Mouse mammary fat pad interacts with mitogens to stimulate epithelial growth *in vitro*

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ABSTRACT

We have examined the ability of co-cultured mouse mammary fat pad (MFP) to modulate the response of mouse mammary epithelial cells to a number of mitogens *in vitro*. In a series of 4 experiments, COMMA-1D cells were cultured with or without epithelium-free MFP obtained from the abdominal mammary glands of 23-day old virgin, female BALB/c mice. Final cell number was quantified as total DNA after 7 days. The main effect of MFP was significant in all experiments, MFP alone inducing a 6.1 to 8.2-fold increase in cell number relative to basal medium (BM) controls ($P<0.001$). Furthermore, this stimulatory effect of MFP synergised ($P<0.02$) with the mitogenic effect of 10% foetal calf serum (FCS). Examination of the response to several mammogenic hormones indicated that insulin (10 μg/ml) alone did not increase cell number ($P>0.05$), and interacted with the effect of MFP ($P<0.001$). There was no effect of prolactin (2.5 μg/ml) either alone or with MFP, whereas hydrocortisone (2.5 μg/ml) attenuated the response to MFP ($P<0.001$). In the absence of MFP, insulin-like growth factor-I (IGF-I; 75 ng/ml) did not increase cell number ($P>0.05$), whilst epidermal growth factor (EGF; 25 ng/ml) significantly increased cell number 3.7-fold. Both of these growth factors markedly interacted with the effect of MFP ($P<0.001$). When cultured with the combination of these growth factors and MFP, the final cell number was 29.2 times that of BM alone (25.14 ± 0.84 vs. 0.86 ± 0.12 μg DNA/well). There was no effect of prostaglandin E_2 (PGE_2; 0.5 μg/ml) or indomethacin (5 μg/ml),an inhibitor of prostaglandin synthesis, in the absence or presence of MFP. These findings demonstrate that a diffusible factor(s) from the MFP interacts with several regulators of mammary growth to markedly stimulate epithelial proliferation *in vitro*. Such a factor appears distinct from a number of classical mitogens, and may be involved in the regulation of mammary development *in vivo*.

Keywords: mouse; mammary fat pad; epithelium; mitogens; growth.

INTRODUCTION

Development of the mammary gland involves the proliferation of epithelial cells within a matrix of adipose and connective tissue, the mammary "fat pad". Mammary epithelium will only develop *in vivo* if implanted in such a fat pad (reviewed by Hoshino, 1978). Milk production is highly correlated with secretory cell number (Tucker, 1981). Therefore, by understanding how the MFP regulates epithelial cell growth, it may be possible to increase mammary development and subsequent milk production.

It has been suggested that the MFP modulates epithelial cell growth by several mechanisms. Non-diffusible factors such as substratum components (Bissell and Hall, 1987) and cell-cell contact (Levine and Stockdale, 1984) have been implicated, the former influencing the responsiveness of epithelium to mammogenic hormones (Salomon *et al.*, 1981). Furthermore, the MFP may serve as a source of diffusible factors which stimulate epithelial cell growth. Rodent adipocytes may release unsaturated fatty acids (Kidwell and Shaffer, 1984) which increase epithelial cell growth both *in vivo* (Welsch and O'Connor, 1989) and *in vitro* (Wicha *et al.*, 1979). Such a response may be due to a variety of mechanisms including the synthesis of fatty acid-derived prostaglandins (reviewed by Welsch, 1987). Other mammogenic factors such as transforming growth factor-α (Liscia *et al.*, 1990), fibroblast growth factor (L.C. Kotolski, pers. comm.) and a mammary fibroblast-derived factor (Enami *et al.*, 1983) may also be derived from the MFP. Whether the growth of mammary epithelial cells in response to various mammogenic factors can be modulated by these, or other diffusible factors derived from the MFP has received limited investigation.

The objectives of these experiments were: 1) to determine if the growth of COMMA-1D epithelial cells can be stimulated by MFP *in vitro*, 2) to examine if MFP can modulate the response of epithelial cells to a number of mammogenic hormones, and 3) to ascertain whether the observed response to MFP can be attributed to the presence or synthesis of prostaglandins.

MATERIALS AND METHODS

Cell culture

The COMMA-1D mouse mammary epithelial cell line (Danielson *et al.*, 1984; passage 34) was used in these experiments. Cells were routinely maintained in growth medium (GM) comprised of Dulbecco's Modified Eagle's Medium (DMEM): Ham's F12 buffered with 44 mM sodium bicarbonate, and supplemented with 2% foetal calf serum (FCS), insulin (6 μg/ml), EGF (5 ng/ml), bovine serum albumin (BSA; 0.3 mg/ml) and penicillin-streptomycin (P/S; 1% v/v). The hormone free basal medium (BM) for all experiments was DMEM containing 44 mM sodium bicarbonate and 1% P/S.

