Identification and Partial Characterization of Mesenchyme-Derived Growth Factor That Stimulates Proliferation and Inhibits Functional Differentiation of Mouse Mammary Epithelium in Culture

MICHIYOSHI TAGA¹⁾, TERUYO SAKAKURA²⁾ AND TAKAMI OKA³⁾

¹⁾Dept. of Obstetrics and Gynecology, Yokohama City Univ. School of Medicine, Yokohama, Japan.

²⁾RIKEN Tsukuba Life Science Center, The Institute of Physical and Chemical Research, Japan.

³⁾NIDDK, National Institutes of Health, Bethesda, U.S.A.

Abstract

The effect of mesenchyme on both proliferation and differentiation of mammary epithelial cells was investigated in a primary cell culture system. Mammary cells cultured on collagen gel for 4 days produced casein in response to the synergistic action of insulin, cortisol, and prolactin. When mammary epithelial cells were co-cultured with fibroblasts derived from three different kinds of fetal mesenchymal tissues, casein production was suppressed. The addition of conditioned media obtained from cultures of these mesenchymal cells stimulated DNA synthesis and reduced casein synthesis in a dose-dependent fashion in the cultured mammary cells. Although such biological actions are similar to those of epidermal growth factor (EGF), the capability to compete with EGF for EGF receptor was not found in this conditioned medium. Sephadex G-200 column chromatography revealed that molecular weight of the peak which has these biological activities was around 100,000. These results indicate that fetal mesenchymal cells secrete a substance(s) which has a stimulatory effect on proliferation and an inhibitory effect on differentiation of mammary epithelial cells.

Among a variety of cell-to-cell interactions, epithelial-mesenchymal interaction is most important in determining the growth and differentiation of many tissues including epithelial cells. During embryogenesis, the interactions between epithelium and mesenchyme are essential for organogenesis and cytodifferentiation (Fleischmajer and Billingham, 1968; Grobstein, 1967; Sawyer and Fallon, 1983). Even in adult, the

mesenchymal tissue of embryo affects the morphogenesis and cytodifferentiation of the epithelial cells (Cunha *et al.*, 1983; Neubauer *et al.*, 1983; Sakakura *et al.*, 1979). With respect to the mechanism of the interactions, two main possible processes have been considered. One is the diffusion of chemical signals (*e. g.* so-called inducers, hormones and growth factors) either from adjacent cells or from other parts of the organism (Massague, 1987), and the other is related to the changes in the composition,

structure, and function of the extracellular matrix substances (Bernfield and Banerjee, 1978; Hay, 1977; Slavkin *et al.*, 1981). However, the precise mechanism responsible for such biological actions is still unknown.

In the last decade, much has been learned about the role of steroid hormones in the embryonic development and expression of functional activity of various organs. For example, in sex differentiation of sex glands, such as mouse mammary gland (Drews and Drews, 1977; Durnberger *et al.*, 1977) and urogenital organs (Cunha and Lung, 1979; Lasnitzki and Mizuno, 1980), the masculin morphogenesis is triggered by androgen produced by the fetal testes. According to the tissue recombination experiments using androgen receptor negative Tfm/Y mutant mice, such androgen-induced sex differentiation is mediated by the androgen receptors in the mesenchyme. Smith and his colleague (1983) have demonstrated that glucocorticoid induction of surfactant in lung alveolar type II cell and tyrosine aminotransferase in hepatocyte is stimulated by fibroblast pneumonocyte factor and fibroblast hepatocyte factor (Dow *et al.*, 1983), which are produced specifically by embryonic lung and liver fibroblasts, respectively.

Besides hormones, recently several kinds of growth factors have been identified as growth-promoting humoral factors, which are required for cell growth and differentiation. However, there is little evidence for the involvement of growth factors in the control of mechanism of epithelial-mesenchymal interaction.

Previously we reported a primary mouse mammary epithelial cell culture system in which the mammary cells proliferate and differentiate in response to the synergistic action of insulin, cortisol, and prolactin (Taketani and Oka, 1983 a). By using this culture system, we have demonstrated that epidermal growth factor (EGF) and 12-O-tetra-decanoylphorbol 13-acetate (TPA)

stimulate cell growth and inhibit milk protein synthesis in mammary epithelium (Taketani and Oka, 1983 b, c.).

