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A TRANSFORMING GROWTH FACTOR RELATED TO EPIDERMAL GROWTH FACTOR IS EXPRESSED BY FETAL MOUSE SALIVARY MESENCHYME CELLS IN CULTURE

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SUMMARY: Fetal mouse salivary mesenchyme cells secrete a protein with an apparent MW of 15 Kd that is immunologically related to epidermal growth factor (EGF). Conditioned medium collected from these cells in culture stimulates the growth of primary mouse mammary epithelial cells cultured within collagen gels, competes for binding to EGF receptor sites on these mammary epithelial cells and stimulates the anchorage-independent growth of normal rat kidney fibroblast cells within soft agarose. Prior immunoprecipitation of salivary mesenchyme cell conditioned medium with anti-EGF antibodies effectively removes or attenuates all of these effects confirming that an EGF-like factor is involved in these responses.

Transplantation of embryonic mouse salivary mesenchyme into the adult mouse mammary gland specifically induces mammary epithelial hyperplasia (1) and makes the mammary epithelial cells much more susceptible to carcinogen and retrovirus-induced tumor formation in vivo (2). The molecular basis of these mesenchymal-epithelial interactions is unknown. A better understanding of these phenomena on the molecular level would contribute to our basic knowledge of abnormal stromal-parenchymal interactions involved in mammary epithelial tumorigenesis.

EGF has been implicated in mammary epithelial cell growth and transformation. EGF stimulates mammary epithelial proliferation both in vivo (3) and in vitro (4). EGF potentiates chemical transformation in vivo and viral transformation in vitro (5). EGF expression by the submandibular gland correlates with spontaneous mammary epithelial tumor formation in female virgin mice, and sialoadenectomy dramatically reduces tumor incidence and growth in vivo (6). In addition, EGF and transforming growth factor (TGF)-alpha, which are homologous and bind to EGF receptors (7), are known to contribute to the anchorage-independent growth of normal rat kidney (NRK) fibroblast cells in soft agarose (8), a widely accepted criterion for cell

ABBREVIATIONS: EGF, epidermal growth factor; TGF, transforming growth factor; SM, salivary mesenchyme; ME, mammary epithelial; NRK, normal rat kidney.

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transformation. These data provide a strong rationale for the investigation of possible EGF-like growth factors which may be expressed by fetal mouse salivary mesenchyme cells and act as transforming growth factors.

We have devised a three dimensional in vitro collagen gel culture system to better study mouse salivary mesenchyme-mammary epithelial cell interactions under precisely controlled conditions which most closely mimic in vivo growth patterns (9,10). Soluble factor(s) released into the culture medium by mesenchyme cells derived from mouse embryonic salivary glands (SM cells) stimulate the growth of primary mouse mammary epithelium (ME cells) embedded within collagen gels (11; Oliver and Hosick, in preparation). In this study, we have investigated the possibility that EGF-like molecules are involved in the response of normal mammary epithelial cells to salivary mesenchyme, as well as the transforming effects of these growth factors on NRK cells cultured within soft agarose.

MATERIALS AND METHODS

Cell culture: Salivary mesenchyme was isolated from 13-day old BALB/c mouse fetuses and cell lines were derived from explant cultures of this tissue (11; Oliver and Hosick, in preparation). Cell passages 17-30 were used for these studies. SM cells were grown to confluence on tissue culture plastic in DME medium containing 10% Bovine Calf Serum and 5μg/ml insulin. Conditioned medium was collected from SM cells that were washed extensively with DME:F12 (1:1) and then incubated with DME:F12 "defined medium" (containing 5μg/ml insulin, 1μg/ml prolactin, 50ng/ml progesterone, 10μg/ml linoleic acid and 1mg/ml BSA) for 24 hours at 37^{o}C. The medium was then filtered and stored at -80^{o}C. ME cells were isolated from midpregnant BALB/c mice using a collagenase digestion procedure as previously described (10). ME cell aggregates were embedded within type-I collagen (isolated from rat tails) at a plating density of 10^5 cells per 0.5 ml collagen in 24 well tissue culture plates, and cultured in various defined or SM cell conditioned media. The NRK soft agarose assay was performed as previously described (12). All cell cultures were maintained at 37^{o}C in 5% CO_2 in a humidified incubator.

