each uterine horn was flushed with 20 ml of Dulbecco’s Modified Eagle’s medium (DMEM) to obtain the blastocysts, and the trophoderm was dissected free from the embryos, yolk sacs and allantoic membranes. The trophoderm was minced using scissors, and a cell suspension in medium was prepared by passing the minced tissue once through a syringe needle (1.1 mm diam.), followed by repeated gentle pipetting. The cell suspension was washed 2 or 3 times by centrifugation (100 g for 10 min at 4°C) and resuspension in DMEM. Cell cultures were set up in 3.5 cm plastic culture dishes (Nunc) in 2 ml of DMEM with or without 5% (v/v) newborn calf serum, and grown for between 1 and 14 days before use, with a change to fresh medium every 3–4
days. Trophoderm consists of two layers of cells, an outer layer of cuboidal trophoderm cells and an inner layer of somatic mesoderm-derived cells. Cell suspensions consisted largely of small aggregates of cells. When cultured, these aggregates settled onto the dish and then spread as a heterogeneous monolayer.

Freshly isolated trophoderm cell suspensions (in plastic centrifuge tubes) or trophoderm cell cultures were incubated at 37°C or 4°C in 1 ml of binding medium (DMEM containing BSA (1 mg ml⁻¹), 0.1 µM KI and 50 mM BES). Ligand concentrations and incubation times are specified in the figure legends. At the end of the incubation, the tubes or dishes were placed on ice and the cells were washed three times at 4°C with ice-cold phosphate-buffered saline containing BSA (1 mg ml⁻¹) and 0.1 µM KI. The samples were solubilized by adding 0.5 M NaOH, and the radioactivity was counted using a Packard gamma counter. Protein in the solubilized samples was assayed by the method of Bradford (1976) using porcine gamma globulin and BSA as standards, in the presence of equivalent additions (<0.5%, v/v) of 0.5 M NaOH.

The chemical cross-linking of 125I-EGF to receptors in trophoderm cell suspensions was performed using 0.5 mM disuccinimidyl suberate (DSS) as described previously (Blay and Brown, 1985; Corps and Brown, 1988). The products were analysed by SDS–polyacrylamide gel electrophoresis under reducing conditions using 4% (w/v) acrylamide stacking gels and 7.5% (w/v) separating gels (Laemmli, 1970). The gels were fixed in 50% (v/v) methanol in 10% (v/v) acetic acid, dried onto filter paper and exposed to Fuji RX film for between 7 and 28 days at -70°C with intensifying screens.

The cross-linking of 125I-IGF-I to binding proteins released by the cultured trophoderm into the supernatant medium was also performed using DSS, as described previously for binding proteins released from Swiss 3T3 cells (Corps and Brown, 1988). Supernatants from cultures in serum-free DMEM were obtained at various times up to 14 days of culture, centrifuged (1 min at 10.000 g) to remove debris, and stored frozen until required. Supernatants were incubated for 30 min at 37°C with 125I-IGF-I (2.5 ng ml⁻¹), cooled to room temperature, and then incubated for 15 min with 25 µM DSS before adding concentrated (5x) electrophoresis sample buffer and heating to 100°C for 4 min. Portions were subjected to SDS–polyacrylamide gel electrophoresis using 4% (w/v) stacking gels and 12.5% (w/v) separating gels, which were then fixed for autoradiography as described above. For 'ligand blotting', supernatants were subjected to gel electrophoresis under non-reducing conditions, and the proteins were transferred to nitrocellulose using a LKB Novablot apparatus. The nitrocellulose was preblocked, incubated with 125I-IGF-I at 4°C overnight, and washed four times (Hossenlopp et al. 1986), and then dried for autoradiography.

