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Polypeptide growth factors in uterine tissues and secretions*

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Introduction

It has often been speculated that uterine secretions (histotrophe) are involved in regulating the attachment, implantation, nutrition and growth of the conceptus (Corner, 1921; Amoroso, 1952; Roberts & Bazer, 1988; Biggers, 1988). However, the factors responsible and the mechanisms involved have not been clearly defined. Work over the past decade has firmly established the involvement of peptide and polypeptide growth factors in the control of animal cell proliferation (see Evered *et al.*, 1985). These results raise the intriguing possibility that uterus-derived growth factors may play a role in regulating the growth of the conceptus. This could occur via a paracrine pathway involving the secretion into the uterine lumen of factors which, on binding to specific cellular receptors, would directly stimulate DNA synthesis and division in cells of the developing conceptus. Alternatively, the factors could affect aspects of uterine cell proliferation (e.g. angiogenesis and vascularization) which may be important for conceptus attachment, implantation or nutrition. Recent evidence indicates that polypeptide growth factors are indeed present in the uterus and its secretions. Several such factors have been identified and, at least in some cases, synthesis of the factor appears to be regulated by steroid hormones (Table 1).

Epidermal growth factor (EGF)

EGF is a 53-residue acidic polypeptide which is a potent mitogen for a variety of cell types *in vivo* and *in vitro* (see Carpenter & Cohen, 1979). In the female mouse, the highest concentrations (30–50 ng/mg) of EGF are found in the submaxillary glands (Byyny *et al.*, 1972; Rall *et al.*, 1985). Gonzalez *et al.* (1984) detected EGF by radioimmunoassay in the uteri of 16-day-old mice although the concentrations were only about 0·3% of those in the submaxillary gland. EGF concentrations were unaffected by a single injection of oestradiol-17β but a daily injection of the steroid for 7 days produced a 5-fold increase in uterine EGF concentration. EGF values in submaxillary gland and liver were not affected by the treatment. Imai (1982) reported that an EGF-like factor is present in the uterine fluid of rats. At 20 days after the subcutaneous injection of 2 mg oestradiol valerate, EGF concentrations in uterine fluid were 3-fold higher than in untreated animals. Moreover, there was a 24-fold increase in total EGF secretion since oestradiol administration resulted in an 8-fold increase in uterine fluid volume. Submaxillary gland EGF concentrations were significantly reduced in oestradiol-treated animals and there was also a decrease in gland weight. Urinary excretion of EGF from treated animals was not changed compared to untreated controls. DiAugustine *et al.* (1988) have reported that low levels of EGF mRNA were detectable in the uteri

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of immature mice. The concentrations increased at least 2-fold after oestrogen treatment and immunoreactive EGF was present in the uterine fluid of the treated animals. Taken together, these results suggest that oestrogens act locally to stimulate EGF synthesis in the uterus of mice or rats.

The uterus itself may be a target for EGF action since high-affinity receptors for the factor have been identified by incubating ^{125}I-labelled EGF with membrane fractions of rat uterus (Mukku & Stancel, 1985a) or human endometrium (Taketani & Mizuno, 1985) or with homogenates of human endometrium and myometrium (Hofmann *et al.*, 1984). Furthermore, administration of oestradiol-17β to female rats produced a 3-fold increase in ^{125}I-labelled EGF binding apparently due to increased de-novo synthesis of the EGF receptor (Mukku & Stancel, 1985b). Similarly, Taketani & Mizuno (1985) reported that EGF binding to primary cultures of human endometrium was increased after addition of oestradiol to the culture medium. A possible role for EGF in uterine growth is suggested by the finding that EGF stimulates proliferation of rabbit endometrial cells in culture (Gerschenson *et al.*, 1979).