EGF receptor assay: Membranes were isolated from ME cells cultured within collagen gels as described elsewhere (Venkateswaran and Hosick, submitted). DNA fluorometric assays were performed as previously described (13). Receptor assays were carried out using [^{125}I]-EGF labeled by the lactoperoxidase procedure according to the manufacturer's instructions (Bio-Rad). The specific activity of the labeled ligand used was 150-250 μCi/μg protein. Specific binding was determined by incubating ME membranes with titrated concentrations of [^{125}I]-EGF with and without the addition of 100-fold excess unlabeled EGF. The purified EGF used in these and other control experiments was obtained from Upstate Biotechnology, Inc. Competition for EGF receptor sites was assayed by addition of 100μl concentrated SM cell conditioned medium, conditioned medium that had been immunoprecipitated with anti-EGF antiserum or defined medium to membrane aliquots in the receptor assay solution. All media for this assay were concentrated 4X by dialysis against Aquacide III concentrating agent (Calbiochem).

Protein radiolabeling: SM cells cultured as described above were washed twice prior to and after a 1 hour pre-incubation in methionine-free DME medium with 5μg/ml insulin at 37^{o} C. Labeling was carried out in methionine-free DME with 5 μg/ml insulin containing 100μCi/ml [^{35} S]-methionine/cysteine (ICN

Biomedicals Inc., specific activity >1000Ci/mM) for 6 hours at 37°C. The labeled medium was collected, the cells were lysed in 50 mM Tris (pH 7.4) plus 0.1% SDS, 2mM PMSF was added and the samples were stored at -80°C.

Immunoprecipitations: EGF immunoprecipitations were performed using a polyclonal rabbit anti-mouse EGF antiserum from Upstate Biotechnology Inc. For immunoprecipitation of radiolabeled samples, an equal number of trichloroacetic acid (TCA) cpm from each sample was brought to equal volumes and incubated on ice for 8 hours with antiserum at a 1:50 dilution in immunoprecipitation buffer containing 50mM Tris (pH 7.4), 1% Tween-20, 0.1% SDS, 1mM L-methionine, 5mM EDTA and 2mM PMSF. 0.1% Protein A-Sepharose in 50mM Tris was then added and the tubes were agitated for 1 hour at 4°C. The Sepharose beads were then centrifuged at 14,000 rpm for 3 min., washed 3 times with immunoprecipitation buffer and once in buffer without Tween-20. Sepharose pellets were resuspended in 60μl electrophoresis sample buffer, boiled, centrifuged and the supernatants electrophoresed in 18% SDS-PAGE gels under reducing conditions. Gels were stained with Coomassie Blue to visualize MW markers, soaked in Amplify solution (Amersham), dried and overlaid with X-ray film. For immunoprecipitation of non-radiolabeled conditioned medium, anti-EGF antiserum was used at a 1:100 dilution and the Protein A-Sepharose was dissolved in defined medium. After immunoprecipitation, the media were then separated from Sepharose complexes by centrifugation and filtered for use in other assays.

RESULTS AND DISCUSSION

Medium conditioned by SM cells stimulated the growth of ME cells cultured within collagen gels (Figure 1C) in a similar fashion to that seen with the addition of purified EGF to defined medium (Figure 1B). Prior immunoprecipitation of SM cell conditioned medium with rabbit anti-mouse EGF antiserum attenuated the response of ME cells to SM cell conditioned medium (Figure 1D). There was a limited amount of early branching outgrowth by ME cells exposed to immunoprecipitated conditioned medium; however, the growth was not extensive, and at later time points these cells (Figure 1D) looked similar to the controls containing defined medium alone (Figure 1A). These observations indicate that the presence of an EGF-like factor in SM cell conditioned medium is required for the continued growth of ME cells cultured within collagen gels.

