Purification and characterization of heparin-binding growth factors from porcine uterus

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Heparin-binding growth factors present in pig uterine tissue were purified by approx. 50000-fold using a combination of ammonium sulphate precipitation, ion-exchange chromatography and heparin-affinity chromatography. Purification of the uterus-derived growth factors (UDGFs) was monitored by the stimulation of [³H]thymidine incorporation into Swiss 3T3 cells and by a radioreceptor assay using ¹²⁵I-labelled epidermal growth factor (EGF) as the ligand. The latter was shown to be a novel, rapid and reliable assay for heparin-binding growth factors which utilizes their trans-modulation of EGF receptor affinity. UDGFs exhibit strong affinity for immobilized heparin and two forms, named α UDGF and β UDGF, were distinguished by salt gradient elution from heparin-agarose affinity columns. β UDGF activity was eluted from heparin-agarose between 1.5 M- and 1.8 M-NaCl, and was correlated with the elution of a protein doublet of 17.2 kDa and 17.7 kDa. Immunoblotting of heparin-purified β UDGF indicated that the β UDGF doublet is immunologically related to the 146-amino-acid form of bovine basic fibroblast growth factor (bFGF), and that the 17.2 kDa component is an N-terminally truncated form of the 17.7 kDa component. After purification by C₄ reversed-phase h.p.l.c., this doublet was biologically active and > 95% pure as assessed by silver-stained SDS/PAGE. Amino acid composition and sequence analysis confirmed that these β UDGF polypeptides were microheterogeneous forms of bFGF. Fractions containing α UDGF activity were eluted from heparin-agarose in 1.3 M-NaCl. These fractions contained a 16.5 kDa protein which co-migrated on SDS/polyacrylamide gels with recombinant human acidic FGF (aFGF) and which cross-reacted with an antiserum raised against aFGF. The identification of heparin-binding growth factors in porcine uterus at the time of implantation raises the possibility that they function in the reproductive tract during early pregnancy.

INTRODUCTION

There is considerable interest in embryo-maternal interactions during early pregnancy. Pigs have been used extensively in these studies because substantial quantities of both embryonic and uterine material can be recovered for experimental use. During the pre-implantation period in this species, there is a striking change in blastocyst morphology and a high incidence of embryonic mortality (Perry & Rowlands, 1962). Although the underlying causes of these events are poorly understood, it is clear that development and survival of the early pig embryo is influenced by its local environment in early pregnancy (see Roberts & Bazer, 1988).

It has been reported that uterine luminal fluid from pigs or sheep contains compounds which stimulate cellular DNA synthesis (Flint, 1981; Ikeda & Sirbasku, 1984; Simmen *et al.*, 1988; Bird *et al.*, 1988), and that endometrial explants enhance the rate of protein synthesis in blastocyst co-cultures (Rice *et al.*, 1981). Although these observations suggest that uterus-derived factors may influence survival and development of the pre-implantation pig embryo, the nature of the compounds involved is unknown. Since polypeptide growth factors are possible candidates, we have investigated their presence in uterine tissue. We have reported previously that

an anionic polypeptide growth factor, which stimulates DNA synthesis in Swiss mouse 3T3 cell cultures, is present in pig uterine extracts (Brigstock *et al.*, 1986). We now report that the principal Swiss 3T3 cell-growth-promoting factors in the pig uterus bind strongly to heparin and are closely related to the acidic and basic forms of fibroblast growth factor (FGF) (Lobb & Fett, 1984; Gospodarowicz *et al.*, 1984). In addition, we have found that heparin-binding growth factors (HBGFs) have the ability to trans-modulate (Brown *et al.*, 1983, 1984; Collins *et al.*, 1983) the affinity of the receptor for epidermal growth factor (EGF) on Swiss 3T3 cells. This finding has allowed us to detect HBGFs by a radioreceptor assay using ¹²⁵I-labelled EGF as the ligand, and has provided a simple and rapid method of monitoring HBGF purification.

EXPERIMENTAL

Animals and materials

[methyl-³H]Thymidine, Na¹²⁵I and recombinant basic FGF (bFGF) were from Amersham International (Amersham, Bucks., U.K.). EGF was prepared from mouse submaxillary glands by the method of Savage & Cohen (1972). ¹²⁵I-labelled EGF was prepared as de-

Abbreviations used: UDGF, uterus-derived growth factor; aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; HBGF, heparin-binding growth factor; ECGF, endothelial cell growth factor; EGF, epidermal growth factor; IGF-I, insulin-like growth factor I; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PTH, phenylthiohydantoin.