Results and Discussion

Binding of 125I-EGF to trophoderm

The specific binding of 125I-EGF to freshly isolated trophoderm cell suspensions was time- and concentration-dependent. As has been found in other cell types, binding at 37°C was faster than at 4°C and reached a maximum value (Fig. 1A). Somewhat unusually, however, after maximal binding had been attained at 37°C there was no decrease in binding to indicate a loss of receptors due to the internalization and processing of EGF-receptor complexes (Fig. 1A). This finding was confirmed in assays using trophoderm cell cultures (Fig. 1B). Consistent with a low level of processing of the EGF-receptor complexes, only a small proportion of cell-associated 125I-EGF was released into the medium as acid-soluble 125I-labelled degradation products (Fig. 1B). In similar experiments using a rat intestinal epithelial cell line, for example, there was an average hourly degradation of 125I-EGF equivalent to about 50% of the maximal 125I-EGF binding (Blay and Brown, 1985).

The binding of 125I-EGF was inhibited by unlabelled EGF in a dose-dependent manner (Fig. 2). The concentration of unlabelled EGF required for 50% inhibition of specific binding of 5 ng ml⁻¹ 125I-EGF was in the range 10–20 ng ml⁻¹ (approx. 1.5–3 nM) for both freshly isolated trophoderm cells (Fig. 2A) and cultured cells (Fig. 2B). The amount of specific binding varied between preparations, but there was no consistent effect of gestational age (15–19 days), or difference between freshly isolated and cultured cells. The addition of high concentrations of other growth factors or hormones, including insulin, IGF-I, PDGF, TGFβ, bFGF, substance P, angiotensin II, vasopressin and bombesin, had no effect on the binding of 125I-EGF (data not shown).
Fig. 2. Inhibition of $^{125}I$-EGF binding to pig trophoderm by unlabelled EGF. Trophoderm cells used either immediately after isolation (A), or after 5 days in culture (B), were incubated for 1 h at 37°C with $^{125}I$-EGF (5 ng ml$^{-1}$) and the indicated concentrations of unlabelled EGF. Binding was determined as described in Materials and methods, and is expressed as a percentage of the binding to control samples. Values are means ± S.E.M. of 4 samples. In A, the inset shows Scatchard analysis of $^{125}I$-EGF binding to freshly isolated trophoderm cells, which were incubated for 1 h at 37°C with $^{125}I$-EGF (0.1–25 ng ml$^{-1}$) in the presence or absence of excess (2 µg ml$^{-1}$) unlabelled EGF. After the incubation, the concentration of free $^{125}I$-EGF was determined by counting an aliquot of the medium from each tube after the first centrifugation of the washing procedure, and specific binding (expressed as pg mg$^{-1}$ protein) was determined as described in Materials and methods. Values are the means of triplicate determinations at each concentration of EGF used.

Scatchard analysis of $^{125}I$-EGF binding to various cell types has generally resulted in curvilinear plots indicating the presence of more than one class of receptor with different affinity states (see Blay and Brown, 1985). A Scatchard plot of $^{125}I$-EGF binding to freshly isolated pig trophoderm (Fig. 2A, inset) was consistent with two classes of receptor, with estimated dissociation constants of approximately $10^{-10}$ M and $10^{-9}$ M for the high- and low-affinity receptors, respectively. The majority of the $^{125}I$-EGF binding to trophoderm (250–300 pg mg$^{-1}$ protein in the experiment shown in Fig. 2A) was to the lower-affinity receptor class. Similar results were obtained in three experiments.

When $^{125}I$-EGF was cross-linked to trophoderm cell suspensions using DSS, a band corresponding to a relative molecular mass of about 160,000 was detected by SDS–polyacrylamide gel electrophoresis (Fig. 3). The formation of this labelled complex was progressively reduced by the presence of increasing concentrations of unlabelled EGF in the binding incubation prior to cross-linking. The radiolabelled band detected at the top of the gel (Fig. 3) is presumably due to aggregated material that did not enter the gel, while the faint labelling of bands at lower relative molecular mass is probably non-specific since it was relatively insensitive to displacement by unlabelled EGF.