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Cells were plated into 24-well culture plates ($6x10^4$ cells/well) in 0.5 ml GM. After 24h attachment, cells were serum-starved in BM for a further 36h. Treatment media (0.5 ml) was then administered (t=0). A further 0.5 ml treatment medium was added on day 2, and 0.5 ml of the culture medium was replaced with fresh medium on days 4 and 6 of culture. Preliminary experiments indicated that full medium replacement in such a co-culture system induced "step-wise" cell growth associated with the removal and subsequent delay in replenishment of factors in media conditioned by the MFP explant. Hydrocortisone, PGE_2 and indomethacin were prepared as ethanolic stocks, and all treatments within each experiment shared the same concentration of ethanol, which never exceeded 0.15%. Preliminary experiments indicated that ethanol at this concentration did not affect cell growth. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere. Final cell number was quantified after 7 days by assay for DNA as described by Labarca and Paigen (1980).

Co-cultures

Mammary fat pads from which the epithelial component had been dissected (DeOme *et al.*, 1959) were obtained from the abdominal (4^{th}) mammary glands of 23-day old virgin female BALB/c mice. Fat pad tissue was sectioned into 5-10 mg explants (~5mm^3), then rinsed for 5 minutes in BM. Co-cultures of COMMA-1D cells and MFP were prepared by placing one MFP explant on a raft of siliconized lens paper (1cm^2) and floating the explants at the gas:medium interface of the appropriate cultures (Topper *et al.*, 1975).

Experiment 1

In an initial experiment, we examined the responsiveness of COMMA-1D cells to mouse MFP in the absence or presence of a known mitogen, FCS. Treatments examined the main effects of MFP and 10% FCS.

Experiment 2

The growth of epithelial cells in response to mammogenic hormones was investigated in the absence or presence of MFP. Treatment combinations examined the effects of MFP, insulin (10 µg/ml), prolactin (2.5 µg/ml) and hydrocortisone (2.5 µg/ml).

Experiment 3

In this experiment, we investigated if the growth of COMMA-1D cells in response to growth factors could be modulated by MFP. Treatments included the effects of MFP, IGF-I (75 ng/ml) and EGF (25 ng/ml).

Experiment 4

We tested whether the mitogenic effect of MFP could be attributed to the presence of prostaglandin E_2 (PGE_2), and if indomethacin, an inhibitor of prostaglandin synthesis, could suppress this response. Treatment combinations examined the effects of MFP, PGE_2 (0.5 µg/ml) and indomethacin (5 µg/ml).

Statistical Analysis

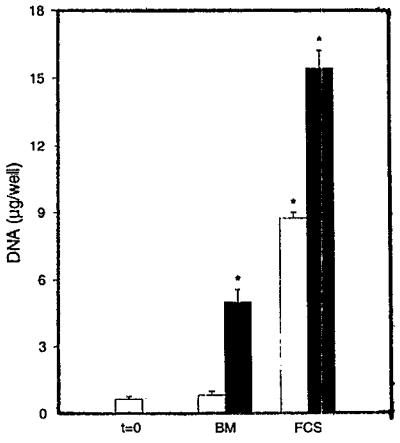
The main effects for each experiment were assigned factorially. Within each experiment, main effects and their interactions were analyzed using the ANOVA procedure of Minitab (1991). Means for individual treatments within each experiment were compared by LSD. Results were considered significant if $P<0.05$. Within an experiment, each replicate for the co-culture treatment combinations represented a separate mouse, with tissue explants from each mouse allocated across all treatments.

RESULTS

Experiment 1

COMMA-1D cells cultured in BM for 7 days did not demonstrate any net increase in cell number (Fig. 1). However, co-culture with MFP elicited a 6.1-fold increase in cell number relative to BM only ($P<0.001$). This response was approximately 50% of that due to the mitogenic effects of 10% FCS. Furthermore, there was a synergistic response to the individual effects of MFP and FCS ($P<0.02$). Therefore, the ability of MFP to stimulate cell growth alone was still apparent in the presence of 10% FCS, a supplement routinely used to maximise cell growth.

FIGURE 1: Growth response of COMMA-1D cells cultured with (■) or without (□) mammary fat pad in either basal medium (BM) or 10% foetal calf serum (FCS). t=0 represents DNA content at commencement of treatments. Values are means ± s.e. (n=6). * Significantly different ($P<0.001$) to BM only treatment.