Table 1. Polypeptide growth factors in uterine tissues and fluids

Growth factor	Uterus	Uterine fluid	Species	Steroid regulation
Epidermal growth factor	+	+	Mouse/rat	E
Transforming growth factor	+	NT	Rat	NT
Insulin-like growth factor I	+	NT	Rat	E
[IGF binding protein(s)]	+	+	Human	P
Colony-stimulation factor 1	+	NT	Mouse	E and P
Oestromedins	+	+	Rat/sheep	E
Uterine luminal fluid mitogen(s)	NT	+	Pig	NT
Fibroblast growth factors (acidic and basic forms)	+	(+)	Pig/human	NT

E, oestrogen increases factor concentration.
P, progesterone increases factor concentration.
NT, not tested.

The mouse fetus and placenta appear not to express EGF (Popliker *et al.*, 1987) yet EGF receptors are abundant on mouse or human placental membranes (Hock & Hollenberg, 1980; Carson *et al.*, 1983; Magid *et al.*, 1985; Smith & Talamantes, 1986). EGF receptors are also present in mouse extra-embryonic tissues (e.g. amnion) and in several organs of the embryo (Adamson *et al.*, 1981).

Transforming growth factor-α (TGF-α)

TGF-α is a 50-residue polypeptide which shows 30% sequence homology to EGF (see Derynck, 1986). TGF-α exerts its biological effects by binding to the EGF receptor (Massague, 1983) and, to date, there has been no convincing demonstration of cellular receptor populations which discriminate between TGF-α and EGF. Since receptors with high affinity for EGF are found in the mouse placenta and embryo, but there is an apparent absence of EGF expression in these tissues (see above), it was proposed that TGF-α (rather than EGF itself) is the true ligand for these receptors. Indeed, evidence for the presence of TGF-α in mouse embryos was obtained using radioimmunoassay and bioassays with a peak of activity observed in Day-7 embryos (Twardzik, 1985). In addition, Lee *et al.* (1985) showed that Day-8 rat embryos contained significant amounts of TGF-α mRNA. However, recently, the same group have used Northern blot and in-situ hybridization to re-examine their earlier findings after more meticulous separation of embryonic and maternal

tissues. They found (Han *et al.*, 1987) that TGF-α mRNA expression occurs primarily in the maternal decidua and not in the embryo or placenta. Expression is detectable by Day 7, peaks on Days 8–9 and has fallen to a low level by Day 15 as the decidua is resorbed. The function(s) of the TGF-α produced during this period is not known, but since EGF/TGF-α receptors are present in the placenta and embryo it is possible that TGF-α produced by the decidua may have a paracrine action on these tissues.

Insulin-like growth factor-I

IGF-I is a 70-residue single-chain polypeptide with approximately 45% sequence homology with insulin (see Blundell & Humbel, 1980). *In vitro*, IGF-I alone is often only a weak stimulator of cell proliferation but the factor markedly potentiates the activity of other mitogens such as EGF or platelet-derived growth factor (Corps & Brown, 1988). *In vivo*, IGF-I appears to be particularly important as the principal cellular mediator of the stimulatory action of growth hormone on longitudinal skeletal growth (see Van Wyk, 1984). Circulating concentrations of IGF-I are largely controlled by the action of growth hormone on IGF-I expression in liver (Roberts *et al.*, 1986; Mathews *et al.*, 1986), although other tissues are able to synthesize much smaller quantities of IGF-I mRNA (Mathews *et al.*, 1986; Murphy *et al.*, 1987a).

Murphy *et al.* (1987a) reported that, liver apart, uterine expression of IGF-I mRNA was higher than in any other tissue tested. A single intraperitoneal injection of oestradiol-17β in pituitary-intact ovariectomized prepubertal rats resulted in a 2-fold increase in uterine IGF-I values within 6 h of hormone treatment with no change in serum IGF-I concentration (Murphy *et al.*, 1987b). A 20-fold increase in the abundance of three major IGF-I mRNA transcripts in uterine tissue was also observed after oestradiol treatment although IGF-I expression in liver and kidney was unchanged (Murphy *et al.*, 1987b; Murphy & Friesen, 1988). These results indicate that oestrogen specifically induces the expression of IGF-I in uterine cells although which cells express the IGF-I has not yet been defined. It remains to be determined whether IGF-I is secreted in uterine fluid although it has been demonstrated that an IGF-binding protein is a major secretory protein of human decidualized endometrium (see Bell, 1988).

