High molecular mass forms of epidermal growth factor in pig uterine secretions

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Accumulating evidence suggests that uterine luminal fluids contain a variety of polypeptide growth factors and cytokines that, it is speculated, have roles in the development, growth and differentiation of the uterus and, during pregnancy, in the growth and survival of the embryo. Although epidermal growth factor (EGF) has previously been identified by radioimmunoassay and immunohistochemistry in the pig uterus, there have been no detailed studies of the secreted EGF protein. EGF was therefore purified from uterine flushings and uterine fluids of nonpregnant pigs of mixed breed using a variety of ion-exchange chromatography steps. Uterine flushings and fluids contained an anionic factor(s) that at 4°C competed with ¹²⁵I-labelled mouse EGF for binding to EGF receptors on an endometrial carcinoma cell line and stimulated DNA synthesis in Balb/c mouse 3T3 fibroblasts. As analysed by gel filtration, uterine fluids contained a 3–6 kDa factor that stimulated 3T3 cell DNA synthesis and was a competitor of cellular ¹²⁵I-labelled EGF binding. Gel filtration further revealed that uterine flushings and fluids contained, respectively, 45 kDa and 40–70 kDa moieties that were mitogenic and that bound to the EGF receptor. SDS-PAGE and western blotting using an antiserum specific for pig EGF revealed immunoreactive forms of EGF of approximately 25 kDa in partially purified uterine flushings. It is concluded that uterine secretory EGF occurs, at least in part, as high molecular mass proteins. The ability of these high molecular mass EGFs to bind to and activate the EGF receptor suggests that they may be authentic ligands for the EGF receptor *in utero*.

Introduction

Uterine luminal fluids contain a variety of largely undefined constituents that are thought to regulate various aspects of uterine and placental function (Roberts and Bazer, 1988). The pig is a useful model for the study of the role of these factors because its uterus, being very large, is particularly suitable for protein biochemical approaches for studying the constituent growth factors in uterine secretory fluids. Previously, the pig uterus has been shown to produce insulin-like growth factors (IGF-I), fibroblast growth factors (FGF), pleiotrophin, and various members of the epidermal growth factor (EGF) family such as EGF, heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor α (TGF- α) and amphiregulin (Tavakkol *et al.*, 1988; Brigstock *et al.*, 1989, 1990, 1996; Letcher *et al.*, 1989; Ogasawara *et al.*, 1989; Simmen *et al.*, 1990, 1992; Diehl *et al.*, 1994; Kennedy *et al.*, 1994; Kim *et al.*, 1995). Of these, IGF-I, EGF, HB-EGF, basic FGF and pleiotrophin have been shown to be components of uterine luminal fluids. Although detailed studies of their production and secretion

during the oestrous cycle and pregnancy are limited to IGF-I (Tavakkol *et al.*, 1988; Letcher *et al.*, 1989; Simmen *et al.*, 1990), the presence of several mitogenic factors in pig uterine fluids suggests interacting autocrine and paracrine pathways through which uterine function is regulated, particularly that of the uterine surface epithelium which is in direct contact with luminal fluids. In addition, the presence of these factors in uterine tissues and fluids suggests that their localization at the attachment site or release into the uterine lumen may represent paracrine mechanisms through which conceptus development is regulated (Brigstock *et al.*, 1994).

EGF occurs at high concentrations (1–1000 ng ml⁻¹) in a variety of body fluids (see Carpenter and Wahl, 1991). Accordingly these fluids are a significant reservoir of EGF and are probably an important means by which EGF is delivered to its target cells *in vivo*. Unfortunately, the lack of homologous probes has severely hampered the study of EGF in the body fluids of pigs. While early studies showed that an antiserum to mouse EGF decreased the stromal cell mitogenic activity of crude pig uterine luminal flushings (Simmen *et al.*, 1988), only with the cloning and expression of the 6 kDa pig EGF (pEGF) molecule (Pascall *et al.*, 1991) was it possible to develop an

