DNA synthesis by ovine mammary alveolar epithelial cells: effects of heparin, epidermal growth factor-related peptides and interaction with stage of pregnancy

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Abstract

Amphiregulin is a heparin-binding member of the epidermal growth factor (EGF) family, which we have recently shown to be expressed in sheep mammary gland. Uniquely among known EGF-like growth factors, its mitogenic activity is inhibited by soluble heparin, but heparin-like molecules on the cell surface and/or in extracellular matrix appear to be necessary for amphiregulin to exert its biological effect. In primary cultures of sheep mammary alveolar epithelial cells, heparin (1–20 mg/l) inhibited DNA synthesis in a dose-dependent manner. The extent of the inhibition was influenced by physiological state, being greater ($P < 0.05$) in mammary cell cultures derived from 5- to 10-week pregnant sheep ($63.1 \pm 8.2\%$, mean $\pm$ S.E.M., $n=8$) than in cultures derived from sheep which were non-pregnant ($35.8 \pm 8.3\%$ inhibition, $n=6$) or late, 20-week, pregnant ($39.8 \pm 5.6\%$,

$n=6$). Both EGF and transforming growth factor- α (TGF- α) significantly ($P < 0.001$) increased DNA synthesis in the presence of heparin. The effect of TGF- α was dose-related, wholly reversing the inhibitory effect of heparin in cell cultures from non-pregnant and 20-week pregnant sheep. DNA synthesis was stimulated by amphiregulin and TGF- α increased the maximum response. The heparin antagonist, hexadimethrine, inhibited DNA synthesis, but, in the presence of amphiregulin, approximately equivalent concentrations of heparin overcame this inhibitory effect. In the presence of heparin, TGF- α showed synergistic interactions with insulin or IGF-I. The results indicate interactive effects of EGF and IGF growth factor families in sheep mammary growth.

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Introduction

Endocrine control of postnatal mammary gland growth is now known to involve the mediation of locally-produced growth factors, acting as autocrine, juxtacrine and paracrine regulators of cell proliferation. An increasing number of growth factors produced by and acting on mammary cells is being identified and an understanding of how these endogenous factors may interact becomes important. Synthesis of DNA in sheep mammary epithelial cells is stimulated by insulin-like growth factor (IGF) (Winder *et al.* 1989) and by transforming growth factor- α (TGF- α) (Moorby *et al.* 1995). We now investigate amphiregulin, another member of the epidermal growth factor (EGF) family expressed in the normal sheep mammary gland (Forsyth *et al.* 1997), and interaction among the IGF and EGF growth factor families. A preliminary report has been made of some of this work (Forsyth & Moorby 1993).

Amphiregulin, a glycoprotein of 84 amino acids, or 78 in a truncated form, is derived from a 252 amino acid transmembrane precursor. Amphiregulin was originally

isolated by Shoyab *et al.* (1988, 1989) from medium conditioned by the MCF-7 human mammary carcinoma cell line, treated with a phorbol ester. It is expressed in normal mammary gland of man (four strains of normal passaged cells, Li *et al.* 1992, nontransformed, immortalized cells, Kenney *et al.* 1993), mouse (Kenney *et al.* 1995) and sheep (Forsyth *et al.* 1997), as well as in human ovary, placenta (Plowman *et al.* 1990, Johnson *et al.* 1991) and keratinocytes (Cook *et al.* 1991).