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scribed (Brown & Blakeley, 1983). Antisera against synthetic peptides of bovine acidic FGF (aFGF) and bFGF were provided by Andrew Baird (Salk Institute, San Diego, CA, U.S.A.). Recombinant human α -endothelial cell growth factor (α ECGF) and antiserum to α ECGF were provided by Michael Jaye (Rorer Biotechnology, Springfield, VA, U.S.A.). [α ECGF is a truncated form of aFGF lacking the first six *N*-terminal residues (Burgess *et al.*, 1986).] Culture medium, trypsin, antibiotics and newborn calf serum were from Flow Laboratories (Irvine, Scotland, U.K.).

Reproductive tracts were removed from gilts (17–19 days pregnant) about 20 min after stunning and exsanguination at a local slaughterhouse. The tracts were transported on ice to the laboratory within 10 min. Uteri were dissected free of ovaries, mesometrium and cervix, and then flushed with 20 ml of Dulbecco's modified Eagle's medium (DMEM) to remove embryonic material. Each uterine horn was cut into 2 cm pieces which were stored at -20°C until processed.

Cell culture

Stock cultures of Swiss mouse 3T3 cells were maintained and passaged as described previously (Brown & Blakeley, 1983). For experimental use, cells were seeded in 24-well cluster plates (Nunc) at a density of 1.25×10^4 cells/well in 0.5 ml of DMEM containing 10% (v/v) newborn calf serum.

The protein content of uterine samples was determined by the method of Bradford (1976) using porcine gamma-globulin as standard. For samples below the sensitivity of this assay, protein content was estimated by comparison with standard proteins on silver-stained SDS/polyacrylamide gels.

DNA synthesis assay and EGF binding assays

Cells were used 6–7 days after seeding, when they had formed a confluent, quiescent monolayer. [*methyl*- ^3H]Thymidine (1 $\mu\text{Ci}/\text{ml}$) and aliquots of test samples [up to 10% (v/v)] were added to the depleted medium in each well and the cells were incubated for 40 h at 37°C . The medium was removed from the wells and the cells were washed twice with 1–2 ml of cold (4°C) phosphate-buffered saline (PBS, 138 mM-NaCl/2.8 mM-KCl/8.0 mM- $\text{Na}_2\text{HPO}_4/1.45$ mM- $\text{KH}_2\text{PO}_4/0.91$ mM- $\text{CaCl}_2/0.49$ mM- MgCl_2 , pH 7.2). The cells were extracted for 30 min with 1–2 ml of cold 5% (w/v) trichloroacetic acid, rinsed with ethanol, air dried and dissolved in 0.5 ml of 0.1 M-NaOH which was transferred to 10 ml of acidified scintillation fluid for counting of ^3H radioactivity.

For measurement of ^{125}I -EGF binding, cell monolayers were rinsed with binding medium (DMEM containing 1 mg of BSA/ml, 0.1 μM -KI and 50 mM-Bes, adjusted to pH 7.0). Each well then received 0.5 ml of binding medium containing ^{125}I -EGF (1 ng/ml) and up to 10% (v/v) of test sample. After incubation at 37°C for 60 min, the cells were washed three times with cold PBS containing 1 mg of BSA/ml and 0.1 μM -KI. The cells were solubilized in 1 ml of 0.5 M-NaOH, and cell-associated ^{125}I -EGF was measured using a gamma counter.

Purification and characterization of UDGFs

Tissue extraction, ammonium sulphate precipitation and ion-exchange chromatography. All procedures were carried out at 4°C . Frozen uteri were thawed rapidly and

approx. 200 g of tissue was homogenized in 400 ml of cold 0.15 M-ammonium sulphate solution in a Waring blender at high speed for 1 min. The homogenate from approx. 1 kg of uterus was pooled, blended again at high speed for 1 min and centrifuged at 5000 *g* for 30 min. The supernatant was filtered through glass wool and adjusted to pH 4.5 with 5 M-HCl. The acidified solution was stirred for 2 h and then centrifuged at 5000 *g* for 30 min. Ammonium sulphate precipitation and CM-Sephadex C-50 chromatography were performed as described by Gospodarowicz *et al.* (1984).

Heparin-agarose affinity chromatography. Biologically active fractions from the CM-Sephadex column were pooled and applied directly to a heparin-agarose (Sigma) column (1.6 cm $\times$ 11 cm) which had been equilibrated with 20 mM-Tris/HCl (pH 8.0) containing 0.5 M-NaCl. Typically, the CM-Sephadex eluate from about 0.5 kg of starting material was applied to the heparin-agarose column in a single run. The column was then washed with 100 ml of equilibration buffer before elution by a 0.5–2 M-NaCl gradient in 20 mM-Tris/HCl (pH 8.0). A GP250 Gradient Programmer (Pharmacia) was used to generate the salt gradient and to maintain a pumped flow rate of 0.5 ml/min. Conductivity of the column output was monitored using a conductivity cell (adapted to flow-through use) linked to a conductivity meter. Absorbance (280 nm) was monitored using a u.v. column monitor (LKB 2238 Uvicord SII). Fractions (10 ml) were collected over the entire gradient and tested for biological activity.