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antisera to pEGF for use in a radioimmunoassay that provided clear evidence for the presence of immunoreactive EGF in pig uterine fluids (Vaughan *et al.*, 1992; Diehl *et al.*, 1994). Although additional studies using the reverse-transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry have verified uterine pEGF expression and production (Kennedy *et al.*, 1994), no studies have addressed the molecular structure of the active ligand. Since members of the EGF family are initially synthesized as large precursor proteins that become embedded in the plasma membrane from which the mature growth factor is released by extracellular proteolytic cleavage (Prigent and Lemoine, 1992), there are numerous points in the biosynthesis and processing of EGF that may serve to regulate its bioavailability and specific activity. With the recognition that the extracellular regulation of growth factor structure and function is a key component of growth factor activity and mode of action *in vivo* (Flaumenhaft and Rifkin, 1992), we have performed a structural and functional analysis of EGF proteins in pig uterine luminal fluids.

Materials and Methods

Animals

Uteri were obtained at random from nonpregnant prepubertal and pubertal pigs of mixed breed of up to about 8 months old from a local abattoir. Uteri were transported on ice to the laboratory where the broad ligament was removed. Uterine luminal fluid was collected by allowing each uterine horn to drain under gravity. Uterine horns were then flushed with PBS as described by Kim *et al.* (1995) and Brigstock *et al.* (1996). Fluid and flushings were either used immediately or stored at -20°C . Samples were centrifuged at $13\,500\text{ g}$ for 30 min, and the supernatant passed through glass wool before analysis.

BioRex cation-exchange and heparin-Sepharose affinity chromatography

Multiple cationic heparin-binding growth factors are present in pig uterine secretions (Brigstock *et al.*, 1989, 1996; Kim *et al.*, 1995). These factors were removed by subjecting the uterine fluid and flushings to cation-exchange chromatography or heparin-affinity chromatography or both processes. Flushings were passed at 2.5 ml min^{-1} through a BioRex 70 cation exchange column ($2 \times 2.5\text{ cm}$; Bio-Rad Labs, Richmond, CA) that had been pre-equilibrated with $10\text{ mmol Tris-HCl l}^{-1}$, $0.1\text{ mol NaCl l}^{-1}$ (pH 7.4). The flow-through fraction was then passed at 2 ml min^{-1} through a heparin-Sepharose column ($1.5 \times 2.7\text{ cm}$; Pharmacia Biotech, Pisectaway, NJ) that had been pre-equilibrated with $10\text{ mmol Tris-HCl l}^{-1}$, $0.1\text{ mol NaCl l}^{-1}$ (pH 7.4). Uterine fluid was subjected directly to heparin-affinity chromatography after either being diluted tenfold in $10\text{ mmol Tris-HCl l}^{-1}$ (pH 7.4) or mixed with one-tenth volume of $100\text{ mmol Tris-HCl l}^{-1}$ (pH 7.4). The flow-through fraction from the flushings and fluid samples, containing non-heparin-binding substances, was selected for further analysis.

DEAE anion exchange chromatography

The flow-through fraction from heparin-affinity chromatography of uterine flushings was applied at 2 ml min^{-1} to a DEAE Sepharose CL-6B column ($2.5 \times 3.5\text{ cm}$; Pharmacia Biotech) that had been pre-equilibrated with $10\text{ mmol Tris-HCl l}^{-1}$, $0.1\text{ mol NaCl l}^{-1}$ (pH 7.4). After application of the sample, the column was washed with 150 ml of equilibration buffer and bound proteins were eluted using a 100 ml gradient of $0.1\text{--}2\text{ mol NaCl l}^{-1}$ in $10\text{ mmol Tris-HCl l}^{-1}$ (pH 7.4). The eluate was collected in fractions that were then tested for their ability to stimulate DNA synthesis in quiescent cultures of Balb/c 3T3 cells and to inhibit cellular binding of ^{125}I -labelled EGF. Uterine fluid was processed similarly except that the pH of the sample and buffers was adjusted to 8.5 for anion-exchange chromatography to facilitate binding of growth factor to the DEAE-Sepharose column. The active eluate was re-adjusted to pH 7.4 before further anion-exchange chromatography.