Colony-stimulating factor-1

Bradley *et al.* (1971) and Rosendaal (1975) showed that extracts from mouse uteri during the second part of gestation possessed 50-times the activity of extracts from non-pregnant uteri or other tissues in an assay which measured colony growth of cultured mouse bone marrow cells. This activity has now been shown to be due to colony-stimulating factor-1 (CSF-1), a glycosylated homodimeric polypeptide required for the survival, proliferation and differentiation of cells of the mononuclear phagocyte lineage (see Stanley *et al.*, 1983). Specific radioimmunoassay has demonstrated that, by Day 5 of gestation, CSF-1 concentrations in mouse uteri are 5-fold higher than on the day of mating (Bartocci *et al.*, 1986). By term this increase has risen to 1000-fold and the CSF-1 content of the uterus far exceeds that of other tissues (Bartocci *et al.*, 1986). Pollard *et al.* (1987) used Northern blot analysis to demonstrate large increases in CSF-1 mRNA in the uteri of pregnant mice, indicating that the increase in uterine CSF-1 was due to local synthesis rather than uptake from the circulation.

Bartocci *et al.* (1986) showed that the concentration of mouse uterine CSF-1 was increased by treating the animals with human chorionic gonadotrophin (hCG) and that this increase was abolished in ovariectomized animals. This suggested that the effect of hCG on CSF-1 production was mediated by ovarian hormones. A role for progesterone was indicated by the inhibition of uterine CSF-1 production by the anti-progestagen RU 486 (Pollard *et al.*, 1987). Furthermore,

uterine CSF-1 concentrations were regulated by the synergistic action of oestradiol-17β and progesterone although the hormone treatment did not increase serum CSF-1. In-situ hybridization studies using a CSF-1 cRNA probe showed that CSF-1 mRNA is principally synthesized in the columnar cells of the endometrial glandular epithelium of pregnant uteri. Since the mouse placenta has been shown to express the CSF-1 receptor mRNA (Muller *et al.*, 1983), these findings suggest that CSF-1, synthesized and secreted by the uterus in response to steroid hormones, has a role in regulating placental growth. This proposal is supported by the finding that CSF-1 stimulates DNA synthesis in cells of fetal origin derived from mouse placenta (Athanassakis *et al.*, 1987).

Oestromedins

The growth of various mammary, pituitary and kidney tumours is oestrogen-dependent but, because no direct mitogenic effect is seen when oestrogens are added directly to cultures of cells from these tumours, it has been proposed that oestrogens induce growth factors *in vivo* which are then responsible for tumour formation (see Sirbasku, 1978). Uterus, kidney and pituitary have been investigated for the presence of oestrogen-induced tumour-forming growth factor or 'oestromedin' (Sirbasku, 1978; Sirbasku *et al.*, 1981; Ikeda *et al.*, 1982). An oestromedin was isolated from sheep uteri by a 5-step procedure which yielded a single Coomassie blue-stained band of apparent M_r 4200 on SDS–polyacrylamide gels (Ikeda & Sirbasku, 1984). The preparation was reported to show a marked specificity in stimulating oestrogen-responsive tumour cell lines (Ikeda & Sirbasku, 1984).

A preliminary report from Simmen *et al.* (1986) has indicated that pig uterine luminal fluid contains a mitogen for a kidney-derived epithelial cell line. Levels of activity were increased in the fluid of oestrogen-primed sows. The mitogenic activity was eluted from a Sephadex G-100 column with an apparent M_r of 86 000 which is unusually high compared with other known growth factors. Further purification and amino acid sequence analysis of both oestromedin and the uterine luminal fluid mitogen will be necessary before it will be possible to assess fully their relationship to other growth factors.