Trophoderm cells thus possess receptors for EGF that appear similar to those from other sources in their molecular size and their specificity and affinity for EGF, suggesting that they will be fully functional. As such, they could be expected to mediate responses to either EGF or the related molecule transforming growth factor-α (TGF-α), but the nature and source of the physiologically relevant ligand remains to be established. Although low levels of EGF and TGF-α have been shown to be present in the uterus or uterine fluid of rats and mice (Imai et al. 1982; Gonzalez et al. 1984; Han et al. 1987; DiAugustine et al. 1988), we have been unable to detect EGF-related ligands in extracts of...
porcine uterus or uterine fluid by radioreceptor assay. However, this assay may not be sufficiently sensitive to detect low levels of the factors. Heterologous radioreceptor assay is of limited value as an alternative method of EGF measurement because of significant species differences in EGF sequence and very limited cross-reactivity of antisera. In any case, EGF radioreceptor assay would not detect related factors such as TGF-α.

**Binding of $^{125}$I-IGF-I to trophoderm**

Trophoderm cells incubated at 37°C were found to secrete binding proteins for IGFs (see below). In order to avoid interference by these proteins, the cellular binding of $^{125}$I-IGF-I was studied only at 4°C. In comparison with the binding of $^{125}$I-EGF, a higher proportion of the total binding of $^{125}$I-IGF-I was non-specific (typically 25–30%, compared with less than 10% for $^{125}$I-EGF binding). Nevertheless, saturable, specific binding of $^{125}$I-IGF-I to both freshly isolated and cultured trophoderm cells was observed (Fig. 4). Binding was inhibited by either IGF-I or insulin (Fig. 4). Insulin gave a dose-dependent inhibition of $^{125}$I-IGF-I binding over the range 1–100 μg ml$^{-1}$ in both freshly isolated cells (Fig. 4C) and cell cultures (not shown). However, the inhibition by IGF-I showed a marked difference between the freshly isolated cells and cell cultures. In freshly isolated cells (Fig. 4A) the inhibition of binding by IGF-I at 1 μg ml$^{-1}$ was small, being equivalent to that given by insulin at 1 μg ml$^{-1}$; in cultured cells, on the other hand, IGF-I at 1 μg ml$^{-1}$ gave a similar inhibition to that given by a maximally effective dose of insulin (Fig. 4D and data not shown), indicating a difference of potency of about 100-fold between IGF-I and insulin, consistent with the presence of type I IGF receptors. Other, unrelated, growth factors or hormones did not affect $^{125}$I-IGF-I binding (results not shown).

The inhibition of $^{125}$I-IGF-I binding by insulin implies that the receptors involved are insulin and/or type I IGF receptors rather than type II IGF receptors, which do not bind insulin (Rechler and Nissley, 1985), and that insulin, IGF-I and IGF-II are therefore potential ligands. Whether the receptors are type I IGF receptors or insulin receptors remains to be determined: since both receptors have a homologous βαββ tetrameric structure, specific antibodies rather than chemical cross-linking would be needed to distinguish between them unequivocally. A previous study (D’Ercole et al. 1976) showed that both $^{125}$I-IGF-I and $^{125}$I-insulin could bind to the fetal portion of the placenta from pigs at later gestational ages, with similar amounts of each receptor at the earliest date examined (24 days), but increasing relative amounts of IGF-I binding at later dates. Our results show that IGF-I binding can be demonstrated as early as day 15. The amount of binding, expressed per mg of cell protein (see, for example, Fig. 4B) is similar to that on Swiss 3T3 cells, which are mitogenically responsive to IGFs (Corps and Brown, 1988).