EconoPac Q and Mono Q anion exchange chromatography of uterine flushings

Active fractions from seven, 11, or 15 individual DEAE purifications of uterine flushings were pooled, diluted three- to fourfold in $20\text{ mmol Tris-HCl l}^{-1}$ (pH 7.4), and pumped onto an EconoPac Q column ($0.7 \times 3.6\text{ cm}$; Bio-Rad Labs) at 2 ml min^{-1} . The column was then washed with $20\text{ mmol Tris-HCl l}^{-1}$, $0.2\text{ mol NaCl l}^{-1}$ and developed with a 50 ml gradient of $0.2\text{--}1.0\text{ mol NaCl l}^{-1}$ in $20\text{ mmol Tris-HCl l}^{-1}$ (pH 7.4) using a fast protein liquid chromatography (FPLC) system (Pharmacia Biotech). Fractions were collected during gradient formation and assayed. Active fractions from each EconoPac Q purification were pooled, diluted sevenfold in $20\text{ mmol Tris-HCl l}^{-1}$ (pH 7.4), filtered, and applied to a Mono Q HR 5/5 FPLC column (Pharmacia Biotech) that had been equilibrated with $20\text{ mmol Tris-HCl l}^{-1}$, $0.1\text{ mol NaCl l}^{-1}$ (pH 7.4). Bound proteins were eluted using a 40 ml gradient of $0.1\text{--}0.75\text{ mol NaCl l}^{-1}$ in $20\text{ mmol Tris-HCl l}^{-1}$ (pH 7.4), and collected into $160\text{--}250\text{ }\mu\text{l}$ fractions. Active fractions were subsequently assayed and subjected to SDS-PAGE and western blotting.

HiLoad Q anion exchange chromatography of uterine fluid

Active fractions from two individual DEAE purifications of uterine fluid were pooled (60 ml) and diluted to 300 ml with $20\text{ mmol Tris-HCl l}^{-1}$ (pH 7.4). The sample was then applied at 4 ml min^{-1} to a HiLoad Q Sepharose FPLC column ($10 \times 26\text{ cm}$; Pharmacia Biotech) that had been pre-equilibrated with $500\text{ ml } 20\text{ mmol Tris-HCl l}^{-1}$, $0.1\text{ mol NaCl l}^{-1}$ (pH 7.4). The column was washed with 60 ml of equilibration buffer and bound proteins were eluted at 4 ml min^{-1} using a 320 ml gradient of $0.1\text{--}1.0\text{ mol NaCl l}^{-1}$ in $20\text{ mmol Tris-HCl l}^{-1}$ (pH 7.4). The eluate was collected into 4 ml fractions.

Gel filtration chromatography

Active fractions from EconoPac Q purification of uterine flushings or from HiLoad Q purification of uterine fluid were

pooled, passed through a 0.45 μm membrane, and concentrated to 0.45–1.5 ml using 5000–10 000 M_r cut-off membranes (Amicon, Beverly, MA and Alltech Associates, Deerfield, IL). Each concentrated sample was then filtered through a 0.2 μm Acrodisc filter (Gelman Sciences, Ann Arbor, MI) and applied to a G2000 SW gel filtration column (0.8 $\times$ 30 cm, 1000–100 000 M_r fractionation range; TosoHaas, Woburn MA) that was equipped with a SW guard column (0.8 $\times$ 4 cm; TosoHaas). Proteins were eluted from the column with PBS containing 0.5 mol NaCl l^{-1} . The flow rate was 0.5 ml min^{-1} and fractions of 200 μl were collected. The column was calibrated with mouse EGF (M_r 6000), lactalbumin (14 200), trypsin inhibitor (20 100) and ovalbumin (45 000).

DNA synthesis and EGF radioreceptor assay

Column fractions were tested for their ability to stimulate [^3H]thymidine incorporation into the DNA of confluent quiescent Balb/c 3T3 cells that had been cultured in 96-well plates for 7–14 days in Dulbecco's Modified Eagles Medium containing 10% calf serum (200 μl per well) as described by Besner *et al.* (1990) and Kim *et al.* (1995). Aliquots from column fractions were also tested in an EGF radioreceptor assay (RRA) developed in this laboratory using HEC-1-B human endometrial carcinoma cells (Besner *et al.*, 1992; Kim *et al.*, 1995). Briefly, cells were grown in 48-well plates and then incubated in 150 μl of binding medium containing 0.5 ng ^{125}I -labelled mouse EGF ml^{-1} (^{125}I -EGF; DuPont NEN, Boston, MA) for 2 h at 4°C in the presence or absence of the test samples. Nonspecific ^{125}I -labelled EGF binding was determined in the presence of 500 ng unlabelled EGF ml^{-1} . DNA synthesis and EGF radioreceptor assays were performed as single determinations for screening column fractions and as single or duplicate determinations for generating dose-response curves, with the data computed as means $\pm$ SD.