Amphiregulin shares with EGF and TGF- α the ability to interact with the EGF (ErbB-1) receptor (Shoyab *et al.* 1989). Unlike EGF and TGF- α , amphiregulin binds heparin, a property conferred by a highly basic 40 amino acid N-terminal extension (Thorne & Plowman 1994). Several studies show that soluble heparin and other sulphated glycosaminoglycans attenuate amphiregulin-dependent mitotic activity in keratinocytes (Cook *et al.* 1991, Piepkorn *et al.* 1994) and in human mammary epithelial cells (Li *et al.* 1992). It is thought that this results from prevention of the interaction of amphiregulin with high-affinity EGF receptors. By contrast, the biological

activity of other members of the EGF family is either unaffected (EGF and TGF- α) or enhanced (heparin-binding EGF, Cook *et al.* 1995a) by heparin. Nevertheless, further studies indicate that extracellular heparan sulphate glycosaminoglycans are necessary for amphiregulin mitogenic signalling in MCF-10A cells (Johnson & Wong 1994) and keratinocyte cell lines (Cook *et al.* 1995b). By using heparin and the heparin antagonist, hexadimethrine (Kimura *et al.* 1959) in primary cultures, we have attempted to assess the physiological significance of endogenous amphiregulin in stimulating DNA synthesis in sheep mammary gland and to study interaction among members of the EGF and IGF growth factor families.

Materials and Methods

Materials

Human TGF- α , human EGF and human IGF-I are recombinant peptides, purchased from Bachem (UK) Ltd (Saffron Walden, Essex, UK). Recombinant human amphiregulin was from R&D Systems Europe (Abingdon, Oxon, UK). Sigma (Poole, Dorset, UK) supplied bovine insulin, heparin (tissue culture tested) from porcine intestinal mucosa, hexadimethrine bromide and powdered Medium 199, with Earle's salts and without phenol red. Medium 199 was prepared in double-distilled water and supplemented with Hepes (25 mmol/l), L-glutamine (0.68 mmol/l), anhydrous sodium acetate (5 mmol/l), sodium bicarbonate (1.1 g/l), BSA fraction V (5 g/l), kanamycin monosulphate (128 mg/l) and gentamycin sulphate (10 mg/l) as described by Moorby *et al.* (1995). [Methyl- ^3H]Thymidine (185 GBq/mmol) and ([^{125}I]iodotyrosyl) TGF- α (human recombinant) were from Amersham International plc (Little Chalfont, Bucks, UK). Hexadimethrine is a polymeric polycation (polybrene) found to neutralize the effects of heparin *in vivo* and *in vitro* (Kimura *et al.* 1959). It inhibits the mitogenic effects of amphiregulin on keratinocytes, without any apparent toxic effects (Cook *et al.* 1995b).

Animals

Welsh Mountain ewes were kept under standard husbandry conditions. They were non-pregnant, 5-, 10-, 15- or 20-weeks pregnant (average pregnancy about 22 weeks) or had suckled lambs for 10 weeks. Sheep were killed by an intravenous overdose of Lethobarb (Duphar, Southampton, Hants, UK). Udder tissue was immediately removed under sterile conditions and used for isolation by collagenase/hyaluronidase digestion of clumps of mammary epithelial cells (Winder *et al.* 1992).

Cell culture

Ovine alveolar mammary epithelial cells were cultured under serum-free conditions on gels of rat tail collagen as

described by Winder *et al.* (1992). Cell clumps attached overnight to the collagen in the presence of fibronectin (Sigma, 4 $\mu\text{g}/\text{ml}$). Treatments, replicated 3 or 4 times, began on the following day and the medium was changed every 24 h. Cultures were terminated at subconfluence to avoid detachment of gels (Winder *et al.* 1992). This was on day 3 for cultures from 15- to 20-week pregnant sheep and days 4–5 for other stages. Synthesis of DNA was determined by incorporation of [^3H]methyl thymidine added for the last 24 h of culture (Winder *et al.* 1989). Concentration of DNA was measured using 4% (w/v) 3,5-diaminobenzoic acid (Hinegardner 1971) and results expressed as d.p.m./ μg DNA. Unless stated otherwise, n =number of independent experiments each carried out on cells prepared from a different sheep. To reduce the resulting variance, some data have been normalised by expression as percentage of DNA synthesis by cells cultured in medium only, or were $\log_{10}$ transformed for analysis (Tables 1 and 2).