In order to assess the possible involvement of mesenchyme in the regulation of epithelial function, we investigated the effects of mesenchyme on both cell proliferation and differentiation of epithelium in this system. In this paper, we will report the identification of a new growth factor(s) which is produced and secreted by mesenchyme and regulates the mammary epithelial cell function, and also report the partial characterization of this substance(s).

Materials and Methods

Materials

Materials were obtained as follows: C3H/HeN mice from the Animal Breeding Facility, National Institutes of Health; L-³H amino acid mixture (1 mCi/ml) from ICN; methyl-³H thymidine (77 Ci/mmol) and ¹²⁵I-epidermal growth factor (EGF) from New England Nuclear; Medium 199 (Hanks' salt), fetal bovine serum and trypsin from GIBCO; mouse EGF (receptor grade and culture grade) from Collaborative Research; trypsin from Pierce Chemical Co.; trypsin inhibitor (soybean) from Calbiochem; crystalline porcine zinc insulin from Lilly Research Laboratories; bovine prolactin (Lot No. B5) from the Hormone Distribution Program, NIADDK, NIH; and Sephadex G-200 from Pharmacia.

Primary Culture of Mammary Epithelial Cells

The primary culture of mammary epithelial cells of lactating mice, 3 to 5 days post-partum, was performed as described previously (Taketani and Oka, 1983 a). Cell culture was carried out for 4 days with a daily medium change. Cells were cultured with Medium 199 supplemented with 10% fetal bovine serum for the first 24 h and with Medium 199 containing 5% fetal bovine serum, insulin (5 µg/ml), cortisol (3 µM) and prolactin (5 µg/ml), with or without conditioned media obtained from cultures of mesenchymal cells, for the last 3 days.

Primary Culture of Embryonic Mesenchymal Cells

Mouse embryonic tissues were obtained from three different sources: the salivary gland, the mammary gland and the skin, and placed in Medium 199 containing 10% fetal bovine serum. The capsular mesenchyme of the salivary rudiments taken from 13–14 day fetuses was removed mechanically with cataract knives. The fat pad precursor tissues were removed from the mammary gland of 17 day female fetuses by the dissection technique described before (Sakakura *et al.*, 1982). Skin without prospective mammary fatty stroma was excised from 13–15 day fetuses. Separation of dermis and epidermis was achieved by a slightly modified trypsin digestion technique (Sakakura *et al.*, 1976). Skin was incubated in 2.5% trypsin for 40–60 min at room temperature. The mesenchymal components were dissected from the epithelium and were then placed in Medium 199 containing 50% fetal bovine serum for 2 h for the trypsin inactivation. Each mesenchymal tissue was transferred to a tissue culture flask. Culture was continued with Medium 199 containing 10% fetal bovine serum at 37°C in humidified air with 5% CO₂ with periodical medium changes. The conditioned media were collected and kept frozen until assay for their biological activities.

Co-culture of Mammary Epithelial Cells with Embryonic Mesenchymal Cells

Three different kinds of mesenchymal tissues were obtained from 14 day mouse embryos by the procedures described above. Cells of each mesenchyme were prepared by trypsin treatment and placed onto collagen gel-coated Falcon plastic tissue culture plates; salivary mesenchyme at a density of 1.7×10^5 /dish, mammary mesenchyme 1×10^5 /dish, and skin mesenchyme 1×10^5 /dish. After they were cultured with Medium 199 containing 10% fetal bovine serum for 2 days, mammary epithelial cells from lactating mice were added on the layer of mesenchymal cells at a density of 1×10^5 /dish according to the procedures as described in primary culture of mammary epithelial cells. Co-culture was continued in the presence of 10% fetal bovine serum for one day and 5% fetal bovine serum, insulin (5 µg/ml), cortisol (3 µM) and prolactin (5 µg/ml) for 43 h. Culture media were changed and culture was continued for another 43 h in the same fresh media.