SDS-PAGE and western blotting

Active fractions from Mono Q chromatography were subjected to SDS-PAGE under reducing conditions using 18% polyacrylamide gels. Successive fractions (205 μl) were combined as pairs (410 μl total volume) and diluted to 1.5 ml with 10 mmol Tris-HCl l^{-1} (pH 7.5). Samples were desalted and concentrated by passage through 10 μl beds of DEAE-Sepharose which were then washed in 10 mmol Tris-HCl l^{-1} (pH 7.5) and extracted using 35 μl SDS-PAGE sample buffer. Fifteen microlitres of each sample was loaded into the lanes of duplicate gels. After electrophoresis, proteins in the gels were transferred to nitrocellulose which was then blocked using 10 mmol Tris-HCl l^{-1} (pH 7.4) containing 0.1% BSA and 0.15 mol NaCl l^{-1} . Blots were subsequently incubated overnight at 4°C in this solution containing either 1:1000 dilution of normal rabbit serum or 1:1000 dilution of a rabbit antiserum raised against 6 kDa recombinant pEGF (Vaughan *et al.*, 1992). This antiserum was kindly donated by K. D. Brown (Babraham Institute, Cambridge, UK). Immune complexes were visualized using alkaline phosphatase-conjugated anti-rabbit IgG followed by 5-bromo-4-chloro-3-indonyl phosphate and nitroblue

Fig. 1. Stimulation of 3T3 cell DNA synthesis by pig uterine flushings or fluid. 3T3 cell DNA synthesis dose-response curves to (●) uterine fluid (40 ml total volume from 10 uteri) or (○) flushings (500 ml from 20 uteri) before chromatography and after sequential passage of the uterine flushings through (△) a BioRex-70 cation exchange column and (□) a heparin-Sepharose column. Values for [^3H]thymidine incorporation at each dose are single determinations (●) or mean $\pm$ SD of duplicate determinations (○, △, □).

tetrazolium chloride as described (Kim *et al.*, 1995; Brigstock *et al.*, 1996).

Results

Uterine flushings and fluid stimulated 3T3 cell DNA synthesis in a dose-dependent manner (Fig. 1). To establish if this mitogenic activity was due entirely to platelet-derived growth factor (PDGF), FGFs, HB-EGF or pleiotrophin (all of which are present in uterine luminal flushings (Brigstock *et al.*, 1989, 1996; Kim *et al.*, 1995)), samples were passed through BioRex 70 resin or heparin-Sepharose beads since both of these matrices are known to strongly bind all of these growth factors. Uterine flushings retained mitogenic activity after passage through both types of column (Fig. 1), suggesting that one or more anionic, non-heparin-binding mitogens were present in the partially purified sample. Similar data were obtained for uterine fluid (data not shown). The anionic property of the residual mitogenic activity was then verified by DEAE-anion exchange chromatography. Treatment of the DEAE column with a salt gradient after application of partially purified flushings or fluid resulted in the elution of a peak of mitogenic activity by, respectively, 0.3–0.6 mol NaCl l^{-1} (Fig. 2a) or 0.3–0.8 mol NaCl l^{-1} (Fig. 2b). EGF is a highly anionic growth factor that stimulates 3T3 cell mitosis, does not bind to heparin (Besner *et al.*, 1990), and is present in the uterine tract of pigs (Vaughan *et al.*, 1992; Diehl *et al.*, 1994; Kennedy *et al.*, 1994); therefore, an EGF radioreceptor assay of fractions from the DEAE column was performed using HEC-1-B cells. A competitor of ^{125}I -labelled EGF binding was eluted from DEAE columns over the same NaCl concentration range as 3T3 cell mitogenic activity (Fig. 2a, b).