Binding of TGF- α to mammary microsomes

Microsomes prepared from mammary tissue of non-pregnant, pregnant and lactating sheep were pooled as described by Moorby *et al.* (1995). Binding assays were carried out in triplicate by the method of Moorby *et al.* (1995) using the pooled microsomes at 300 μg protein per tube and 20 000 c.p.m. ^{125}I -labelled TGF- α in a total volume of 400 μl . Heparin was added at 0, 0.1, 0.5, 1.0, 1.5 and 2.0 $\mu\text{g}/\text{tube}$.

Statistical analysis

The significance of differences in response was tested either by Student's *t*-test or by analysis of variance (ANOVA).

Results

Effect of heparin on DNA synthesis

Heparin inhibits EGF-independent growth of human mammary epithelial cells, an effect attributed to prevention of interaction between endogenous amphiregulin and the EGF receptor (Li *et al.* 1992). Heparin (1–20 mg/l) similarly inhibited DNA synthesis by sheep mammary alveolar epithelial cells in serum and growth factor-free medium, with a maximum effect at 5–10 mg/l (Fig. 1). The amount of DNA per well was reduced from 0.86 ± 0.14 $\mu\text{g}/\text{well}$ (mean $\pm$ s.e.m.) in medium to 0.53 ± 0.08 $\mu\text{g}/\text{well}$ ($n=29$, $P<0.001$, paired Student's *t*-test).

If heparin inhibition reflects a role for endogenous amphiregulin in mammary growth, then the greatest effect may be expected in cells derived from pregnant sheep. In

Figure 1 Effect of heparin on DNA synthesis ($[^3\text{H}]$ methyl thymidine incorporation, d.p.m./ μg DNA) by sheep mammary alveolar epithelial cells grown on collagen gels. Cells were prepared from a non-pregnant (●), 5-week pregnant (■) or 20-week pregnant (▲) sheep. Values are means $\pm$ S.E.M. of four replicates.

Figure 2 Effect of physiological state on the response of DNA synthesis to heparin (10 mg/l) in cultured sheep mammary alveolar epithelial cells. Values (means $\pm$ S.E.M.) are DNA synthesis (%) compared with control cultures without heparin. The number of sheep contributing to each observation is shown within each bar. * $P < 0.05$ compared with non-pregnant and 20-week pregnant sheep.

mammary cells from sheep 5- to 10-weeks pregnant, DNA synthesis was reduced to 35% of control values (Fig. 2; 65% inhibition). This was significantly ($P < 0.05$, Student's *t*-test) different from the reduction to about 60% of control (40% inhibition) when cells were derived from

non-pregnant or late, 20-week, pregnant sheep. In mammary cells from 15-week pregnant sheep, the effect of heparin was intermediate. Cells from lactating sheep behaved similarly to those from non-pregnant sheep.

Effects of TGF- α or EGF on heparin inhibition

We have previously shown that TGF- α approximately doubles DNA synthesis by sheep mammary epithelial cells. EGF displaces ^{125}I -TGF- α from sheep mammary microsomes, but shows no consistent effect on DNA synthesis (Moorby *et al.* 1995). As heparin does not inhibit the biological activity of TGF- α or of EGF (Cook *et al.* 1995b), their ability to reverse the inhibitory effect of heparin on DNA synthesis was tested.

