Mitogenic activity of lamb mammary fat pad *in vitro* is distinct from that of mouse mammary fat pad

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ABSTRACT

Our previous findings have shown that a diffusible factor(s) from the mouse mammary fat pad (MFP) markedly enhances the growth of COMMA-1D mouse mammary epithelial cells in response to insulin-like growth factor-I (IGF-I) and epidermal growth factor (EGF). In these experiments we examined whether MFP from prepubertal ewe lambs is a source of a similar diffusible mitogenic activity. MFP tissue was from lambs slaughtered at either 1 or 5 weeks of age. COMMA-1D cells were plated into 24-well plates at $5x10^4$ or $1x10^5$ cells/well, and quiesced in DMEM basal medium (BM). COMMA-1D cells were co-cultured with MFP by floating an explant of MFP in the appropriate cultures for 5 days. Conditioned medium (CM) was prepared by incubating MFP tissue in BM for 48 hours; cells were then cultured in CM for 3 days. In all experiments lamb MFP increased cell number by 2-3 fold relative to BM controls ($P<0.015$). This mitogenic effect was additive to that of 10% foetal calf serum. Cell number was increased by IGF-I (100 ng/ml), EGF (25 ng/ml) or their combination when added to BM ($P<0.03$). In contrast to the marked interactions with mouse MFP, these growth factors were typically additive with co-cultured lamb MFP ($P<0.05$), although there was a slight but significant interaction of IGF-I and MFP ($P=0.048$). Cell number increased with the proportion of CM added to cultures either alone, or with the addition of IGF-I, EGF or IGF-I + EGF. Bovine somatotropin (1 (μg/ml) in culture medium did not stimulate growth in the absence or presence of lamb MFP ($P>0.05$). These results show that the MFP of prepubertal lambs is the source of a diffusible mitogen(s). This activity differs to that from mouse MFP as it does not markedly interact with specific growth factors to stimulate the growth of COMMA-1D cells.

Keywords: lamb; mammary; fat pad; mouse; epithelium; growth factors.

INTRODUCTION

Postnatal development of the mammary gland involves the proliferation of epithelial cells into a matrix of adipose and connective tissue, the mammary fat pad (MFP). By manipulating this local environment, which is a potential source of growth regulating factors, it may be possible to increase the number of epithelial cells, and hence, potential milk production.

In rodents, MFP is predominantly comprised of adipocytes containing a high proportion of unsaturated fatty acids. Our previous studies have shown that MFP is a source of a diffusible mitogen(s) which markedly enhances the proliferative effect of specific growth factors on mouse mammary epithelial cells *in vitro* (Hovey *et al.*, 1994a,b).

In contrast to rodents, MFP of ruminants has a greater proportion of connective tissue, and adipocytes contain a high proportion of saturated fatty acids. However, there has been limited investigation into the role of the ruminant MFP as a regulator of epithelial growth. The objectives of this study were to determine if a mitogenic activity was present in neonatal ovine MFP, and to examine if this factor(s) interacted with other mitogens to stimulate mammary epithelial cell growth *in vitro*.

MATERIALS AND METHODS

The COMMA-1D mouse mammary epithelial cell line (Danielson *et al.*, 1984) was used. Cells were incubated at 37°C in a humidified 95% air: 5% CO_2 atmosphere. Cells were plated into 24-well culture plates ($5x10^4$ or $1x10^5$ cells/well) and quiesced in DMEM basal medium (BM) for 48 hours. For co-culture experiments, 0.5 ml treatment medium was added at day 0, and a further 0.5 ml added at day 3; co-cultures were terminated at day 5. For conditioned medium (CM) experiments, cells were cultured in 1 ml treatment medium for 3 days. Final cell number was quantified as total DNA (Labarca and Paigen, 1980).

Two Coopworth ewe lambs (aged 1 and 5 weeks) were slaughtered to provide mammary tissue which was dissected into the parenchymal and fat pad components. Co-cultures were prepared by placing an explant of MFP (~5 mm^3, 5-10 mg) on a raft of siliconized lens paper and floating the explants at the gas:medium interface of the appropriate cultures. To prepare CM, explants of MFP were briefly rinsed in BM and were then incubated in BM for 48 h (7.5 mg MFP/ml BM). CM was filtered (0.2 μm) and added to the appropriate cultures. Control BM was treated similarly in the absence of MFP explants. All hormone treatment combinations were added after the conditioning period.

The GLM procedure of SAS (1990) was used to test for significance of main effects and interactions in experiments 1, 2 and 3. Individual means comparisons were made by either t-test (experiment 1) in order to address variance heterogeneity, or by least significant difference (LSD). Means within CM dose response curves were compared by one-way ANOVA.

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RESULTS

Co-culture of COMMA-1D cells with lamb MFP increased final cell number by 2.4-fold relative to BM only (Table 1, Expt 1), whilst supplementation with 10% foetal calf serum (FCS) increased final cell number by 9-fold. A non-significant interaction of MFP with 10% FCS indicated that the mitogenic effects of these two factors were additive. Addition of recombinant bovine somatotropin (bST; 1 μg/ml) to BM did not alter final cell number (Table 1, Expt 2). Furthermore, the presence of MFP did not induce a response to bST.

TABLE 1: Growth of COMMA-1D cells (μg DNA/well) in response to foetal calf serum (FCS), bovine somatotropin (bST; 1 μg/ml), insulin-like growth factor-I (IGF-I; 100 ng/ml) or epidermal growth factor (EGF; 25 ng/ml) with or without co-cultured lamb mammary fat pad (MFP). Data are means ± s.e.m. Means comparisons by t-test (Expt 1) or LSD.