The anionic growth factor from uterine flushings was further purified by subjecting active fractions from multiple DEAE-Sepharose column purifications to EconoPac Q anion exchange chromatography. A typical chromatogram is shown (Fig. 3a),

Fig. 2. DEAE-anion exchange chromatography of pig uterine flushings and fluid. (a) Uterine flushings (900 ml) from 29 pigs were subjected to BioRex-70 and heparin-affinity chromatography. The flow-through fraction was then applied at 2 ml min^{-1} to a DEAE-Sephacrose column that was then washed with 150 ml $10 \text{ mmol Tris-HCl l}^{-1}$, $0.1 \text{ mol NaCl l}^{-1}$ and developed with 100 ml of $0.1\text{--}2 \text{ mol NaCl l}^{-1}$ in $10 \text{ mmol Tris-HCl (pH 7.4)}$. Seventy-five fractions (approx 1.3 ml each) were collected and tested at $7 \mu\text{l}$ per well for their stimulation of 3T3 cell DNA synthesis ($\bullet$) and at $15 \mu\text{l}$ per well for their inhibition of ^{125}I -labelled epidermal growth factor (EGF) binding ($\circ$). (b) Filtered uterine fluid (160 ml) was diluted to 1.5 l with $10 \text{ mmol Tris-HCl l}^{-1}$, $0.1 \text{ mol NaCl l}^{-1}$ ($\text{pH } 7.4$) and passed through a heparin-affinity column. The flow-through fraction was then applied to a DEAE-Sephacrose column that was washed and developed as described in (a) above. Sixty-two fractions (approximately 1.6 ml each) were collected and tested at $10 \mu\text{l}$ per well in the 3T3 cell DNA synthesis assay ($\bullet$) and in the ^{125}I -labelled EGF radioreceptor assay ($\circ$).

and demonstrates the elution of 3T3 cell mitogenic activity as two peaks, one by $0.2\text{--}0.3 \text{ mol NaCl l}^{-1}$ and a second, smaller peak by $0.35\text{--}0.4 \text{ mol NaCl l}^{-1}$. Since active fractions from the first peak also inhibited ^{125}I -labelled EGF binding (Fig. 3a), they were concentrated and further purified by gel filtration chromatography. Peaks of mitogenic activity and ^{125}I -labelled EGF competition co-eluted in fractions corresponding to M_r 45 000 (Fig. 3b). No evidence for a 6 kDa form of EGF was obtained.

The presence of high molecular mass forms of EGF in the first EconoPac Q peak was verified by further purification of the corresponding fractions from a different EconoPac Q purification by Mono Q chromatography and analysis by SDS-PAGE and western blotting. Mitogenic activity was eluted from the Mono Q column by $0.3 \text{ mol NaCl l}^{-1}$ (Fig. 3c) and was correlated with the presence of several proteins of $M_r \sim 25\ 000$ that were detected by a pEGF-specific rabbit antiserum but not by normal rabbit serum (Fig. 3c, inset). Immunoreactive EGF proteins of 6 kDa were not detectable in the active fractions (Fig. 3c). Recombinant 6 kDa mouse and human EGF were subjected to SDS-PAGE under identical conditions to ensure that 6 kDa EGF migrated as predicted under the SDS-PAGE conditions used in this study, with the result that they both migrated close to a 6.5 kDa aprotinin standard (data not shown). Further analysis of the second mitogenic peak from EconoPac Q chromatography of uterine flushings was not possible in view of the low amounts of activity that were recovered (Fig. 3a). In addition, western blot analysis of the gel filtration fractions of high molecular mass were inconclusive, also owing to low recoveries of the bioactive protein(s).