DNA Synthesis

The extent of DNA synthesis was determined by allowing cells to incorporate ³H-thymidine (0.5 µCi/ml) into trichloroacetic acid-insoluble materials for 24 h (Taketani and Oka, 1983 a).

Casein Synthesis

The extent of casein synthesis was determined by allowing cells to incorporate L-³H amino acid mixture (10 µCi/ml) for 24 h. The amounts of casein released into media were measured by an indirect immunoprecipitation method (Ono and Oka, 1980).

Column Chromatography

Two ml of conditioned medium obtained from culture of mesenchymal cells of fetal skin was applied to a Sephadex G-200 column, 1.5 × 50 cm, and eluted in 2-ml fractions with 1 M acetic acid at a flow rate of 5 ml/h at 4°C. After absorbance was measured at 280 nm in a Gilford Spectrometer, each fraction was lyophilized and dissolved in Medium 199 containing 5% fetal bovine serum, insulin (5 µg/ml), cortisol (3 µM) and prolactin (5 µg/ml). The solution was added to the primary mammary epithelial cell culture in order to test its biological activities.

¹²⁵I-EGF Binding Assay

EGF binding assay was carried out slightly modified as described previously (Taketani and Oka, 1983 b). The incubation was performed at room temperature for 2 h with constant shaking. Each tube contained 2×10^6 mammary epithelial cells suspended in 250 µl of Medium 199 containing 0.25% of bovine serum albumin, 250 µl of diluted conditioned media (0–100%) obtained from culture of fetal skin mesenchyme or 250 µl of EGF (0–100 ng/ml) and 100 µl of ¹²⁵I-EGF (approximately 1.2×10^6 cpm) with or without 10 µl of unlabeled EGF (1 µg). Specific ¹²⁵I-EGF binding was measured by subtracting the amount of radioactivity bound nonspecifically in the presence of excess unlabeled hormone. These assays were conducted in triplicate.

Results

In order to examine the effects of mesenchymal cells on casein production by

mammary epithelial cells, three different kinds of embryonic mesenchymal cells were co-cultured with mammary epithelial cells for 4 days. Fig. 1 shows the amounts of casein which were produced in the last 43 h of these co-cultures. The presence of embryonic mesenchymal cells in direct contact with mammary epithelial cells markedly reduced the amounts of casein released into culture medium. All kinds of embryonic mesenchymal cells, *i.e.*, those from salivary gland, mammary gland and skin, had this inhibitory action on casein production regardless of their sources. Among these three mesenchymal tissues, mammary mesenchyme had the strongest inhibitory effect on milk protein synthesis.

The effects of conditioned media obtained from three different kinds of mesenchymal cell cultures on DNA synthesis and casein production in mammary epithelial cells are shown in Fig. 2. The addition of conditioned media into culture medium of mammary epithelial cell culture enhanced DNA synthesis 5–8 fold (I) and reduced casein synthesis by about a half (II). Al-

Fig. 1. Inhibition of casein synthesis in mammary epithelial cells by embryonic mesenchymal cells. Culture was performed as described in *Materials and Methods* under the following culture conditions: mammary epithelial cells alone (A), co-culture of mammary epithelial cells with salivary mesenchyme (B), co-culture with mammary mesenchyme (C), and co-culture with skin mesenchyme (D). The amounts of casein produced are expressed as cpm per 1×10^5 cells of each mesenchyme.

Fig. 2. Effects of conditioned media from embryonic mesenchymal cell cultures on DNA synthesis (I) and casein synthesis (II) in mammary epithelial cells. Mammary cells were cultured in medium containing 5% fetal bovine serum, insulin, cortisol, and prolactin (A) or in medium containing these agents and conditioned medium derived from salivary mesenchymal culture (B), mammary mesenchyme culture (C), or skin mesenchyme culture (D). ^3H -amino acid mixture or ^3H -thymidine was added into the medium in the final 24 h of culture.

though the conditioned medium obtained from culture of fetal skin mesenchyme had the strongest activity, it was difficult to quantitatively compare the activities of the three different kinds of embryonic mesenchymal cells because the conditioned media were not always obtained under the same culture conditions. Fig. 3 shows a clear dose-dependent relationship between the

amount of conditioned media obtained from culture of fetal skin mesenchyme and the stimulation of DNA synthesis (I) as well as the inhibition of casein synthesis (II).