Experiment 2

The growth of cells cultured with insulin alone appeared to be stimulated, although final cell number was not significantly different to the BM control (Fig. 2). There was no significant effect of either prolactin or hydrocortisone alone. MFP alone increased cell number 8.2-fold ($P<0.001$), and markedly enhanced the growth of cells in response to insulin ($P<0.001$). Prolactin did not influence the response to MFP ($P>0.05$). Growth in response to MFP was reduced by hydrocortisone ($P<0.001$). Hydrocortisone also evoked a negative interaction with insulin in both the presence and absence of MFP ($P<0.001$). Overall, the effect of either MFP or insulin was to stimulate growth ($P<0.001$), whilst hydrocortisone was inhibitory ($P<0.001$).

Experiment 3

Cell growth in the presence of IGF-I appeared to be enhanced, although final cell number was not significantly different to that in the BM control (Fig 3). Cells cultured with

FIGURE 2: Growth response of COMMA-1D cells cultured with (■) or without (□) mammary fat pad and combinations of basal medium (BM), insulin (Ins; 10 µg/ml), prolactin (Prl; 2.5 µg/ml) and hydrocortisone (HC; 2.5 µg/ml). Values are means ± s.e. (n=5). *Significantly different (P<0.001) to respective BM treatment.

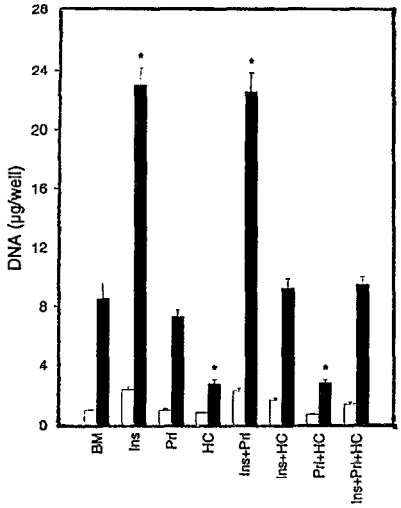
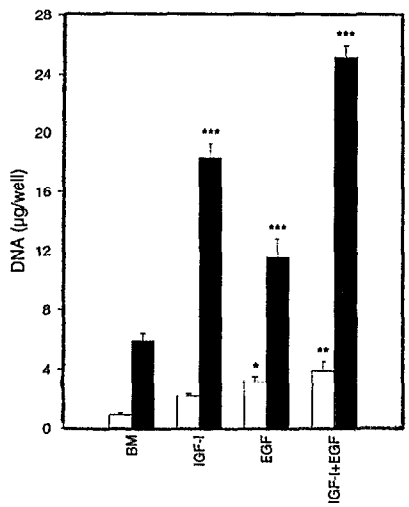


FIGURE 3: Growth response of COMMA-1D cells cultured with (■) or without (□) mammary fat pad and combinations of basal medium (BM), insulin-like growth factor-I (IGF-I; 75 ng/ml) and epidermal growth factor (EGF; 25 ng/ml). Values are means ± s.e. (n=5). Means significantly different *(P<0.05), **(P<0.025), *** (P<0.001) to respective BM treatment.

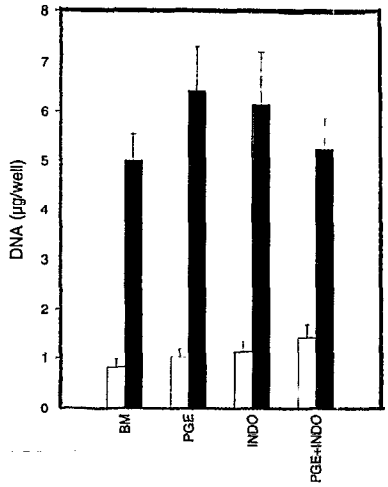


either EGF alone or the combination of IGF-I and EGF increased significantly in cell number by 3.7 and 4.6-fold respectively. Both of the growth factors markedly synergised with the effect of MFP (P<0.001). Furthermore, these individual interactions were additive, resulting in a final cell number 29.2 times that in BM alone. Overall, main effects of MFP, IGF-I and EGF were significant (P<0.001).

Experiment 4

When COMMA-1D cells were cultured with either PGE_2 or indomethacin, or the combination of both, there was no significant difference in final cell number (Fig. 4). Cell number was increased by co-culture with MFP (P<0.001), but this response was not altered by the presence of PGE_2 and/or indomethacin (P>0.05).

FIGURE 4: Growth response of COMMA-1D cells cultured with (■) or without (□) mammary fat pad and combinations of basal medium (BM), prostaglandin E_2 (PGE; 0.5 µg/ml) and indomethacin (INDO; 5 µg/ml). Values are means ± s.e. (n=6). Means not significantly different (P>0.05) to respective BM treatment.