Heparin-binding growth factors (HBGFs)

We have previously shown that extracts of pig endometrium, obtained from pregnant or non-pregnant animals, produced a dose-dependent stimulation of DNA synthesis in Swiss mouse 3T3 cells (Brigstock *et al.*, 1983). Vascular perfusion of the uterine horns with saline at autopsy did not reduce the level of biological activity in the extract, indicating that the active component(s) was present in the tissue rather than derived from blood contamination (Fig. 1). Partial purification (~2000-fold) indicated that the activity was due to the presence of a growth factor with properties that were clearly different from those of EGF, IGF-I or platelet-derived growth factor. The properties of the uterus-derived growth factor (UDGF) suggested a similarity with the acidic and basic fibroblast growth factors (FGFs) previously purified from bovine brain and pituitary respectively by a complex multi-chromatographic process (Bohlen *et al.*, 1984; Thomas *et al.*, 1984). FGFs stimulate the proliferation of a wide variety of mesoderm-derived cell types in culture (see Gospodarowicz *et al.*, 1987). Much current interest in FGFs centres on their actions as potent angiogenic agents which stimulate vascular endothelial cells in culture, and neovascularization *in vivo* (see Folkman & Klagsbrun, 1987).

Purification of acidic and basic FGF was simplified considerably by the observation that the factors have a remarkably high affinity for heparin, allowing heparin–agarose affinity chromatography to be used as a key step in their isolation (Gospodarowicz *et al.*, 1984; Lobb & Fett, 1984). When the partly purified UDGF was applied to a column containing heparin–agarose, it was found

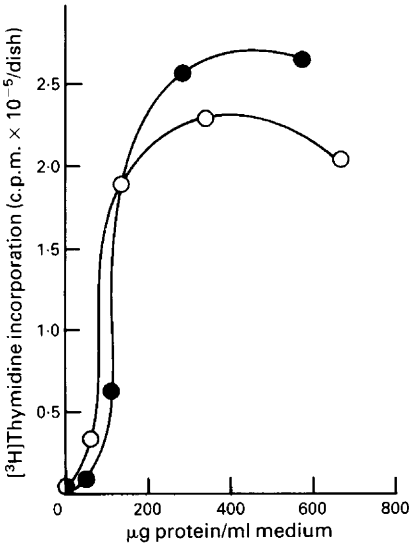

Fig. 1. Pig endometrial extract stimulates DNA synthesis in Swiss mouse 3T3 cells. A gilt was killed on Day 14 of pregnancy, the uterus was removed and one horn was immediately perfused with sterile saline (0·15 M-NaCl). Samples of endometrial tissue from the perfused (●) and non-perfused (○) horns were homogenized (Polytron) in Dulbecco's modified Eagles' medium (DMEM: Flow Laboratories). The extract was clarified by centrifugation and tested for its ability to stimulate [^{3}H]thymidine incorporation. Values are means for duplicate determinations for separate dishes of cells.

that the biological activity bound to the column and a concentration of >1 M-NaCl was required for elution. Because of the similarity between the uterus-derived activity and the FGFs, the procedures of Gospodarowicz *et al.* (1984), Lobb & Fett (1984), and Gimenez-Gallego *et al.* (1985) for the purification of acidic FGF and basic FGF from bovine brain, pituitary or hypothalamus were adapted for the purification of UDGF. The essential features of this purification are ammonium sulphate precipitation of the uterine extract, followed by cation-exchange chromatography over CM-Sephadex followed by a heparin–agarose affinity step. In the presence of 0·5 M-NaCl, FGFs are retained on a heparin–agarose column whilst other factors such as EGF or PDGF do not bind (Shing *et al.*, 1984). The heparin-binding proteins can then be eluted from the column by using a salt gradient.

The biologically active fractions obtained from CM-Sephadex chromatography of a uterine extract prepared from a pig on Day 17 of pregnancy were applied to a column containing heparin–agarose. Most (90%) of the detectable protein passed straight through the column or was eluted as a large protein peak when the column was washed with buffer containing 0·5 M-NaCl: 2 peaks of growth factor activity were eluted by the salt gradient. One peak of activity, named α-uterus-derived growth factor (α-UDGF) was eluted in approximately 1·3 M-NaCl whilst the second peak of activity, named β-UDGF, was eluted later between 1·5 and 1·8 M-NaCl. The salt concentrations at which α- and β-UDGF were eluted from the heparin–agarose column are characteristic of those required for elution of acidic and basic FGF respectively (see Folkman & Klagsbrun, 1987).