The results of experiments on the stability of this substance are summarized in Table 1. The treatment of conditioned medium obtained from culture of fetal skin mesenchyme with various doses of trypsin

Fig. 3. Effect of different amounts of conditioned medium from culture of fetal skin mesenchyme on DNA synthesis (I) and casein synthesis (II) in mammary epithelial cells. Mammary cells were cultured in medium containing 5% fetal bovine serum, insulin, cortisol, prolactin, and the amount of conditioned medium indicated. ^3H -amino acid mixture or ^3H -thymidine was added into the medium in the final 24 h of culture.

Table 1. Effects of treatment of conditioned medium from fetal skin mesenchyme with trypsin or heating on DNA synthesis in cultured mammary epithelial cells. Mammary cells were cultured in medium containing fetal bovine serum, insulin (I), cortisol (F), and prolactin (P) with or without conditioned medium (C. M.). Conditioned media were treated with trypsin at concentrations indicated or with heating at 60°C for 30 min.

Culture Conditions	Thymidine Incorporation cpm/dish/24 h
IFP	(4) 4098 $\pm$ 251
IFP + C. M.	(4) 43514 $\pm$ 1652
IFP + C. M. treated with 0.1 mg/ml of trypsin	(4) 33542 $\pm$ 683
IFP + C. M. treated with 0.5 mg/ml of trypsin	(4) 28129 $\pm$ 779
IFP + C. M. treated with 1.0 mg/ml of trypsin	(4) 13969 $\pm$ 302
IFP	(4) 3805 $\pm$ 311
IFP + C. M.	(4) 45124 $\pm$ 801
IFP + C. M. heated at 60°C for 30 min	(4) 29770 $\pm$ 726

Fig. 4. Sephadex G-200 column chromatography of conditioned medium from a culture of fetal skin mesenchyme. Each fraction was tested for DNA synthesis (●—●), casein synthesis (○---○) and absorbance at 280 nm (•—•). The column was calibrated by using the following molecular weight standards: aldolase (A), 158,000 MW; bovine serum albumin (B), 68,000 MW; cytochrome C (C), 12,500 MW; and insulin (D), 6,000 MW. Blue dextran was used to identify the void volume of this column (V. V.).

Fig. 5. Effect of conditioned medium from culture of fetal skin mesenchyme on the amount of specific ¹²⁵I-EGF binding of mammary epithelial cells. Mammary cells were incubated in medium containing 0.25% bovine serum albumin and ¹²⁵I-EGF (approximately 1.2×10^5 cpm per tube) with diluted conditioned media or EGF standards.

decreased its stimulatory activity on DNA synthesis in a dose-dependent fashion. The heating of conditioned medium from culture of fetal skin mesenchyme at 60°C for 30 min reduced its activity by 34%.

Fig. 4 shows the elution profile of Sephadex G-200 column chromatography of conditioned medium obtained from a culture of fetal skin mesenchymal cells. The peak having the activity of stimulating DNA synthesis was observed in fractions around 17 indicating that its molecular weight was approximately 100,000. In these fractions, the activity of casein synthesis was suppressed. The slightly inhibitory effect on DNA synthesis was detected in fractions around 27, in which the production of casein was also inhibited.

The addition of all diluted conditioned media obtained from a culture of fetal skin mesenchymal cells did not change ^{125}I -EGF specific binding in mammary epithelial cells in spite of their strong ability to enhance DNA synthesis and to inhibit casein production, indicating that this substance cannot compete with EGF for EGF receptor (Fig. 5).

Discussion

The present study has shown that fetal mesenchymal cells secrete a substance(s) which can enhance DNA synthesis and inhibit casein production in mouse mammary epithelial cells in culture. These effects of this substance are very similar to those of EGF in mammary epithelial cells in the same culture system, which was previously reported in our laboratory. However, it appears that this substance is quite different from EGF based on its molecular weight. Furthermore, the addition of anti-EGF antibody did not abolish these biological actions of this substance and it did not compete with EGF for EGF receptor in mammary epithelial cells. Sephadex G-200

column chromatography revealed that the peak which has activity capable of inducing DNA synthesis and reducing casein synthesis has a molecular weight of approximately 100,000.