Uterine fluid was of higher specific activity than uterine flushings (Fig. 1) and contained a similar growth factor activity (Fig. 2); therefore, subsequent experiments were conducted with fluid samples. When DEAE-purified uterine fluid was subjected to large-scale HiLoad Q anion exchange chromatography, two mitogenic peaks were eluted from the column, both of which were correlated with individual peaks of competition for ^{125}I -labelled EGF binding (Fig. 4a). These peaks, which were eluted by $0.4 \text{ mol NaCl l}^{-1}$ (Peak 1) and $0.45 \text{ mol NaCl l}^{-1}$ (Peak 2), were consistently observed in several HiLoad Q purifications. Subsequent analysis of Peak 1 by gel filtration under dissociating, non-reducing conditions showed that its mitogenic and ^{125}I -labelled EGF-competing properties co-eluted in fractions corresponding to M_r 3000–6000 (Fig. 4b). However, under the same conditions, Peak 2 was separated into moieties of two molecular masses corresponding to M_r 3000–6000 and 40 000–70 000 (Fig. 4c). Although further purification of these various EGF-like proteins was achieved using C_8 reverse-phase high performance liquid chromatography (data not shown), their structural characterization was unsuccessful due to the continued presence of contaminating proteins.

Discussion

Although EGF-like factors have been detected in uterine luminal fluids of mice and rats by radioreceptor assay or radioimmunoassay (Imai 1982; DiAugustine *et al.*, 1988), the molecular size of the secreted EGF protein(s) has not been reported for these species and has been hampered by the lack of fluid available for analysis. A uterine secretory EGF-like factor in pigs was first demonstrated by Simmen *et al.* (1988), who showed that the mitogenic activity of uterine flushings was partially decreased by an antiserum to mouse EGF. Although immunoreactive EGF has since been detected in the pig uterus, oviduct and uterine flushings by homologous and heterologous radioimmunoassays or immunohistochemistry

(Vaughan *et al.*, 1992; Diehl *et al.*, 1994; Kennedy *et al.*, 1994; Swanchara *et al.*, 1995), these reports have not addressed qualitative aspects of the uterine luminal EGF protein. While our data confirm the presence of non-heparin-binding, anionic, EGF-like growth factors in uterine flushings and fluid, they further demonstrate their occurrence as high molecular mass as well as 6 kDa proteins. The structural relationship between the 45 kDa biologically active protein observed by gel filtration and the 25 kDa immunoreactive EGF proteins observed by

SDS-PAGE in uterine flushings has not yet been established. For example, (i) the different molecular masses may be the result of the same molecule migrating at different rates under the non-reducing dissociating conditions used in gel filtration as opposed to the reducing and denaturing conditions used in SDS-PAGE; (ii) the gel filtration data may reflect the presence of a carrier protein for the 25 kDa form of EGF that was dissociated under the conditions of SDS-PAGE or (iii) distinct molecular forms of EGF may have been detected by each technique. Whatever the explanation, the data show that uterine secretions contain biologically active forms of EGF that are of appreciably higher molecular mass than mature EGF.

Since the 3–6 kDa factor in uterine fluid was not characterized by western blotting, the possibility that it is a low molecular mass ligand for the EGF receptor (EGF-R) that is distinct from EGF itself cannot be excluded. One such molecule would be TGF- α which is produced by the pig uterus (Kennedy *et al.*, 1994) and is of M_r 6000 in its mature form (Carpenter and Wahl, 1991). Alternatively, the reactivity of the high molecular mass proteins with a highly specific pig EGF antiserum (Vaughan *et al.*, 1992) coupled with their lack of heparin-binding supports the notion that these are genuine forms of EGF rather than other members of the EGF family such as TGF- α , HB-EGF or amphiregulin. Although the basis for the mass of these proteins requires further study, possibilities such as complexes of EGF with itself or other proteins seem unlikely given the dissociating or denaturing conditions used in gel filtration or SDS-PAGE. However, an alternative