	Treatment	- MFP	+ MF	Signif.
Expt 1. (n=4)	BM	0.92 ± 0.06^{a}	2.2 ± 0.25^{a}	*
	10% FCS	8.2 ± 0.29^{b}	10.56 ± 1.21^{b}	n.s.
Expt 2. (n=4)	BM	1.02 ± 0.05^{a}	2.25 ± 0.08^{a}	***
	bST	0.85 ± 0.05^{a}	2.29 ± 0.17^{a}	***
Expt 3. (n=3)	BM	1.03 ± 0.03^{a}	2.62 ± 0.17^{a}	***
	IGF-I	1.91 ± 0.04^{b}	4.66 ± 0.44^{b}	***
	EGF	1.83 ± 0.22^{b}	3.47 ± 0.33^{c}	***
	IGF-I + EGF	2.41 ± 0.20^{b}	4.35 ± 0.19^{b}	***

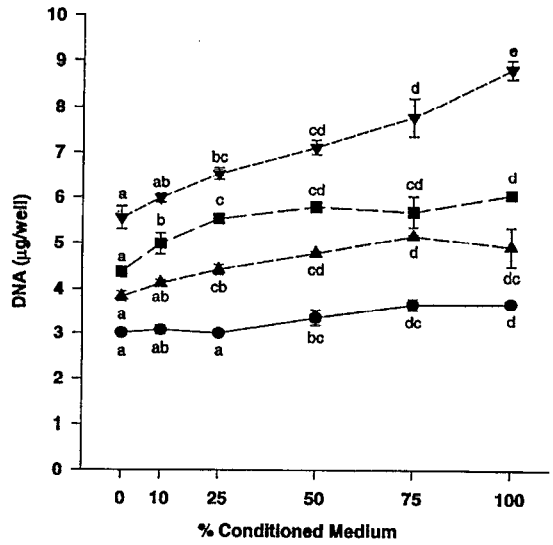
^{a,b,c} Within columns, means with different superscripts are different (P<0.05). Within rows, * P<0.05, *** P<0.001.

A third experiment tested if the effect of co-cultured lamb MFP could markedly interact with the mitogenic activity of insulin-like growth factor-I (IGF-I; 100 ng/ml) and epidermal growth factor (EGF; 25 ng/ml). Added alone, IGF-I, EGF or their combination increased cell number by 86%, 78% and 135% respectively (Table 1, Expt 3). There was no interaction between the effects of lamb MFP and EGF (P>0.05). There was a slight but significant interaction between the effects of MFP and IGF-I (P=0.048). Furthermore, individual responses to MFP with IGF-I or EGF were not additive when the combination of IGF-I and EGF was tested. A final experiment utilised CM to demonstrate dose responsiveness to the mitogenic activity of lamb MFP. For all treatment combinations there was an increase in final cell number with dose of lamb MFP conditioned medium (Fig. 1).

DISCUSSION

These results demonstrate that the mammary fat pad of the neonatal ovine is a source of a diffusible mitogen(s) which stimulates the growth of COMMA-1D mouse mammary epithelial cells. Similar to mouse MFP, the effect of lamb MFP was additive to that of 10% FCS, suggesting that diffusible mitogenic activity from the mouse and lamb MFP may have common properties. However, differences in their ability to modulate the effects of IGF-I and EGF suggest that these two mitogens are distinct. This differential responsiveness may be reflective of the fatty acid composition of these mammary fat pads. The mouse MFP contains a high proportion of unsaturated fatty acids which have been demonstrated

FIGURE 1: COMMA-1D cell growth in response to increasing proportions of lamb mammary fat pad conditioned medium either alone (●) or supplemented with IGF-I (■;100 ng/ml), EGF (▲; 25 ng/ml), or IGF-I + EGF (▼). Data are means (± s.e.m. (n=3).



to stimulate the growth of mouse mammary epithelial cells (Wicha *et al.*, 1979). Linoleic acid also enhances the proliferative effects of EGF by activating the protein kinase C signal transduction pathway, whereas saturated fatty acids attenuate this action (Sylvester *et al.*, 1994). In contrast, the ruminant fat pad contains a high proportion of saturated fatty acids which inhibit the growth of mouse mammary cells (Wicha *et al.*, 1979). Therefore, it is possible that both fat pads contained levels of unsaturated fatty acids adequate to evoke a growth response in the presence of BM and FCS, but that lamb MFP contained excessive levels of saturated fatty acids which may have inhibited the interactive effect with IGF-I and EGF. However, there may also be a differential presence of other mitogenic and anti-mitogenic factors in the two mammary fat pads which could account for these results.

The growth of mouse mammary cells is stimulated by the direct action of growth hormone *in vivo* (Silberstein and Daniel, 1987), and in organ culture *in vitro* (Plaut *et al.*, 1993). However, mammary cells cultured alone are not responsive to growth hormone, suggesting that one or more components of the MFP may be required to elicit a proliferative response to growth hormone. The presence of diffusible factors from the lamb MFP was unable to initiate a response to bST. However, a prerequisite in such a system may be the presence of other physical or soluble extracellular factors.

In conclusion, the neonatal ovine mammary fat pad is a source of a diffusible mitogen(s) which differs to that from the mouse MFP in the way it modulates the effects of exogenous growth factors on COMMA-1D growth. The cause of these differences remains to be elucidated.

ACKNOWLEDGEMENTS

R. Hovey is the recipient of a Massey University Postgraduate Scholarship. This research was funded by the Foundation for Research in Science and Technology.

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