In order to characterize heparin-purified UDGs by h.p.l.c. or SDS/PAGE, it was necessary to concentrate and desalt the samples. This was achieved by passing individual fractions through small columns (4 mm $\times$ 10 mm) of Vydac C_4 bonded-silica particles (15–20 μm). Columns were first washed with 1 ml of 20% acetonitrile/0.1% trifluoroacetic acid and retained proteins were then eluted in 1 ml of 80% acetonitrile/0.1% trifluoroacetic acid. This eluate was dried in a Speed-Vac concentrator (Savant) and reconstituted in either 20% acetonitrile/0.1% trichloroacetic acid (for h.p.l.c.) or complete sample buffer (for SDS/PAGE).

Reversed-phase h.p.l.c. Reversed-phase h.p.l.c. was performed using a Waters Associates chromatography system and a Vydac C_4 column (0.46 cm $\times$ 25 cm; Technicol, Stockport, U.K.). Before sample application, the column was equilibrated with 20% acetonitrile/0.1% trifluoroacetic acid.

SDS/PAGE. UDGf samples were subjected to SDS/PAGE under reducing conditions. Samples were applied to a 15% polyacrylamide resolving gel with a 4% stacking gel and electrophoresed at 20 mA for 4–5 h. Protein bands were detected by silver staining according to the method of Wray *et al.* (1981).

Immunoblotting. Heparin-purified UDGf samples were blotted from gels on to nitrocellulose sheets by electrotransfer (Towbin *et al.*, 1979) at 4°C for 90 min at an initial current of 0.7 A (rising to 1.2 A). Blots were blocked by soaking them for 60 min at room temperature in MTA buffer, which is composed of PBS containing 5% (w/v) non-fat dry milk, 0.2% (v/v) Tween 20 and 3 mM-sodium azide (Glenney, 1986). Blots were then incubated overnight at 4°C in MTA buffer containing

Fig. 1. CM-Sephadex C-50 chromatography of UDGFs

Proteins extracted from 1.2 kg of uterine tissue by ammonium sulphate precipitation were dissolved, dialysed and loaded on to a CM-Sephadex C-50 column which was equilibrated, washed and eluted as described in the Experimental section. The flow rate was 2.5 ml/min and fractions of 10 ml were collected and assayed for UDG activity. The broken line indicates the absorbance (280 nm) of the column eluate. The effects of the addition of 2% (v/v) of the fractions on [^3H]thymidine incorporation (●) and ^{125}I -EGF binding (■) by Swiss 3T3 cells were determined.

Fig. 2. Heparin-agarose affinity chromatography of UDGFs

Active fractions from the 0.65 M-NaCl eluate obtained by CM-Sephadex C-50 chromatography were pooled and applied to a heparin-agarose column. The column was washed with 20 mM-Tris/HCl buffer (pH 8.0) containing 0.5 M-NaCl and developed with a linear salt gradient of 0.5–2 M-NaCl. The flow rate was 0.5 ml/min and 10 ml fractions were collected. The effects of the addition of 2% (v/v) of the fractions on [^3H]thymidine incorporation (●) and ^{125}I -EGF binding (■) were determined. The solid line and the dashed line represent the conductivity and absorbance respectively (at 280 nm) of the column output.

primary antiserum. Immunoreactive proteins were revealed by successive incubations with biotinylated goat anti-rabbit IgG, a pre-formed streptavidin-biotin-horseradish peroxidase complex (both from Amersham International) and diaminobenzidine.

Amino acid composition and sequencing. Amino acid compositions were determined using an LKB 4151 Alpha Plus analyser. *N*-Terminal sequencing was performed

using an Applied Biosystems 470A gas phase sequencer. Phenylthiohydantoin (PTH) derivatives were identified using a 120A on-line PTH analyser.

RESULTS

We have observed previously that extracts of pig uterus contain mitogenic activity for Swiss 3T3 cells (Brigstock *et al.*, 1986). Vascular perfusion of the uterine

Fig. 3. Dose-response to UDGFs at different stages of purification

UDGF samples at different stages of purification were assayed for their ability to stimulate [^3H]thymidine incorporation (●) and to inhibit ^{125}I -EGF binding (■) in Swiss 3T3 cells. Curve A, 40–80% ammonium sulphate precipitate; B, 0.65 M-NaCl eluate from CM-Sephadex C-50 column; C, 1.2 M-NaCl eluate from heparin-agarose column (α UDGF); D, 1.5–1.8 M-NaCl eluate from heparin-agarose column (β UDGF). The apparent reduction in the maximal stimulation of [^3H]thymidine incorporation by heparin-purified UDGFs is due to the high salt concentrations of the samples.

horn at autopsy with approx. 500 ml of 0.9% (w/v) NaCl before dissection failed to remove biological activity, indicating that the factor(s) responsible was probably not derived directly from blood. Preliminary characterization (results not shown) indicated that the uterine growth factor(s) had several similarities with the FGFs. Since FGFs have been purified by a combination of ion-exchange chromatography and affinity chromatography on heparin-Sepharose (Lobb & Fett, 1984; Lobb *et al.*, 1986), we have adopted this procedure for the purification of the uterine factors.