Immunoblotting of the heparin-purified β-UDGF preparation, using antisera raised against basic FGF, identified a doublet of immunoreactive proteins at M_r 17 700 and 17 200 (Fig. 2). The protein doublet was purified to homogeneity by h.p.l.c. and subjected to amino-terminal sequence analysis. Up to 4 different residues were identified in each of the 40 degradative cycles, and after 10 cycles it became apparent that the residues obtained in each cycle fitted a model of 4 microheterogeneous forms of the same polypeptide sequence whose amino-terminal residues were at positions

1, 3, 7 and 11. When the sequences of these 4 proteins were aligned to give a β-UDGF consensus sequence, it was evident that 27 of the first 30 amino-terminal residues were identical to those of bovine basic FGF (Brigstock, 1988). Although the remaining 3 residues could not be ascribed unequivocally, these results strongly indicate that β-UDGF is basic FGF of the pig. It is not known whether microheterogeneity of the β-UDGF is artefactually introduced during processing and/or storage of the samples, but amino-terminal cleavage has frequently been observed in basic FGF preparations (Baird *et al.*, 1985a, 1986; Gospodarowicz *et al.*, 1985, 1986; Klagsbrun *et al.*, 1987).

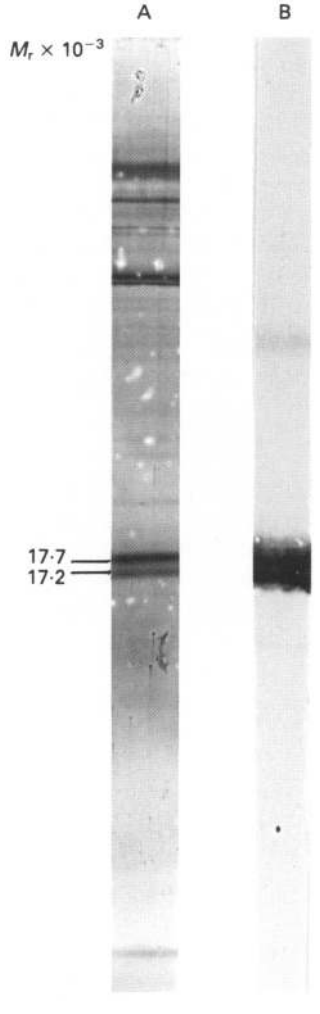

Fig. 2. An antiserum to bovine basic FGF detects a protein doublet in heparin-purified β-UDGF. Heparin-purified β-UDGF was electrophoresed on a 15% polyacrylamide gel from which a strip was removed and silver-stained (A). Proteins from a duplicate strip of gel were electrophoretically transferred to nitrocellulose (Glenney, 1986) and probed using an antiserum raised against a synthetic peptide corresponding to residues 106–120 of the bovine basic FGF sequence (B). Immunoreactive proteins were visualized by successive incubations with biotinylated goat anti-rabbit IgG, a pre-formed streptavidin–biotin–horseradish peroxidase complex and diaminodenzidine.

Immunoblotting of the heparin-purified α-UDGF preparation, using antiserum raised against acidic FGF, identified an immunoreactive protein at an M_r of 16 500 which is very similar to the M_r of acidic FGF on polyacrylamide gels (Lobb & Fett, 1984). This protein was not reactive with anti-basic FGF. The elution position of the protein of M_r 16 500 from heparin–agarose and reversed-phase h.p.l.c. columns was closely correlated with the presence of biological activity in the fractions. These results indicate that α-UDGF is related to the acidic form of FGF. Amino acid sequence analysis will be required to establish whether α-UDGF is in fact the acidic FGF molecule of the pig.