Heparin was first shown to have no effect on binding of TGF- α to pooled sheep mammary microsomes. Binding of ^{125}I -labelled TGF- α was 9.6%. On addition of heparin, 0.1, 0.5, 1.0, 1.5 or 2 $\mu\text{g}/\text{tube}$, (final concentration 0.25–5 mg/l), binding was 9.3, 9.6, 10.0, 9.8 and 9.4% respectively. The effect of TGF- α (0.1, 1.0, 10 or 100 $\mu\text{g}/\text{l}$) or EGF (1, 10 and 100 $\mu\text{g}/\text{l}$) on DNA synthesis was tested in the presence and absence of heparin (10 mg/l) in mammary cells derived from sheep ($n=5$ at each stage) which were non-pregnant, 10-, 15- or 20-weeks pregnant (Tables 1 and 2). Heparin significantly ($P < 0.001$) inhibited DNA synthesis and there were significant interactions of pregnancy stage with heparin treatment (see also Fig. 2). There was a significant ($P < 0.001$) dose-related effect of TGF- α on DNA synthesis in both the presence and absence of heparin, but there was no interaction between pregnancy stage and response to TGF- α , in confirmation of previous results (Moorby *et al.* 1995). At 10 $\mu\text{g}/\text{l}$ (Fig. 3) and 100 $\mu\text{g}/\text{l}$, TGF- α was able completely to overcome the inhibitory effect of heparin in cells from non-pregnant and 20-week pregnant ewes.

EGF significantly increased DNA synthesis in the presence and absence of heparin (Table 2, $P < 0.001$), but the effect was smaller and not clearly dose-related compared with TGF- α .

Effect of amphiregulin and interaction with TGF- α

To determine if amphiregulin is a sheep mammary gland mitogen, its effect on DNA synthesis was tested in epithelial cells from 20-week pregnant sheep. TGF- α was more potent (Fig. 4a), but maximum DNA synthesis was the same in response to TGF- α and to amphiregulin. Surprisingly, a maximally effective dose of TGF- α (10 $\mu\text{g}/\text{l}$) significantly ($P < 0.02$, paired Student's *t*-test) increased the response to amphiregulin (1 mg/l) (4330 ± 323 vs 8224 ± 842 d.p.m./ μg DNA, $n=4$).

Effects of hexadimethrine

The heparin antagonist, hexadimethrine, is selective in its effects on the mitogenic activity of EGF-related growth

Table 1 Effect of TGF- α on the synthesis of DNA by sheep mammary alveolar cells without (–) or with (+) heparin (10 mg/l). Results (means) are shown (upper panel) normalized as % of control DNA synthesis by cells in medium only. Control DNA synthesis (d.p.m./ μ g DNA, mean $\pm$ s.e.m.) at 0, 10, 15 and 20 weeks of pregnancy was 18 913 $\pm$ 12 161, 1552 $\pm$ 426, 5318 $\pm$ 2302 and 8504 $\pm$ 3986 respectively. Cells derived from five sheep were studied at each stage of pregnancy. The significance of differences was calculated (lower panel) by ANOVA using values for DNA synthesis expressed as d.p.m./ μ g DNA, and log₁₀ transformed to reduce variance

	Stages of pregnancy (weeks)							
	0		10		15		20	
	–	+	–	+	–	+	–	+
Dose of TGF- α (μ g/l)								
0	100	69.8	100	44.3	100	46.5	100	61.6
0.1	118.3	78.6	137.2	45.7	144.9	62.4	131.1	94.0
1.0	133.8	121.9	223.2	101.2	201.6	95.5	153.6	135.5
10	154.7	132.7	292.4	139.3	250.3	123.7	179.5	185.2
100	168.1	155.6	309.8	123.8	197.9	116.7	165.0	154.1

	Degrees of freedom	P value
Source of variation		
Dose of TGF- α	4	<0.001
Stage of pregnancy	3	<0.05
Heparin	1	<0.001
Dose $\times$ stage	12	>0.1
Stage $\times$ heparin	3	<0.001

Table 2 Effect of EGF on the synthesis of DNA by sheep mammary alveolar cells without (–) or with (+) heparin (10 mg/l). Results (means) are shown (upper panel) normalized as % of control DNA synthesis by cells in medium only. For values at 100%, see Table 1. Cells derived from five sheep were studied at each stage of pregnancy. The significance of differences was calculated (lower panel) by ANOVA using values for DNA synthesis expressed as d.p.m./ μ g DNA, and log₁₀ transformed to reduce variance