Fig. 3. Characterization of anionic growth factors in pig uterine flushings. (a) Active fractions from 15 individual DEAE purifications of uterine flushings (approximately 12 l starting material from 383 pigs) were pooled (200 ml total volume), diluted to 800 ml with 20 mmol Tris-HCl l⁻¹ (pH 7.4), and applied as two 400 ml samples to an EconoPac Q column. In each run, the column was washed with 20 mmol Tris-HCl l⁻¹, 0.2 mol NaCl l⁻¹ (pH 7.4) and developed with a 50 ml gradient of 0.2–1.0 mol NaCl l⁻¹. Fifty fractions (1 ml each) were collected and tested at 7 µl per well in the 3T3 cell DNA synthesis assay (●) and at 15 µl per well in the ¹²⁵I-labelled epidermal growth factor (EGF) binding assay (○). (b) Fractions 7–12 from the two EconoPac Q runs described in (a) above were pooled (12 ml), and concentrated to 450 µl using a 5000- M_r cut-off membrane. The sample was then injected on to a G2000 SW column that was then washed with PBS/0.5 mol NaCl l⁻¹ at 0.5 ml min⁻¹. One hundred and three fractions (200 µl each) were collected and tested at 5 µl per well for stimulation of 3T3 cell DNA synthesis (●) and at 10 µl per well for inhibition of ¹²⁵I-labelled EGF binding (○). (c) Fractions 7–12 from two EconoPac Q purifications, obtained from 18 samples of uterine flushings as described in (a) above, were pooled, diluted and applied to a Mono Q FPLC column that was subsequently washed with 0.1 mol NaCl l⁻¹ in 20 mmol Tris-HCl l⁻¹ (pH 7.4). Bound proteins were then eluted at 0.5 ml min⁻¹ using a 40 ml gradient of 0.1–0.75 mol NaCl l⁻¹ in 20 mmol Tris-HCl l⁻¹ (pH 7.4) and collected into fractions of 250 µl. The figure shows the 3T3 cell mitogenic activity of the fractions when assayed at 15 µl per well. The inset shows a western blot of paired mitogenic fractions using 1:1000 dilutions of pig EGF antiserum or normal rabbit serum. Lanes A and K contained pre-stained molecular mass standards. Lanes B, C, D, E, F, G, H, I, J, L, M, N and O contained fractions 40–41, 42–43, 44–45, 46–47, 48–49, 50–51, 52–53, 54–55, 56–57, 58–59, 60–61, 62–63 and 64–65, respectively.

explanation is the incomplete processing of the EGF precursor. While the pig EGF precursor has been only partially characterized (Pascall *et al.*, 1991), that of other species is initially synthesized as a ~ 1200 -residue transmembrane protein from which mature 6 kDa EGF is released by extracellular proteolytic cleavage. Despite this well characterized processing

mechanism, size heterogeneity has nevertheless been documented for human and rat EGF. Human urine was shown to contain a high molecular mass form of EGF that contained 28 kDa and 33 kDa components after SDS-PAGE under reducing conditions (Hirata and Orth, 1979). Analysis of multiple human body fluids by gel filtration and radioimmunoassay further showed that urine, milk, seminal plasma, saliva, tears, amniotic fluid and breast sweat contained high molecular mass forms of EGF of M_r 20 000, 70 000, 150 000 and ≥ 300 000 (Pesonen *et al.*, 1987). Whereas the N-terminus of mature 6 kDa human EGF commences at Asn971 of the EGF precursor, structural characterization of human urinary 30–33 kDa EGF proteins showed that their N-termini commenced at Met828 or Val829 of the precursor molecule (Mount *et al.*, 1987; Tsukumo *et al.*, 1987). Similarly by comparison with the mouse EGF precursor, the N-terminus of a biologically active 45 kDa form of rat urinary EGF was shown to start at Asn837 (Nexo *et al.*, 1990). More recently human milk and urine have been shown by western blotting to contain a 160–170 kDa form of the EGF precursor that was biologically active as shown by its ability to stimulate tyrosine phosphorylation of the EGF receptor (Mroczkowski and Reich, 1993). The secreted 170 kDa EGF precursor was shown to bind to heparin (Mroczkowski and Reich, 1993), whereas 6 kDa EGF (Besner *et al.*, 1990) and the EGF proteins of high molecular mass detected in this study do not. In addition, 33 kDa urinary human EGF had 26% of the receptor-binding activity as 6 kDa human EGF (Hirata and Orth, 1979). These data suggest that the additional amino acid sequence in EGF molecules of high molecular mass may serve to regulate their bioavailability or biological activity. Although the factors that lead to production of high molecular mass forms of EGF in the uterus have yet to be defined, it may be relevant that the pig uterus produces protease inhibitors (Fazleabas *et al.*, 1982; Roberts and Bazer, 1988) that may result in incomplete processing of the EGF precursor.