Uterine flushings were demonstrated to contain high levels of 3T3 cell growth-promoting activity (Fig. 3). Therefore, it was of interest to determine whether HBGFs were present in the flushings. When flushings from gilts between Days 15 and 19 of pregnancy were subjected to heparin–agarose affinity chromatography, 2 peaks of cell-growth promoting activity were eluted by a salt gradient (Fig. 4). One activity peak was eluted by 0·75–1 M-NaCl (Fractions 7–11) and a second active peak was eluted by 1·6–1·8 M-NaCl (Fractions 18–21). The earlier eluting active fractions contained two proteins (M_r 21 000 and 22 000) which reacted (on immunoblots) with an antiserum against acidic FGF but not with an antiserum to basic FGF (Fig. 5). In marked contrast, the second peak of biological activity (1·6–1·8 M-NaCl) contained 3 proteins (M_r 17 000–18 500) which reacted with anti-basic FGF but not with anti-acidic FGF (Fig. 5). Although several other lightly-staining protein bands were also visible on the blots, their elution profile did not correspond with the elution profile of biological activity (compare Figs 4 & 5). Nevertheless, further experiments will be required to investigate the structure and activity of the FGF-immunoreactive proteins in the flushings.

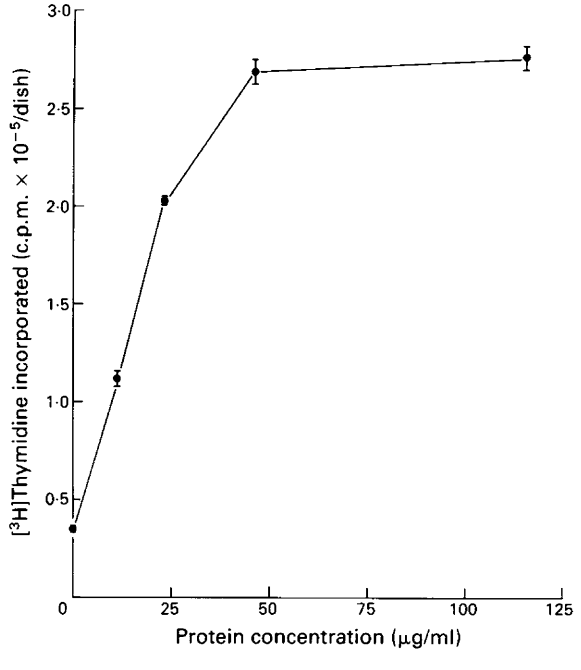

Fig. 3. Cell growth-promoting activity in pig uterine flushings. Uterine horns were separated and individually flushed using ~20 ml cold DMEM. The fluid was clarified by centrifugation and tested for its ability to stimulate [^{3}H]thymidine incorporation into quiescent cultures of Swiss mouse 3T3 cells. Values are mean ± s.e.m. for 3 determinations from separate dishes of cells.

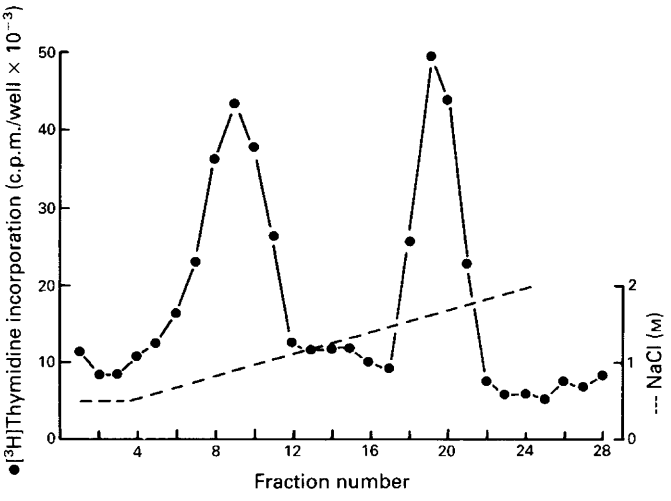

Fig. 4. Heparin–agarose affinity chromatography of pig uterine flushings. The uterine horns from twelve 15- to 19-day-pregnant gilts were flushed with cold DMEM. The flushings were separated from blastocyst tissue by decantation and clarified by centrifugation. Sodium chloride was added to the supernatant to a final concentration of 0·5 M and the solution was applied to a heparin–agarose affinity column. The column was washed with 20 mM-Tris–HCl (pH 8·0) containing 0·5 M-NaCl and then developed using a 0·5–2·0 M-NaCl gradient in the same buffer (broken line). The ability of the collected fractions to stimulate [^{3}H]thymidine incorporation into quiescent cultures of Swiss mouse 3T3 cells was tested (●).