	Stages of pregnancy (weeks)							
	0		10		15		20	
	–	+	–	+	–	+	–	+
Dose of EGF (μ g/l)								
0	100	69.8	100	44.3	100	46.5	100	61.1
1	124.0	97.2	187.8	61.5	152.2	76.1	139.7	133.3
10	122.9	123.7	208.9	72.2	162.2	87.0	129.0	129.7
100	118.8	84.7	147.7	75.1	180.5	78.2	124.5	101.3

	Degrees of freedom	P value
Source of variation		
Dose of EGF	3	<0.001
Stage of pregnancy	3	<0.05
Heparin	1	<0.001
Dose $\times$ stage	9	>0.1
Stage $\times$ heparin	3	<0.01

factors, inhibiting amphiregulin-induced DNA synthesis by keratinocytes, but enhancing or having no effect on the activity of EGF (Cook *et al.* 1995*b*). The suggested mechanism is disruption of interaction between amphiregulin and cell surface sulphated proteoglycans. In alveolar epithelial cells from 20-week pregnant sheep, hexadimethrine

was inhibitory at doses of 1 mg/l (57.5 $\pm$ 0.6% of DNA synthesis in medium only, mean $\pm$ s.e.m., *n*=3) and 10 mg/l (22.7 $\pm$ 5.2%) in the presence of amphiregulin (500 μ g/l, *P*<0.01, paired Student's *t*-test). Similar inhibition was obtained in basal medium (51.3% and 21.2% respectively). Heparin was able to reverse the inhibitory

Figure 3 Effects of heparin (10 mg/l, open bars), heparin+TGF- α (10 µg/l, hatched bars) and TGF- α (solid bars) on DNA synthesis by cultured mammary alveolar epithelial cells from sheep which were non-pregnant or 10-, 15- or 20-weeks pregnant. Values are means $\pm$ S.E.M. for cells from five sheep, expressed as a percentage of [3 H]methyl thymidine incorporation by cells cultured in medium only in each experiment.

effect of hexadimethrine on amphiregulin-stimulated DNA synthesis, although heparin became inhibitory again when present in excess (Fig. 5).

Effect of TGF- α on the response of DNA synthesis to insulin or IGF-I: influence of heparin

The dose-dependent effect of both insulin (Fig. 4b) and IGF-I (Moorby *et al.* 1995) is enhanced by TGF- α . Cultures were carried out in the absence and presence of heparin to test statistically whether these interactions are additive or synergistic, using maximally effective doses of TGF- α (10 µg/l), insulin (100 µg/l) and IGF-I (10 µg/l). The ratio was calculated: (response to insulin or IGF-I in the presence of TGF- α) to (response to insulin or IGF-I plus response to TGF- α) (Moorby *et al.* 1995). The ratio was corrected for the double contribution of basal incorporation in the denominator, using experimentally determined values in the presence or absence of heparin in each culture. A ratio greater than 1.0 indicates a synergistic interaction. The results are shown in Fig. 6a (insulin) and 6b (IGF-I). The ratio is significantly affected by heparin ($P < 0.05$, ANOVA) for both the interaction between insulin and TGF- α and IGF-I and TGF- α . The effect of pregnancy stage was not significant, but the highest mean ratios were in tissue from 10-week pregnant sheep in the presence of heparin (4.45 for insulin and 3.02 for IGF-I, Fig. 6a,b).

The overall mean ratios for interaction of insulin and TGF- α were 1.44 ± 0.64 (S.D.) in the absence and

Figure 4 Response of DNA synthesis to (a) TGF- α (open bars) or amphiregulin (hatched bars), $n=3$, and (b) insulin (○) or insulin+TGF- α (●, 10 µg/l), $n=6$, in mammary alveolar epithelial cells from 15- to 20-week pregnant sheep. Values are means $\pm$ S.E.M. * $P < 0.05$ compared with medium only.