CM-Sephadex C-50 and heparin-agarose chromatography

When the redissolved and dialysed ammonium sulphate precipitate was loaded on to the CM-Sephadex C-50 column, most of the protein passed unretarded through the column and contained only low levels of biological activity (Fig. 1). A major peak of protein was eluted by buffer containing 0.2 M-NaCl, but this peak contained only low levels of cell-growth-promoting activity. However, a large peak of growth factor activity was eluted from the column by buffer containing 0.65 M-NaCl (Fig. 1). Material which inhibited the binding of ^{125}I -EGF to the cells was found to co-elute with the mitogenic activity (Fig. 1).

Most (90%) of the protein in the active fractions from the CM-Sephadex column was either unretarded by the heparin-agarose column or was eluted as a major protein peak when the column was washed with 20 mM-Tris/HCl (pH 8.0) containing 0.5 M-NaCl. An additional small protein peak was eluted at the beginning of the 0.5–2 M-NaCl gradient. Thereafter the absorbance of the column output remained below the detection limit of the column

Table 1. Purification of HBGFs from porcine uterus

Purification step	Protein recovered (mg)	ED ₅₀ * (ng/ml)	Activity recovered† (%)	Purification factor
Ammonium sulphate precipitate	14000	1×10^5	100	0
0.65 M-NaCl eluate from CM-Sephadex C-50	118	3500	24	29
1.3 M-NaCl eluate from heparin-agarose (α UDGF)	0.05	16	2	6200
1.5–1.8 M-NaCl eluate from heparin-agarose (β UDGF)	0.02	2	7	5×10^4

* Concentration required to reduce ^{125}I -EGF binding by 50%; monitored using ^{125}I -EGF binding assay.

† Calculated by comparison of the total activity at each purification step with the total activity in the ammonium sulphate precipitate. Approx. 1 kg of uterine tissue was used for this purification.

monitor (Fig. 2). When the fractions eluted by the salt gradient were assayed, two major peaks of 3T3 cell-stimulating activity were found; one (named α UDGF) was eluted at approx. 1.3 M-NaCl and the other (named β UDGF) was eluted between 1.5 M- and 1.8 M-NaCl (Fig. 2). Both α - and β -UDGF stimulated DNA synthesis in 3T3 cells and inhibited ^{125}I -EGF binding to these cells. The dose-response curves for these effects at different stages of purification are shown in Fig. 3 and summarized in Table 1.

Characterization of heparin- and h.p.l.c.-purified UDGFs

SDS/PAGE and reversed-phase h.p.l.c. When heparin-purified β UDGF was subjected to SDS/PAGE, a prominent doublet which migrated at 17.7 kDa and 17.2 kDa was observed after silver staining (Fig. 4a). When heparin-purified β UDGF was purified further by reversed-phase h.p.l.c., the elution profile of biologically active material correlated with the elution profile of this protein doublet (Figs. 4b and 4c). SDS/PAGE and silver staining of heparin-purified α UDGF showed that this material contained numerous proteins, and it was not possible to correlate biological activity with the elution of a particular protein(s) after C_4 h.p.l.c. (results not shown).

Immunoblotting. The 17.2/17.7 kDa protein doublet observed in heparin-purified β UDGF preparations by silver staining (Fig. 4a) was detected on immunoblots probed using an antiserum to bFGF-(106–120) but not by an antiserum to aFGF-(1–15) (Fig. 5a). The levels of this immuno-stained doublet were maximal in fractions 18–20, which possessed maximal biological activity (see Fig. 2), although lower levels of the doublet were detected in earlier fractions (Fig. 5a). This same protein doublet also reacted with several other antisera raised against