We tested to see if medium conditioned by SM cells was able to effectively compete with [^{125}I]-EGF for binding to EGF receptor sites in a receptor assay using cell membranes isolated from ME cells cultured within collagen gels. Our results showed that SM cell conditioned medium significantly inhibited the binding of [^{125}I]-EGF to EGF receptors on ME cell membranes whereas the control medium did not (Figure 2; bars 4 and 2, respectively). Prior immunoprecipitation of SM cell conditioned medium with anti-EGF antiserum completely removed the ability of SM cell conditioned medium to compete for EGF receptor binding sites (Figure 2, bar 5). These results indicate that a factor present in medium conditioned by SM cells is capable of binding specifically to EGF receptors present on ME cells.

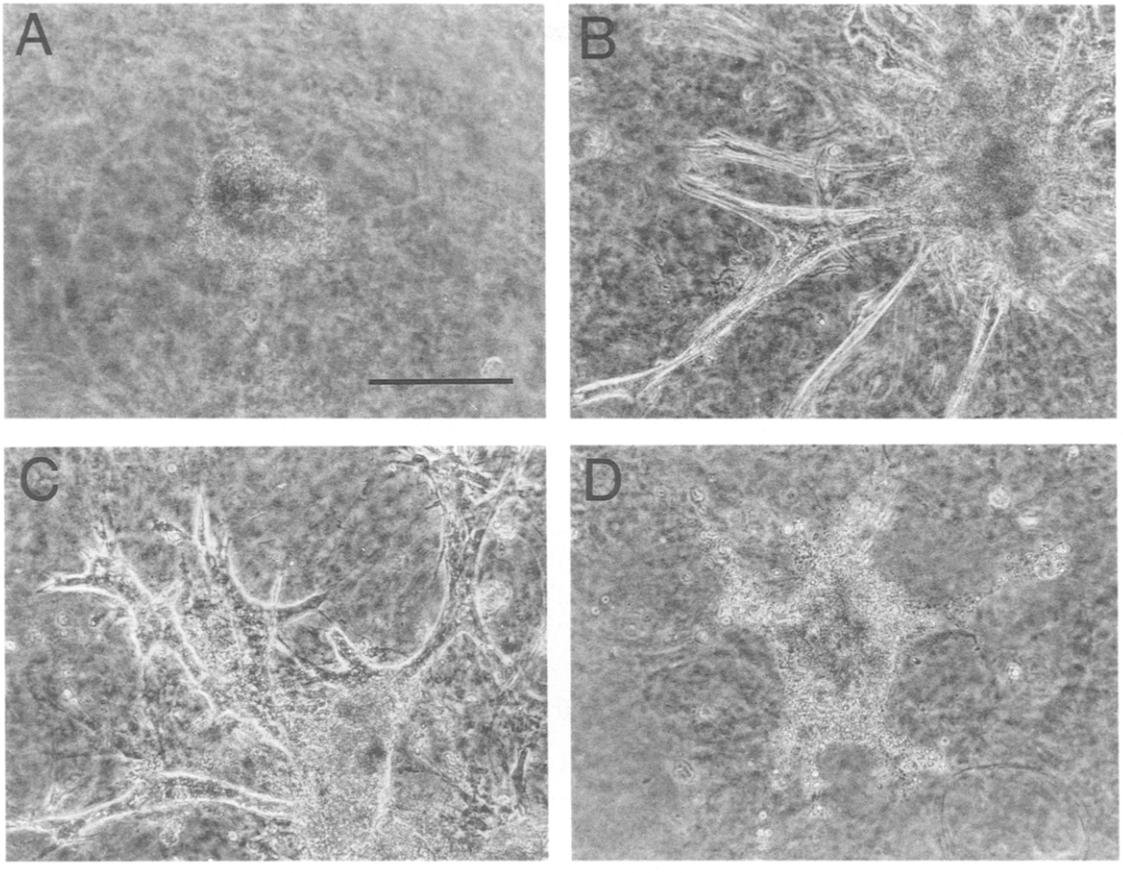

Figure 1. Growth stimulation of ME cells in collagen gels by SM cell conditioned medium. ME cells were embedded within collagen gels and cultured for 7 days either in the presence of defined medium (A), 10 ng/ml purified EGF (B), medium conditioned by SM cells (C) or SM cell conditioned medium that had been immunoprecipitated with anti-EGF antibodies (D). Bar = 500 μm.

In order to identify any EGF related proteins in SM cell conditioned medium, we immunoprecipitated media and lysate fractions collected from [^{35}S]-methionine/cysteine labeled SM cells cultured on plastic and collagen gel substrata. SDS-PAGE of these immunoprecipitated samples showed that a protein with an apparent MW of 15 Kd was secreted by