2.96 ± 2.94 in the presence of heparin, both significantly different from 1.0 ($P < 0.01$, degrees of freedom 18). For interaction of IGF-I and TGF- α , the ratios were 1.15 ± 0.56 ($P > 0.1$, degrees of freedom 19) without heparin and 2.09 ± 1.80 ($P < 0.02$, degrees of freedom 19) in the presence of heparin respectively.

Figure 5 Effect of amphiregulin (500 µg/l)+hexadimethrine (5 mg/l) in the presence of increasing amounts of heparin (0–20 mg/l) on DNA synthesis (d.p.m./µg DNA) in mammary alveolar epithelial cells from a 20-week pregnant sheep (mean ± S.E.M. of 3 replicates). The dose–response to amphiregulin in cells from the same sheep is also shown. The experiment was repeated in cells derived from two more sheep with similar results.

Discussion

Local synthesis of growth factors plays an important role in mammary growth. Expression of both the insulin-like growth factor and epidermal growth factor families has been reported in mammary gland (see Forsyth 1996, for references) and both are mitogens for mammary epithelial cells. In ruminants, IGF-I and IGF-II are potent mitogens (Shamay *et al.* 1988, Baumrucker & Stemberger 1989, Winder *et al.* 1989, McGrath *et al.* 1991, Peri *et al.* 1992) thought to act via the type 1 IGF receptor and stimulating a 5- to 10-fold increase in DNA synthesis. Amphiregulin and TGF- α , members of the EGF family, approximately double DNA synthesis (Fig. 4a, Zurfluh *et al.* 1990, Woodward *et al.* 1994, Moorby *et al.* 1995). In primary cultures of sheep mammary gland (Moorby *et al.* 1995) and in the MAC-T bovine mammary epithelial cell line (Woodward *et al.* 1994), EGF itself is reported to be without effect. Expression of EGF is not known so far in any ruminant tissue, although EGF is an effective competitor for TGF- α binding to sheep mammary microsomes (Moorby *et al.* 1995). The mouse mammary gland expresses at least four EGF-like peptides, EGF, TGF- α , amphiregulin and cripto-1 (Kenney *et al.* 1995), but their respective roles have still to be established. Multiple expression of the EGF family may also occur in ruminants, as the bovine mammary gland expresses TGF- α (Zurfluh *et al.* 1990) and the sheep amphiregulin (Forsyth *et al.* 1997).

Amphiregulin and TGF- α belong to the group of EGF agonists which bind to the EGF (ErbB-1) receptor (Shoyab *et al.* 1989, Cook *et al.* 1991, Beerli & Hynes 1996). There are four known members of the type 1/ErbB family of receptor tyrosine kinases. Ligand binding induces receptor dimerization and it is now known that heterodimers as well as homodimers can be formed (Beerli & Hynes 1996). It might have been anticipated that human recombinant amphiregulin would be a competitive inhibi-

tor of TGF- α given its low potency on sheep mammary epithelial cells (Fig. 4a). However, the maximum response to TGF- α could be increased by amphiregulin. Alternative methods of intracellular signalling not shown by TGF- α have been proposed for amphiregulin, including interaction with defective EGF receptors lacking tyrosine kinase activity and targeting of amphiregulin to the nucleus (Cook *et al.* 1995b, Modrell *et al.* 1992). In addition, several studies now indicate that amphiregulin must associate with extracellular matrix to exhibit biological activity, a property shared with some members of the fibroblast growth factor family (Cook *et al.* 1995b). A part of the evidence for this has come from studying the effect of the heparin antagonist, hexadimethrine, a polymeric polycation. Hexadimethrine inhibits the mitogenic activity of amphiregulin in two mouse keratinocyte cell lines. It appears to affect the interaction between amphiregulin and cellular heparin-like glycosaminoglycans which facilitate interaction with an EGF receptor, an effect that can be overcome by equivalent concentrations of heparin (Cook *et al.* 1995b). Essentially similar effects of hexadimethrine have been obtained with sheep mammary epithelial cells in the present study.