Fig. 4. SDS/PAGE of heparin- and HPLC-purified β UDGF

β UDGF-containing fractions from heparin-agarose chromatography were pooled, desalted and dried. The sample was dissolved in 20% acetonitrile/0.1% trifluoroacetic acid and applied to a Vydac C_4 column which was eluted using a linear acetonitrile gradient (broken line in c) of 0.66%/min at a flow rate of 1.5 ml/min. Fractions of 1.5 ml were collected, dried and dissolved in 100 μ l of 10 mM-Tris/HCl (pH 8.0) for assaying activity. (a) SDS/PAGE of heparin-purified β UDGF. (b) SDS/PAGE of successive fractions eluted from the C_4 column (44–49% acetonitrile). Gels were silver-stained. Marker proteins were carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), myoglobin (17.2 kDa) and lysozyme (14 kDa). (c) Aliquots (25 μ l) of the h.p.l.c. fractions were tested for their ability to stimulate [^3H]thymidine incorporation (●) and inhibit ^{125}I -EGF binding (■).

basic FGF peptide sequences including bFGF-(1–24) (Fig. 5b, lane 1), bFGF-(73–87) (Fig. 5b, lane 2) and bFGF-(139–146) (Fig. 5b, lane 3). In contrast, control

antisera failed to stain the proteins (Fig. 5b, lanes 4 and 5). Although the upper (17.7 kDa) band of the doublet was stained with an antiserum against bFGF-(1–10), the lower (17.2 kDa) band was not detected using this antiserum (Fig. 5b, lane 6), suggesting that it may be an *N*-terminally truncated variant of the 17.7 kDa band.

When blots of individual fractions from heparin-agarose columns were probed using an antiserum raised against aFGF-(1–15), proteins at 21 and 16.5 kDa were stained (Fig. 5a, lower blot). The 21 kDa protein was not correlated with elution of α UDGF activity, since it was apparently present at similar levels in all fractions. However, the 16.5 kDa protein was detected only in fractions 14–15, which were shown to possess α UDGF mitogenic activity (see Fig. 2). Because the staining of the 16.5 kDa protein was rather faint, blots of the α UDGF-containing fractions from a heparin-agarose column were also probed with an antiserum raised against α ECGF which corresponds to aFGF-(7–140) (Burgess *et al.*, 1986). The 16.5 kDa protein reacted strongly with this antiserum (Fig. 5c, lanes 2 and 3). Furthermore, the immuno-stained band was found to co-migrate on SDS/PAGE with recombinant α ECGF (Fig. 5c, lane 7). In contrast, the 16.5 kDa protein was not detected using non-immune serum (Fig. 5c, lane 6) or an antiserum to bFGF-(73–87) (Fig. 5c, lane 5). The latter antiserum caused light staining of protein(s) of a slightly higher molecular mass (17–18 kDa) as would be expected from the known presence of the β UDGF doublet in these fractions (see Fig. 5a).

In addition to the 17.2/17.7 kDa protein doublet, several other bFGF-immunoreactive proteins were detected in fractions from the heparin column (Fig. 5a). Two proteins (14 kDa and 32 kDa) were detected in fractions 18–20, and a third immunoreactive protein (27 kDa) was eluted earlier in fractions 12–14 at a NaCl concentration of 0.9–1.1 M. The 14 kDa protein was always weakly stained and is possibly a breakdown product from the β UDGF doublet. The 32 kDa band may be due to dimerization of doublet proteins, since an immuno-stained band at this position was also observed in lanes loaded with high concentrations (100 ng) of purified recombinant bFGF (results not shown). In addition, the 32 kDa band was not detected by an antiserum raised against the peptide bFGF-(–8 to +1), corresponding to the nine additional residues recently identified in an *N*-terminally extended form of bFGF (Ueno *et al.*, 1986). In contrast, the 27 kDa protein in fractions 12–14 reacted as strongly with this antiserum as with anti-bFGF-(106–120) (results not shown). The possibility that this band is due to a high-molecular-mass bFGF-related protein merits further study.

Inhibition of ^{125}I -EGF binding by UDGs and recombinant FGFs. Several peptide and polypeptide mitogens, as well as tumour-promoting phorbol esters, are known to cause a temperature-dependent reduction in the affinity of the EGF receptor (Brown *et al.*, 1984; Schlessinger, 1986). This effect, which has been termed trans-modulation, is caused by phosphorylation of the EGF receptor at Thr⁶⁵⁴ by protein kinase C, although additional mechanisms cannot be excluded (Lin *et al.*, 1986; Davis & Czech, 1986, 1987; Walker & Burgess, 1988). Scatchard analysis suggested that the inhibitory action of β UDGF on ^{125}I -EGF binding was due to a reduction of receptor affinity with little or no change in