SM cells when cultured on either substrata (Figure 3A, lanes 1 and 2). The intracellular fractions failed to show the presence of this protein (Figure 3A, lanes 3 and 4), indicating that the relative amount of this protein inside the cells was not detectable within the limits of this assay. EGF has a predicted MW of 6.1 Kd (14), and purified samples of EGF migrate slightly above the 6.5 Kd MW marker in these gels. This indicates that a previously uncharacterized protein immunologically related to EGF, but with a different MW, is secreted by SM cells in culture. It has been shown that differentially processed forms of pro-TGF-alpha with apparent Mr of 15-20 Kd are expressed by certain transformed fibroblast cell lines in culture, and in some cases these factors are released into the medium with low

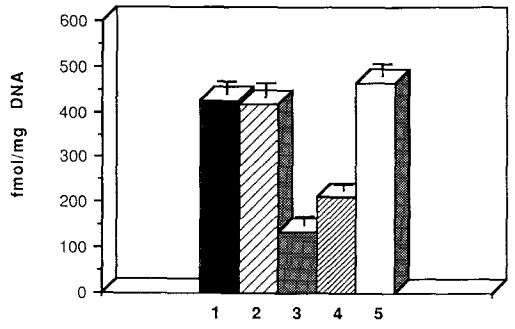

Figure 2. SM cell conditioned medium competes for EGF receptor binding sites on ME cell membranes. Cell membranes isolated from ME cells cultured within collagen gels were used for an EGF receptor competitive binding assay. Bar 1, without media addition-total [^{125}I]-EGF bound; Bar 2, plus defined medium; Bar 3, plus purified EGF; Bar 4, plus SM cell conditioned medium; Bar 5, plus SM cell conditioned medium that had been immunoprecipitated with anti-EGF antibodies. The standard deviation for triplicate samples is indicated.

efficiency (15-18). Further characterization is underway to determine if this EGF-like protein is a differentially processed form of TGF-alpha or a product of EGF gene expression.