Taken together, these results indicate that pig uterine tissues, and uterine flushings from early pregnant gilts contain acidic and basic HBGFs. The basic factors from the tissues and fluids were very similar and probably represent microheterogeneous forms of basic FGF which is known to be present in a number of animal tissues (see Baird *et al.*, 1986; Gospodarowicz *et al.*, 1987). In contrast, the acidic FGF-immunoreactive proteins in flushings were clearly different (higher M_r and earlier elution from heparin–agarose) from that extracted from uterine tissue. Until recently, the acidic form of FGF was thought to be restricted to neural tissues, having been detected only in brain and retina (Lobb & Fett, 1984; Thomas *et al.*, 1984; Bohlen *et al.*, 1985; Baird *et al.*, 1985b). However, acidic FGF has now been purified and sequenced from human prostate (Crabb *et al.*, 1986) and bovine kidney (Gautschi-Sova *et al.*, 1987). These findings, together with our detection of an acidic FGF-related factor in pig uterus, suggest that the tissue distribution of acidic HBGFs is wider than originally supposed.

The question of FGF secretion is interesting and somewhat controversial because neither basic nor acidic forms contain classical signal peptides (Jaye *et al.*, 1986; Abraham *et al.*, 1986a, b). The question of whether the factors we have detected in the uterine flushings are secreted from the uterus therefore requires careful consideration. It is possible that the proteins are of embryonic origin or leaked from uterine cells which were damaged during the flushing procedure. It has been demonstrated (Baird & Ling, 1987; Vlodavsky *et al.*, 1987a) that FGFs become deposited in the extracellular matrix produced by cultured endothelial cells. When incorporated into the extracellular matrix, FGFs are probably maintained in a stable but functionally inactive state by binding to heparin sulphate which constitutes 90% of the side chains of subendothelial glycosaminoglycans of the extracellular matrix (see Gospodarowicz *et al.*, 1987; Vlodavsky *et al.*, 1987a, b). Sequestration of FGFs in this manner might be an important mechanism by which FGF activity is tightly controlled (see Folkman & Klagsbrun, 1987). Mobilization of FGFs from the extracellular matrix is effected by the action of heparin sulphate degrading enzymes such as heparanase, heparinase, or heparitinase (Baird & Ling, 1987) and this presumably enables FGFs to interact