Investigation of growth factors acting via the EGF receptor in ruminant mammary tissue is limited by the lack of availability of cross-reacting antibodies with anti-biological activity. We have, therefore, studied the effects of soluble heparin on DNA synthesis in primary cultures of sheep mammary epithelial cells. Heparin prevents binding of amphiregulin, but not TGF- α or EGF, to EGF binding sites (Cook *et al.* 1991) and inhibits amphiregulin signalling via the human EGF receptor transfected into murine fibroblasts (Piepkorn *et al.* 1994). It inhibits the growth, independent of added EGF, of normal human mammary epithelial cells which express amphiregulin, an effect which can be overcome by exogenous EGF (Li *et al.* 1992). In these cells functional EGF receptors are necessary for

Figure 6 Interaction of effects of (a) insulin (100 µg/l) or (b) IGF-I (10 µg/l) and TGF-α (10 µg/l), on DNA synthesis measured by incorporation of [³H]methyl thymidine in cell cultures of sheep mammary alveolar epithelial cells. The vertical axis shows the ratio of (response to insulin or IGF-I in the presence of TGF-α) to (response to insulin or IGF-I+response to TGF-α). Cultures were carried out in the absence (–) or presence (+) of heparin (10 mg/l). A ratio significantly greater than 1 indicates synergism (see text). Tissues from five sheep were used at each pregnancy stage.

EGF-independent growth. Similar results have been reported for human keratinocytes (Piepkorn *et al.* 1994). The present study shows that DNA synthesis in sheep

mammary epithelial cells is inhibited in a dose-dependent manner by heparin. The inhibition can be partially or completely reversed by TGF-α. EGF also has an effect, although unlike the effect of TGF-α it does not increase with increasing dose. Moreover, the extent to which DNA synthesis is inhibited by heparin is related to the physiological state of the sheep from which the cells are derived. Although the growth of the mammary gland in pregnancy is exponential (see Fowler *et al.* 1990), thymidine labelling shows that proliferative activity is greatest in the first half of pregnancy (Battersby & Anderson 1988). Inhibition of DNA synthesis by heparin was greater in cultures of mammary cells from sheep at 10–15 weeks of pregnancy than from non-pregnant or late (20-week) pregnant sheep. This might reflect the developmental changes in cell proliferation and/or changes in mammary gland amphiregulin expression. Information is limited, but Western blotting suggested amphiregulin expression may be greater in pregnant than in non-pregnant or lactating sheep (Forsyth *et al.* 1997).

Our previous study (Moorby *et al.* 1995) found that exogenous IGF-I and TGF-α have an additive effect on DNA synthesis by sheep mammary epithelial cells. This was established by calculating the ratio: (response to IGF-I in the presence of TGF-α) to (response to IGF-I plus response to TGF-α) and showing it to be not significantly different from 1. In the present study this ratio was similarly calculated to study the interaction of both insulin and IGF-I with TGF-α and, to take account of a possible contribution of amphiregulin, in the absence and the presence of heparin. Insulin showed a synergistic interaction with TGF-α which was amplified when cultures contained heparin. The additive response to IGF-I and TGF-α was confirmed, but this became synergistic in the presence of heparin. Blocking of endogenous amphiregulin by heparin would have the effect of reducing the denominator in the calculated ratio by removing interaction with exogenous insulin or IGF-I, a possible explanation for this result.

Confirmation of a role for endogenous amphiregulin in the growth of the normal sheep mammary gland in pregnancy will require further studies. However, together with the demonstration that amphiregulin is expressed by sheep mammary epithelial cells (Forsyth *et al.* 1997) and stimulates their DNA synthesis (Figs 4a, 5), the present results are consistent with such a role. The results also indicate the potential for complex interactions between the different members of the IGF and EGF growth factor families. In view of the importance of ovarian steroids in mammary growth, it is of interest that amphiregulin is oestrogen-inducible (Martinez-Lacaci *et al.* 1995).

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