The presence and binding properties of pig endometrial EGF-R were initially described by Zhang *et al.* (1992a). More recently, RT-PCR was used to show that mRNAs encoding EGF and EGF-R are present in pig endometrium on days 4, 13, 15 and 20 of the oestrous cycle and on days 10, 12, 14, 18 and 22 of pregnancy (Kennedy *et al.*, 1994). In addition, using the same anti-pEGF antiserum with which we performed western

Fig. 4. Characterization of anionic growth factors in pig uterine fluid. (a) Active fractions from individual DEAE-Sepharose purifications of two uterine fluid samples (100 ml fluid each) were pooled, diluted 1:6 in 20 mmol Tris-HCl l⁻¹ (pH 7.4) (300 ml final volume), and applied to a HiLoad Q FPLC column at 4 ml min⁻¹. The column was washed with 60 ml of 20 mmol Tris-HCl l⁻¹, 0.1 mol NaCl l⁻¹ (pH 7.4) and bound proteins were eluted at 4 ml min⁻¹ using a 320 ml gradient of 0.1–1.0 mol NaCl l⁻¹ in 20 mmol Tris-HCl l⁻¹ (pH 7.4). Ninety-five fractions (4 ml each) were collected. Selected fractions were tested at 15 µl per well for their stimulation of 3T3 cell DNA synthesis (●). The ¹²⁵I-labelled epidermal growth factor (EGF) binding data (○) were generated from a comparable run in which fractions were assayed at 10 µl per well. Active fractions from (b) Peak 1 or (c) Peak 2 from six HiLoad Q purifications were pooled, concentrated, and applied to a G2000 SW gel filtration column as described in Fig. 3b. Fractions were tested at 3 µl per well in the DNA synthesis (●) and EGF radioreceptor (○) assays.

blotting, these investigators demonstrated that EGF immunoreactivity was detected primarily in the glandular epithelial cells of endometrium from pregnant and non-pregnant animals (Kennedy *et al.*, 1994). Moreover, their immunohistochemical data provided evidence for a membrane-associated form of EGF, with apparently higher amounts of EGF on the luminal surfaces. Although the EGF-R and its ligands are steroidally regulated in the uteri of other species (Brigstock, 1991), further research is required to clarify the role of oestrogen and progesterone in regulating mRNAs encoding EGF and EGF-R and protein content in the pig uterus. In addition, since our studies were conducted using uterine fluids and flushings from prepubertal pigs as well as pigs at random stages of the cycle, it is yet to be established whether the production of high molecular mass forms of EGF is associated with the stage of maturity of the uterus, or stage of the oestrous cycle or pregnancy. Although it has been inferred that the pattern of mRNA expression of EGF-R and EGF does not support a primary role for these factors in early pig embryonic development (Kennedy *et al.*, 1994), the results reported here suggest that further studies are required at the protein level to address this point. While this report focussed on non-pregnant animals to prevent contamination of uterine fluids by embryo-derived EGF, embryonic development and survival may be regulated by extrinsic uterine EGF, either by its secretion into the uterine lumen or by its localization at the implantation site (Brigstock *et al.*, 1989, 1994; Pollard, 1990; Brigstock, 1991; Pollard *et al.*, 1991; Simmen and Simmen, 1991; Cullingford and Pollard, 1994). EGF-R in extra-embryonic and placental membranes (Corps *et al.*, 1990; Zhang *et al.*, 1992b) are possible targets for uterine secretory EGF, and this role for uterine secretory EGF may persist for relatively long periods in the pig as this species exhibits non-invasive placentation (Roberts and Bazer, 1988).

In summary, the data presented here suggest that uterine fluids contain variably processed forms of the EGF precursor that bind and activate the EGF receptor. Since body fluids have been proposed as an important reservoir and delivery system for EGF (Carpenter and Wahl, 1991), further biological and structural characterization of uterine secretory EGF of high molecular mass and 6 kDa EGF should prove fruitful for understanding their respective functions in the female reproductive tract.

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