Fig. 5. Immunoblot characterization of α - and β -UDGF

(a) Fractions which were eluted from heparin-agarose between 1 M- and 2 M-NaCl after purification from 0.5 kg of uterus were individually concentrated and desalted. Proteins were separated by SDS/PAGE and electroblotted from the gels to nitrocellulose. The blots were probed using antisera to either bFGF-(106-120) (upper blot) or aFGF-(1-15) (lower blot). The molecular masses of the predominant immunoreactive proteins are indicated. Fraction numbers correspond to those in the heparin-agarose chromatograph shown in Fig. 2. (b) The heparin-purified β UDGF peak from 1 kg of uterus was dissolved in sample buffer and applied across the full width of an SDS/polyacrylamide gel. Proteins were electroblotted from the gel to nitrocellulose paper, which was blocked in MTA buffer (see the Experimental section) and cut into vertical strips which were individually probed using antisera raised against bFGF-(1-24) (lane 1), bFGF-(73-87) (lane 2), bFGF-(139-146) (lane 3), BSA (lane 4) and bFGF-(1-10) (lane 6). Lane 5 was probed with normal rabbit serum. (c) Blots of individual α UDGF-containing fractions from heparin-affinity chromatography were probed using an antiserum to α ECGF (an *N*-terminally truncated form of aFGF) (lanes 1-4), antiserum to bFGF-(73-87) (lane 5) or normal rabbit serum (lane 6). Lane 7 shows recombinant α ECGF (85 ng) probed using the antiserum to α ECGF. The molecular mass of the predominant immunoreactive protein is marked. Fraction numbers correspond to those in the heparin-agarose chromatograph shown in Fig. 2.

receptor numbers (Fig. 6a). However, additional experiments and more detailed analysis will be needed to confirm this point and to quantify the changes involved. In addition, the inhibitory action of UDGFs on 125 I-EGF binding was markedly temperature-dependent, with little inhibition when the binding assay was performed at 4 °C rather than 37 °C (Fig. 6a, inset). These results indicate that UDGFs inhibit 125 I-EGF binding by an

indirect trans-modulation of EGF receptor affinity rather than by a direct competitive interaction.

Magnaldo *et al.* (1986) have reported that acidic and basic FGFs, purified from bovine brain, did not inhibit 125 I-EGF binding to hamster lung fibroblasts. This difference from our results using UDGFs suggested that the ability to inhibit 125 I-EGF binding might be used to distinguish UDGFs from FGFs. We therefore

Fig. 6. Inhibition of ^{125}I -EGF binding by UDGFs and by recombinant FGFs

(a) Confluent monolayers of Swiss 3T3 cells were incubated for 60 min at 37 °C in binding medium containing concentrations of ^{125}I -EGF in the range of 0.05–30 ng/ml with (●) or without (■) heparin-purified β UDGF (20 $\mu\text{l/ml}$). Immediately before washing the cells, 0.1 ml of the binding medium was removed for measurement of unbound (free) ^{125}I -EGF. The cells were washed and cell-associated radioactivity was determined. Each point represents the value obtained from a single dish of cells. Inset: cells were incubated in binding medium containing ^{125}I -EGF (1 ng/ml) and α UDGF (60 $\mu\text{l/ml}$) or β UDGF (60 $\mu\text{l/ml}$) or unlabelled EGF (8 ng/ml) for 60 min at either 37 °C (□) or 4 °C (■). The cultures were washed and cell-associated radioactivity was determined. Each value is the mean of three determinations of ^{125}I -EGF binding to separate dishes of cells. The estimated protein concentrations of the α - and β -UDGF pools were 1.2 and 0.2 $\mu\text{g/ml}$ respectively. (b) Cells were incubated in binding medium containing ^{125}I -EGF (1 ng/ml) and either aFGF (●, ○) or bFGF (■, □) for 60 min at either 37 °C (●, ■) or 4 °C (○, □). The cultures were washed and cell-associated radioactivity was determined. Each value is the mean of three determinations of ^{125}I -EGF binding to separate dishes of cells.

Fig. 7. Reversed-phase h.p.l.c. purification of β UDGF

A total of 11.3 kg of uterine tissue from 21 animals between days 12 and 19 of pregnancy was extracted and purified in six separate experiments. β UDGF-containing fractions from each heparin-agarose run were pooled (total vol. 500 ml) and pumped directly on to a Vydac C_4 reversed-phase column in the presence of 5% acetonitrile and 0.1% trifluoroacetic acid. During sample application

investigated the action of recombinant FGFs on ^{125}I -EGF binding to Swiss 3T3 cells. Both acidic and basic FGF were potent inhibitors of ^{125}I -EGF binding at 37 °C; either induced a maximal inhibition of approx. 80% (Fig. 6b). In contrast, neither factor caused substantial inhibition of binding when the assay was performed at 4 °C (Fig. 6b). A Scatchard plot very similar to that presented in Fig. 6(a) was obtained when ^{125}I -EGF binding was measured in the presence or absence of bFGF (results not shown). Thus both UDGFs and FGFs show a similar ability to inhibit ^{125}I -EGF binding to Swiss 3T3 cells.