**Secretion of IGF-binding proteins by trophoderm cultures**

Various cell types have been shown to synthesize and secrete specific IGF-binding proteins, of relative molecular mass between 25000 and 40000, which can modulate the binding of IGFs to their cellular receptors (Nissley and Rechler, 1984; Baxter and Martin, 1989). Preliminary experiments indicated that supernatants from serum-free cultures of trophoderm cells could inhibit the binding of $^{125}$I-IGF-I to the type I IGF receptors on Swiss 3T3 cells (Corps et al. 1989). To test whether this inhibition was due to the release of IGF-binding proteins by the cultures, we incubated the supernatants with $^{125}$I-IGF-I and DSS, and observed a $^{125}$I-labelled complex of relative molecular mass about
Growth factor receptors on pig trophoderm

**Fig. 5.** Affinity-labelling of proteins in supernatants from pig trophoderm cultures with $^{125}$I-IGF-I. (A) Supernatants from the first 24 h of culture (lanes 1–4) and from days 1–4, 4–7 and 7–10 (lanes 5–7 respectively) were cross-linked to $^{125}$I-IGF-I in the absence of unlabelled ligand (lanes 4–7) or in the presence of IGF-I (100 ng ml$^{-1}$), IGF-II (100 ng ml$^{-1}$) or insulin (100 ng ml$^{-1}$) (lanes 1–3 respectively). (B) Supernatants from the first 24 h of culture (lanes 1–3) and uterine fluid from the same pig (lanes 4–6) were cross-linked to $^{125}$I-IGF-I in the presence of IGF-I at 0 (lanes 1, 4), 10 (lanes 2, 5) or 100 ng ml$^{-1}$ (lanes 3, 6). (C) Supernatants from the first 24 h of culture (lanes 1, 3) or days 10–14 (lanes 2, 4) were electrophoresed, blotted onto nitrocellulose and probed with $^{125}$I-IGF-I in the absence (lanes 1, 2) or presence (lanes 3, 4) of unlabelled IGF-I (100 ng ml$^{-1}$); note that the blot was exposed to film for a longer time for the 24 h supernatant than for the day 10–14 supernatant. The supernatants used in A, B and C were obtained from trophoderm cultures derived from pigs at days 17, 16 and 18 post-coitum respectively.

45 000 on SDS–polyacrylamide gel electrophoresis (Fig. 5). The formation of this complex was blocked by IGF-I or IGF-II but not insulin (Fig. 5A), which is characteristic of IGF-binding proteins derived from other sources (Nissley and Rechler, 1984). The IGF-binding protein produced by the cultures was investigated further using a 'ligand blotting' technique (Hossonlopp et al. 1986). A single broad band was specifically labelled with $^{125}$I-IGF-I; on low exposure of the autoradiograph this was resolved into four components with relative molecular mass between 32 000 and 42 000, the most heavily labelled of which had a relative molecular mass of about 39 000 (Fig. 5C).

Two observations indicated that the IGF-binding protein was secreted by the cultured cells, rather than being derived from the fluid in which the blastocysts had been flushed from the uterus. First, when cultures were maintained for up to 14 days with a change of serum-free DMEM every 3–4 days the supernatants obtained at each change of medium contained similar amounts of IGF-binding activity (Fig. 5A). Second, the diluted uterine fluid itself did not contain detectable amounts of binding protein (Fig. 5B and data not shown).

IGF-binding proteins have recently been detected in the allantoic and amniotic fluids of pigs at much later stages of gestation (Walton and Etherton, 1989). In the present study, we find no evidence for an IGF-binding protein in uterine fluid similar to that secreted by the cultured trophoderm (Fig. 5); however, this fluid substantially inhibited $^{125}$I-IGF-I binding to type 1 receptors on Swiss 3T3 cells (A.N.C., unpublished data), and it is likely that this activity is due to the presence of IGFs in the fluid, synthesised and secreted by the porcine uterus, as indicated by previous studies (Tavakkol et al. 1988; Simmen et al. 1989; Ogasawara et al. 1989). Taken together, these results suggest that the function of the IGF-binding protein could be to modulate the local interaction of uterus-derived IGFs with the trophectoderm. However, we cannot exclude the possibility that the IGF-binding protein might be secreted into the lumen of the blastocyst to perform a similar modulatory role on IGFs produced by the embryo.