Recently, EGF-like transforming growth factors (TGFs) have been identified in mouse and rat embryos (Heine *et al.*, 1987; Matrisian *et al.*, 1982; Twardzik *et al.*, 1982). Twardzik *et al.*, (1982) reported that growth factors, which stimulate anchorage-independent growth of normal mouse and rat fibroblasts and have the ability to compete with ^{125}I -labeled EGF for receptor binding, can be isolated from 12-to 13-day-old normal mouse embryos of various strains. Although this growth factor identified in this study was derived from fetal mesenchymal tissue, it is different from that which Twardzik *et al.* reported because it does not possess the activity to bind to EGF receptor and the molecular weights of these two growth factors are quite different. The molecular weight of this growth factor is greater than those of TGFs isolated so far which have a molecular weight of 6000–25000 (Roberts *et al.*, 1983).

This growth factor is sensitive to trypsin, which is common with TGF reported in rat fetus (Matrisian, 1982), but some properties such as lack of heat stability are different from those of TGFs.

Our cell culture system for mammary epithelial cells offers a sensitive assay for DNA synthesis as well as casein production. By using this system, we could demonstrate that this growth factor strongly inhibits cell differentiation. This finding is very important since there has been little study on the effects of TGFs on the functional aspect of cell differentiation.

There are several mechanism for regulating epithelial-mesenchymal interaction (Cunha *et al.*, 1983; Fleischmajer and Billingham, 1968; Grobstein, 1967; Neubauer *et al.*, 1983; Sakakura *et al.*, 1979; Sawyer and Fallon, 1983). In mammary gland

development, the inductive interactions between epithelium and mesenchyme are essential for normal morphogenesis in the fetal stage (Kratochwil, 1969; Sakakura, 1987) and also in adult life (Sakakura *et al.*, 1979). The dependency of mammary epithelium in its morphology on the mesenchymal stroma is shown. Levine and Stockdale (1984) reported that the interaction of mammary epithelium with adipocytes resulted in a marked increase in the proliferation of mammary epithelium and that extra cellular components may mediate this effect. The following question then arises: What role does the fetal mesenchyme-derived growth factor play in mammary gland development? Our present study raised the possibility that this growth factor controls the cell function of epithelium by exerting a stimulatory effect on cell proliferation and an inhibitory effect on cell differentiation. However, mammary epithelium, which is quite different from other epithelia, proliferates after puberty with each ovarian cycle and further proliferates during each successive pregnancy. Is this growth factor released from fetuses, and does it promote the epithelial cell proliferation of the mother mammary glands through the placenta? To answer this question, further studies are necessary to identify this humoral factor in the serum of the pregnant mouse and to investigate the tissue and function specificity by changing the combinations of epithelial and mesenchymal cells. The present studies antecedently indicate that the growth factor released from fetal mesenchyme seems to have these activities regardless of the origin of tissue: mesenchymal cells from salivary gland and skin also have a biological action on mammary epithelium as mammary mesenchyme does, although this action is not as strong as that of mammary mesenchyme. This suggests the presence of two different types of growth factors in embryonic mesenchyme; one is tissue specific and the other is a tissue non-specific growth factor.

The analysis by column chromatography revealed that conditioned medium from fetal mesenchymal cell culture contained not only stimulatory substance but also inhibitory substance in DNA synthesis, depending on the difference in molecular weight. The physiological role of this inhibitory action of mesenchyme on epithelial proliferation is unknown.

Many newly discovered peptide growth factors are produced and released in the immediate vicinity of their site of action and such a control of cell growth and differentiation is considered to be a primitive regulatory mechanism (Bradshaw and Sporn, 1983). The growth factor identified in our study may exert its effect by this mechanism. This regulatory system probably is fundamentally important for epithelial-mesenchymal interaction as well as for the fetal development.

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