Amino acid analysis of β UDGF. Heparin-purified β UDGF, prepared from 11.5 kg of uterine tissue, was further purified by C_4 reversed-phase h.p.l.c. The β UDGF doublet was eluted from the C_4 column at 48% acetonitrile and was homogeneous when analysed by silver-stained SDS/PAGE (Fig. 7). Since attempts to

the flow rate was 2 ml/min; thereafter, it was reduced to 0.8 ml/min. The column was washed with 5% acetonitrile containing 0.1% trifluoroacetic acid until the absorbance (214 nm) of the column eluate reached baseline levels, and was then eluted with a linear acetonitrile gradient of 0.66%/min (broken line). Fractions of 800 μl were collected and an aliquot (10 μl) of each fraction was dried, redissolved in complete sample buffer and subjected to SDS/PAGE and silver staining to determine which fractions contained the β UDGF doublet. Fractions 13–15 were pooled, reduced in volume, and used for determination of amino acid composition and sequence.

Table 2. Amino acid composition of β UDGF

The amino acid composition of bovine basic FGF-(1-146) was deduced from sequence analysis (Esch *et al.*, 1985). Values for amino acid composition of β UDGF were obtained from a 24 h hydrolysis in 6 M-HCl at 110 °C and calculated assuming a molecular mass of 17.5 kDa. n.d., not determined.

Amino acid	Pituitary bovine bFGF-(1-146)	β UDGF
Asx	12	11.7
Thr	4	3.4
Ser	10	7.1
Glx	12	13.9
Pro	10	11.4
Gly	15	16.4
Ala	9	9.3
Val	7	6.8
Cys	4	n.d.
Ile	4	4.0
Leu	13	11.1
Tyr	7	3.1
Phe	8	7.1
Lys	14	15.1
His	3	3.1
Arg	11	12.7
Met	2	n.d.
Trp	1	n.d.

separate the component bands of the doublet by h.p.l.c. were not successful, the amino acid composition of the protein doublet was determined. The composition was very similar to that of the 146-amino-acid form of bFGF, except that the levels of serine and tyrosine were significantly lower in the β UDGF molecule (Table 2). This apparent difference is probably due to the partial destruction of these amino acids by acid hydrolysis during analysis (see Glazer *et al.*, 1985).

When approx. 450 pmol of the h.p.l.c.-purified β UDGF was subjected to microsequence analysis, up to four PTH-amino acids were identified in each cycle of degradation, indicating that four peptides were being sequenced simultaneously. When the results from the first 19 cycles were analysed, it became apparent that the data were well fitted to a model of four microheterogeneous forms of bFGF whose *N*-terminal residues were positions, 1, 3, 7 and 11 of bFGF-(1-146). These four sequences were aligned to give a consensus sequence for β UDGF of: Pro-Xaa-Leu-Pro-Glu-Asp-Xaa-Gly-Ser-Gly-Ala-Phe-Pro-Pro-Gly-His-Phe-Lys-Asp-Pro-Lys-Arg-Leu-Tyr. Further details supporting this sequence are available on request from the authors. Although we were unable to unequivocally identify the residues at positions 2 and 7, the remaining 22 residues are identical to the sequence of bovine bFGF (Esch *et al.*, 1985). This result, together with the amino acid composition and the immunoblotting data, indicate that β UDGF is porcine bFGF.

DISCUSSION

bFGF has been isolated from several tissues, principally of human or bovine origin (Baird *et al.*, 1986; Gospodarowicz *et al.*, 1987). The identification of

β UDGF as porcine bFGF demonstrates that this angiogenic mitogen is also present in uterine tissues. The high degree of similarity between porcine and bovine bFGF supports previous evidence that bFGF has been very well conserved during evolution. The sequences of human and rat bFGF (predicted from cDNA clones) differ from bovine bFGF by only two and four residues respectively when the 146-amino-acid forms are compared (Abraham *et al.*, 1986; Shimasaki *et al.*, 1988; Kurokawa *et al.*, 1988). Although h.p.l.c.-purified β UDGF was microheterogeneous due to *N*-terminal cleavages, it is not known whether multiple forms are present in the uterus or were generated during purification. Microheterogeneity of bovine bFGF was originally described by Baird and co-workers who isolated an *N*-terminally truncated [des-(1-15)-bFGF] form of bFGF from corpus luteum, kidney and adrenal gland (Baird *et al.*, 1986). In addition, Klagsbrun *et al.* (1987) have reported cleavage of human bFGF between amino acid residues 11 and 12. Extraction of tissues at neutral pH or in the presence of proteinase inhibitors has been reported to prevent acid-activated proteolytic cleavage of bFGF (Mormede *et al.*, 1985; Klagsbrun *et al.*, 1987). However, we were unsuccessful in preventing the formation of the 17.2 kDa β UDGF protein band using these conditions (results not shown). Although four polypeptide chains were detected during sequencing, we observed only two β UDGF bands on silver- or immuno-stained SDS gels of h.p.l.c.-purified samples. It seems probable that either the gels did not fully resolve the microheterogeneous forms of β UDGF present, or that two of the truncated polypeptides were formed during storage of the h.p.l.c.-purified sample before sequencing.