**Stimulation of cultured trophoderm cells by added growth factors**

When EGF was added to cultures of trophoderm cells in low serum (Table 1) or serum-free medium (data not...
shown), there was a stimulation of \(^{3}H\)thymidine incorporation into DNA, indicating that the EGF receptors described above are functional. There was some variability between experiments in the degree of stimulation observed, probably reflecting the stage at which the tissue was isolated or the treatment of the cultures before the experiment. The addition of IGF-I to the cultures did not affect \(^{3}H\)thymidine incorporation in either control or EGF-stimulated cells (Table 1). While this could indicate that the IGF receptors are not functional, further work will be required to distinguish this explanation from at least two other possibilities. For example, the IGF-binding protein secreted by the trophoderm cells may prevent stimulation by added IGF-I. Consistent with this, insulin (which does not bind to the binding protein – see Fig. 5) stimulated \(^{3}H\)thymidine incorporation in the trophoderm cultures (data not shown). Alternatively, the trophoderm cells might also secrete IGFs, such that the signalling pathway triggered by the IGF receptors is active without the addition of exogenous IGF-I. Indeed, we have found that serum-free medium conditioned by trophoderm cultures is able to stimulate DNA synthesis in Swiss 3T3 cells (A.N.C., unpublished data), indicating that growth factor(s) are secreted by the trophoderm cells; whether IGFs are among the factors secreted remains to be established.

The finding that receptors for both EGF and IGF/insulin are present on early trophoderm suggests that these factors might play a role in regulating trophoblast growth and development. A major question that arises is whether these receptors are present on cells derived from the outer ectodermal layer or the inner mesodermal layer of trophoderm, and hence which fluid compartments might contain the relevant growth factors for the activation of the receptors. The culture system described here should be useful for further investigation of growth factor–trophoderm interactions. For example, the stimulation of DNA synthesis by added growth factors could be monitored in individual cells using \(^{3}H\)thymidine autoradiography, or bromodeoxyuridine plus anti-bromodeoxyuridine antibodies (Plickert and Krohner, 1988); it should be possible to use these methods, together with antibodies specific for trophoderm (Whyte et al. 1984), to determine the origin of the cells which are stimulated by the growth factors.

We thank Dr F. Stewart for useful comments on the manuscript.

References


Table 1. Incorporation of \(^{3}H\)thymidine by pig trophoderm cultures

<table>
<thead>
<tr>
<th>Condition</th>
<th>(^{3}H)thymidine (cts min(^{-1})/dish)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25,330±2,228</td>
</tr>
<tr>
<td>EGF (30 ng ml(^{-1}))</td>
<td>42,236±1,958***</td>
</tr>
<tr>
<td>IGF-I (30 ng ml(^{-1}))</td>
<td>30,996±3,278</td>
</tr>
<tr>
<td>EGF+IGF-I</td>
<td>42,942±2,202***</td>
</tr>
</tbody>
</table>

Trophoderm cell cultures were prepared in DMEM containing 5% (v/v) calf serum. After 4 days in culture, the medium was changed to DMEM containing 0.2% (v/v) calf serum plus growth factors at the concentrations indicated. After 48th incubation \(^{3}H\)thymidine (1 \(\mu\)Ci ml\(^{-1}\); 1 \(\mu\)Ci ml\(^{-1}\)) was added, and after a further 24 h the incorporation into DNA was determined as described previously (Brown and Blakeley, 1983). Values are means±S.E.M. from 5 dishes of cells. *** indicates a significant difference from control cultures (P<0.001 by t-test).
Growth factor receptors on pig trophoderm


(Accepted 1 June, 1990)