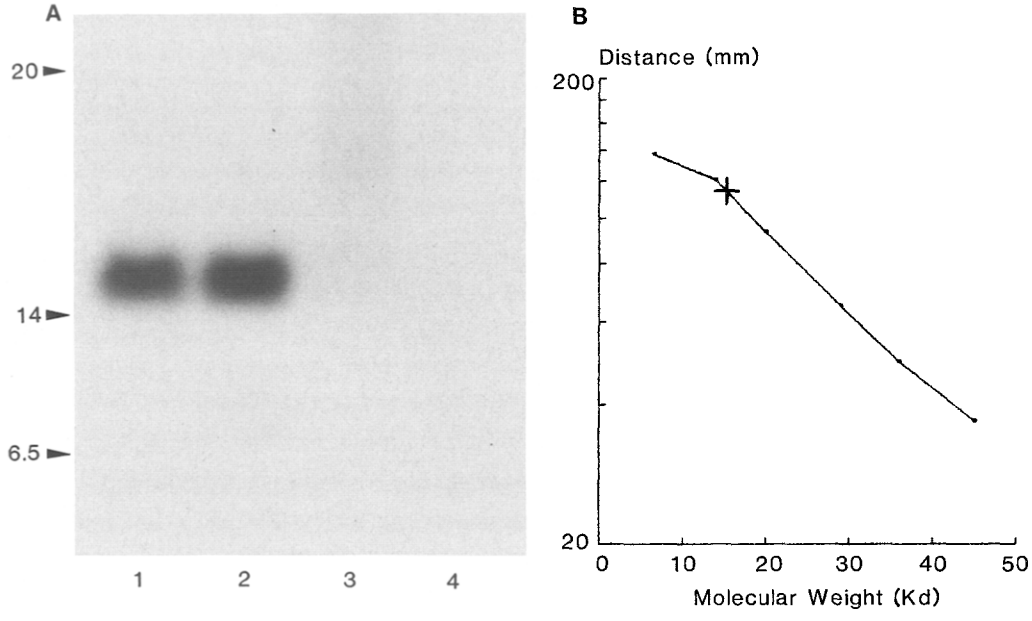

Figure 3. SM cells secrete an EGF-like protein with an apparent MW of 15 Kd. A) SDS-PAGE of EGF immunoprecipitated samples collected from [^{35}S]-methionine/cysteine labeled cultures of SM cells grown on plastic or collagen gel substrata. Radiolabeled samples containing an equal number of TCA precipitable cpm were immunoprecipitated with anti-EGF antiserum and then electrophoresed in 18% acrylamide gels under reducing conditions. Lanes 1 and 2, media samples; Lanes 3 and 4, lysate samples; Lanes 1 and 3, from cells grown on plastic; Lanes 2 and 4, from cells grown on collagen gels. B) Semi-log plot of the MW markers showing the relative position of the immunoprecipitated protein at 15 Kd.

Table 1. Stimulation of NRK cell anchorage-independent growth by SM cell conditioned medium

Media	Colony Formation* (%)	Average Diameter (mm)
a) Defined medium	0.0	0.00
b) + 10 ng/ml EGF	0.0	0.00
c) + 10% Serum	31.6 +/- 15.8	0.15
d) SM cell conditioned medium	50.9 +/- 17.5	0.17
e) - EGF (Immunoprecipitated)	13.3 +/- 3.9	0.13

* The mean average and standard deviation for triplicate samples is shown.

Medium conditioned by SM cells also stimulated the growth of NRK cells in soft agarose (Table 1, row d). The percent colonies induced by SM cell conditioned medium was as great as the maximal responses attained with defined medium containing 10% serum (Table 1, row c). Prior immunoprecipitation of SM cell conditioned medium with anti-EGF antiserum reduced the percent colony formation by 74% (Table 1, row e). Duplicate immunoprecipitated samples from these same experiments completely removed the EGF competitive activity from SM cell conditioned medium in the EGF receptor binding assay (Figure 2, bar 5), which indicates that the EGF-like protein was effectively removed by immunoprecipitation. These results demonstrate that the immunoprecipitated EGF-like protein contributes to the anchorage-independent growth of NRK cells in soft agarose, a definition for transforming growth factors (8). Other transforming growth factors may also be present in SM cell conditioned medium which synergise with this EGF-like protein to cause colony formation by NRK cells in soft agarose. This is consistent with previous reports which show that EGF or TGF-alpha augment colony formation in conjunction with other growth factors such as TGF-beta, which is also required for the growth of NRK cells in soft agarose (19,20). We are presently investigating the possibility that other transforming growth factors are secreted by SM cells, as well as further defining the biochemical nature of the 15 Kd EGF-like transforming growth factor secreted by SM cells.

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