Although bFGF appears to be relatively widely distributed in animal tissues, it was originally thought that anionic HBGFs were present only in brain and retina (Gospodarowicz *et al.*, 1987). However, this appears not to be the case, since aFGF has now been purified from bovine kidney and its identity confirmed by sequencing (Gautschi-Sova *et al.*, 1987). Furthermore, aFGF-like factors have been reported to be present in human prostate (Crabb *et al.*, 1986) and in bovine bone (Hauschka *et al.*, 1986). Unfortunately, we have been unable to obtain amino acid sequence data for α UDGF, the second heparin-binding mitogen identified in porcine uterus. Nevertheless, its properties (affinity for heparin, immunological cross-reactivity) suggest that it is related to aFGF. However, in the absence of sequence data, this evidence must be interpreted with caution since genes encoding at least four additional (putative) HBGFs have recently been cloned, and the family of known HBGFs seems likely to expand (Yoshida *et al.*, 1987; Delli-Bovi *et al.*, 1988; Zhan *et al.*, 1988; Rubin *et al.*, 1989).

There is currently considerable interest in the possible existence of high-molecular-mass forms of bFGF. *N*-Terminal extensions of 8, 11 and 16 amino acids have been characterized (Ueno *et al.*, 1986; Sommer *et al.*, 1987; Klagsbrun *et al.*, 1987) and heparin-binding bFGF-immunoreactive proteins of 25 kDa (Moscatelli *et al.*, 1987) and 22 kDa (Presta *et al.*, 1988) have been reported. Our results suggest the presence, in uterine tissue, of a 27 kDa bFGF-related protein which is eluted from heparin-agarose by approx. 1 M-NaCl and reacts with several antisera raised against bFGF-peptides, including bFGF-(−8 to +1). We do not know whether

the 27 kDa protein has mitogenic activity, although the fractions from heparin-agarose columns which contain this protein did not stimulate DNA synthesis when tested on Swiss 3T3 cells at 2% (v/v).

Factors that trans-modulate EGF receptor affinity commonly stimulate phosphoinositide hydrolysis and cause activation of protein kinase C leading to phosphorylation of the receptor. However, some reports have indicated that both protein kinase C-dependent and -independent pathways may be involved in the regulation of EGF receptor affinity by platelet-derived growth factor (Davis & Czech, 1987; Olashaw & Pledger, 1987; Friedman & Rosner, 1987). The mechanism by which HBGFs alter EGF receptor affinity remains to be defined. Whatever the mechanism involved, the effect on EGF receptor affinity has allowed us to develop a heterologous radioreceptor assay using ^{125}I -EGF as a radioligand. This assay is advantageous because it enables very rapid screening of column fractions for biological activity.

Mitogenic activity has been reported to be present in uterine tissue extracts or uterine fluids from several species (Brigstock *et al.*, 1989). Ikeda & Sirbasku (1984) isolated a growth factor from sheep uteri which stimulated DNA synthesis in several tumour cell lines. Recent microsequencing studies on similar material purified from pig uteri have demonstrated that the activity is due to two *N*-terminally truncated forms of insulin-like growth factor I (IGF-I) which possess potent biological activity (Ogasawara *et al.*, 1989). In addition, low levels of mRNAs for IGF-I (Murphy & Friesen, 1988) and EGF (DiAugustine *et al.*, 1988) have been identified in rat uteri, and high levels of colony-stimulating factor I are produced by mouse endometrial cells, apparently under the control of sex steroids (Pollard *et al.*, 1987). To our knowledge, the presence of FGFs in uterine tissues has not been demonstrated previously. However, it has been known for many years that uterine tissues display angiogenic activity when implanted in the anterior chamber of the eye or on chick chorioallantoic membranes (Markee, 1940; Abel, 1985; Fuchs *et al.*, 1985). Since both acidic and basic FGFs are potent angiogenic factors (see Folkman & Klagsbrun, 1987), it is possible that the angiogenesis observed was related to the presence of FGF in the tissue. However, in addition to angiogenic activity, FGFs affect the growth and differentiation of various cell types (see Gospodarowicz *et al.*, 1987) as well as inducing mesoderm formation in early embryos (Slack *et al.*, 1987; Kimelman *et al.*, 1988). Furthermore, bFGF has been reported to regulate steroid hormone metabolism in ovarian granulosa cells (Baird & Hsueh, 1986). Taken together, these results suggest that FGFs could influence various aspects of uterine function.

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