DISCUSSION

These results demonstrate that a diffusible factor(s) from the mouse MFP stimulates the proliferation of COMMA-1D mouse mammary epithelial cells. Growth in response to such a factor(s) is not masked by the array of mitogens present in FCS; rather, it interacts with this and several other stimulants to markedly enhance epithelial cell growth.

It has previously been reported that both co-culture with mouse MFP (Carrington and Hosick, 1985) and medium conditioned by mouse MFP (Beck and Hosick, 1988; Beck *et al.*, 1989) stimulates the proliferation of normal and preneoplastic mammary epithelium *in vitro*. However, such studies have utilised a basal medium containing mitogenic factors such as insulin and/or EGF, thereby not addressing the possibility that MFP derived factors interact with such mitogens. We speculated that *in vivo*, the MFP may release diffusible factors into the local environment of the epithelial cell which modulates its responsiveness to a variety of factors implicated during mammogenesis. We have begun to investigate this possibility utilising the COMMA-1D mammary epithelial cell line and a hormone-free BM.

The growth of mammary epithelial cells in response to mammogenic hormones *in vitro* is much less than that observed *in vivo* (Yang *et al.*, 1980). Our findings demonstrate that the mouse MFP releases a diffusible factor(s) which not only stimulates the growth of epithelial cells *in vitro*, but also markedly enhances the growth of cells in response to several factors which have been shown to stimulate mammary development in the mouse. Such a factor(s) would appear to have a permissive role, facilitating the response of epithelial cells to a variety of mitogenic signals. The diffusion of such factors from the MFP into the environs of the epithelial cell *in vivo* may well be essential for hormonal responsiveness throughout the course of mammary gland development.

As adipocytes constitute a major proportion of the MFP, and mammary epithelial cells develop in close association with these cells in *vivo*, it is possible that the release of unsaturated

fatty acids from the mouse MFP is involved in the stimulation of epithelial cell growth. Beck *et al.* (1989) proposed that the stimulatory effect of media conditioned by either MFP or isolated MFP adipocytes was attributable to the presence of unsaturated fatty acids. There is limited evidence suggesting that unsaturated fatty acids increase the response of mammary epithelial cells to other mitogenic factors. The EGF induced growth of epithelial cells in primary culture can be enhanced and sustained by linoleic acid (18:2 ω 6) and arachidonic acid (20:4 ω 6), and eicosanoids like prostaglandin E_2 and hydroxy-fatty acids (Bandyopadhyay *et al.*, 1987; Bandyopadhyay *et al.*, 1988). Such action may be the result of enhanced signal transduction from cell-surface receptors via increased activity of several kinase pathways (Imagawa *et al.*, 1988; Bandyopadhyay *et al.*, 1993). Although our results suggest that the proliferation of COMMA-1D cells in response to MFP was not due to the presence of PGE_2 or the synthesis of prostaglandins by epithelial cells, other pathways utilising MFP-derived unsaturated fatty acids such as the amplification of growth factor signals may explain the observed interactions between the effects of mitogens and MFP. We are presently investigating the mechanisms by which these responses may be elicited.

It is also possible that other diffusible factors from the MFP may account for such results. It has recently been reported that both the intact and epithelium-free MFP of pregnant mice expresses mRNA for acidic and basic fibroblast growth factor (L.C. Kotolski, pers. comm.), a factor which stimulates the growth of COMMA-1D cells (Riss and Sirbasku, 1987). Given that we observed marked synergistic growth responses to MFP in the presence of insulin, IGF-I and EGF, we postulate that the effect of MFP in these experiments cannot be solely attributed to the diffusion of these polypeptides from the MFP. However, epithelial cell growth in response to MFP in these experiments may be a culmination of the effects of several soluble factors released from the MFP.

Under the conditions tested, there was no growth response to prolactin, whilst hydrocortisone attenuated the response of epithelial cells to stimulatory factors. Such results may reflect the requirement for an extracellular matrix, as the growth of primary mouse epithelial cells within collagen gels is stimulated by prolactin and corticosteroids (Imagawa *et al.*, 1985). Whether diffusible factors from the MFP can enhance the growth response to these hormones in such an environment remains to be elucidated.

CONCLUSIONS

These results demonstrate that the MFP releases a diffusible factor(s) distinct from several other mitogens which enhances the growth of mammary epithelial cells *in vitro* in response to several mammogenic factors. We propose that this factor(s) may be involved in the stimulation of epithelial cell proliferation *in vivo*, and may be important in regulating the response of epithelial cells to several mammogenic hormones.

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