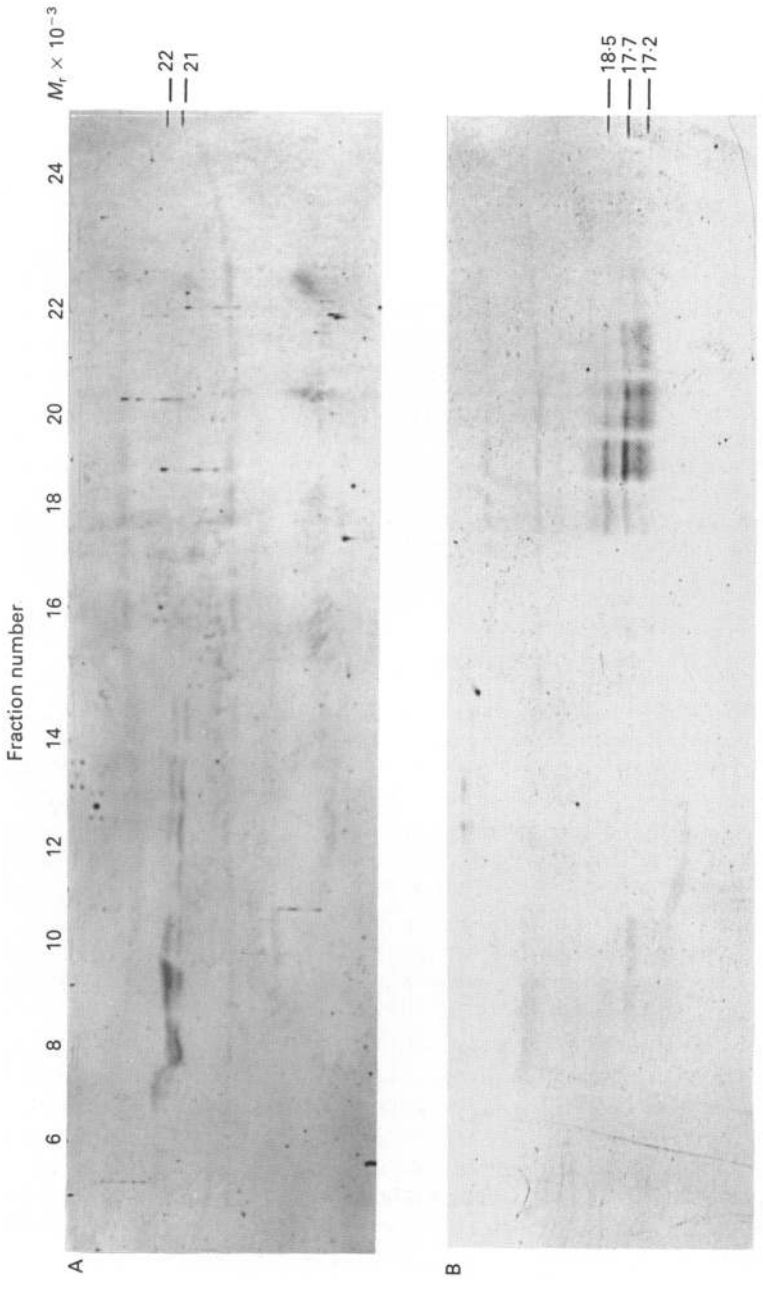

Fig. 5. Immunoblotting of fractions from heparin–agarose chromatography of pig uterine flushings. The fractions which were eluted from the heparin–agarose column (Fig. 4) between 0·5 and 2·0 M-NaCl were individually desalted and concentrated. Portions of each fraction were then electrophoresed on two identical polyacrylamide gels and the separated proteins were electrophoretically transferred to nitrocellulose sheets. One blot (A) was probed with an antiserum raised against a synthetic peptide corresponding to amino acid residues 1–15 of bovine acidic FGF. The other blot (B) was probed with an antiserum raised against a peptide corresponding to amino acid residues 106–120 of bovine basic FGF. The molecular weights ($\times 10^{-3}$) are indicated for the immunoreactive proteins for which the elution profile from the heparin–agarose column correlated with the elution profile of biological activity.

with their cellular receptors. If FGFs are present in uterine extracellular matrix they may be released by uterus- or conceptus-derived degrading enzymes of the extracellular matrix.

Summary and perspectives

In this report we have reviewed what is known about the occurrence of polypeptide growth factors in uterine tissues and fluids. To date, there is no evidence for a uterus-specific growth factor with unique functions. However, a substantial body of data indicates that various well-characterized growth factors, previously isolated from other tissues or cells, are detectable at significant concentrations in the uterus. At least in some cases, uterine synthesis of the factors has been demonstrated and evidence has been obtained indicating a role for steroid hormones in the control of this synthesis. Increased availability of growth factors, their antisera and cDNA probes will undoubtedly generate further, more detailed, studies of the growth factors present in the uterine environment. However, the evidence for a functional role of these factors in uterine growth and/or conceptus development, although compelling in some cases (e.g. CSF-1), remains circumstantial. There is currently intensive effort in pharmaceutical companies to produce peptide antagonists with an ability to inhibit growth factor binding and activity. If specific antagonists are successfully produced, they may provide valuable tools which could be used to study more directly the function of polypeptide mitogens in uterine physiology.

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