Mouse Mammary Epithelium Produces a Soluble Heat-Sensitive Macromolecule that Inhibits Differentiation of 3T3-L1 Preadipocytes (43496)

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Abstract. **Coculture of normal mouse mammary gland (NMMG) epithelial cells with 3T3-L1 preadipocytes resulted in inhibition of triglyceride accumulation. This inhibition was also observed when the NMMG cells were grown in inserts and placed within a 100-mm dish containing confluent 3T3-L1 cells. As the number of NMMG-containing inserts was increased, there was a progressive decline in triglyceride content of the 3T3-L1 cells. Conditioned medium from NMMG cells also resulted in a dose-dependent inhibition of adipocyte formation, and when concentrated 10-fold by passage through a filter with a cutoff of 30 kDa, all of the inhibitory activity was recovered. Heating the concentrated conditioned medium at 98°C for 30 min resulted in complete loss of activity. Of several peptides tested, transforming growth factor-β, platelet-derived growth factor, tumor necrosis factor, interleukin 6, and basic fibroblast growth factor showed inhibitory activity, whereas epidermal growth factor, insulin-like growth factor I, and transforming growth factor-α did not.** [P.S.E.B.M. 1992, Vol 201]

The normal development of a mammary gland involves intricate communication among different cell types. Epithelial and stromal cells (adipocytes, for example) interact with both positive and negative signals hormonally regulated as well as through specific paracrine and possible autocrine interactions (1). During the ovarian cycle, pregnancy, and lactation, the development of parenchymal tissue is usually accompanied by the involution of stromal components of the gland (2).

Several possibilities could account for these interactions: (i) hormones (steroid or peptide) or growth factors may be synthesized and secreted locally or systemically; (ii) hormones may be concentrated locally by the specific receptors of the different cell types; (iii) local metabolism of these substances may result in secretion of new factors; or (iv) these hormonal factors could result in a differential synthesis of extracellular matrix. In turn, this matrix could act as a supportive frame for the newly synthesized structures during the development of the gland and also modify gene expression with alteration of the physiological function of the gland.

Although a cause and effect relation in development has not been as well demonstrated for the extracellular matrix as for the mesenchyme, the phenomenon is easily demonstrable in culture in terms of maintenance of expression of tissue-specific functions (3). Our own investigations provide evidence for a change in the proteoglycan components of the extracellular matrix, during differentiation of 3T3-L1 preadipocytes in culture (4).

The intimate relation between the mammary epithelium and the adjacent adipose tissue has been clearly established *in vivo* as well as *in vitro*.

Kratochwil (5) showed that recombination of isolated mammary epithelium with mammary mesenchyme resulted in a mammary pattern, whereas recom-

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bination with salivary mesenchyme resulted in a pattern consistent with the salivary gland. Interaction of the mammary epithelium with the fat pad precursor appeared to be an obligatory step in determining the characteristic structure of the mammary gland in early embryogenesis (6).

In the present study, we utilized a model system to facilitate the investigation of bidirectional metabolic, humoral, and/or paracrine interactions between epithelial and stromal (adipose) components of mammary gland, by using two well-characterized, established cell lines, normal mouse mammary gland (NMMG) epithelial cells (7, 8) and 3T3-L1 preadipocytes (9–11). These cell lines have been shown to resemble the original when injected into animals (7, 8). The 3T3-L1 cells were the chosen model for several studies on adipocyte function and regulation (12).

We now report evidence for the existence of a soluble protein factor secreted by the mouse mammary epithelial cells involved in the differentiation of 3T3-L1 mouse preadipocytes.

Materials and Methods

Cells and Reagents. NMMG epithelial cells (7, 8), 3T3 cells (fibroblast, Swiss albino mouse), and 3T3-L1 cells (preadipocytes, murine) (9, 10) were obtained from American Type Culture Collection, Rockville, MD. Dulbecco's modified minimum essential (DMEM) medium, with 25 m*M* glucose, and Hanks' balanced salt solution were purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Gibco and from INOVAR (Gaithersburg, MD). Mouse recombinant tumor necrosis factor was purchased from Genzyme Corp. (Cambridge, MA). All the other growth factors and cytokines were purchased from BioSource International (Camarillo, CA).

Cell Culture. Cells were cultured in DMEM medium containing 10% FBS (10% FBS-DMEM), pH 7.4, at 37°C under 5% CO_2. Medium was changed every 48–72 hr, and cells were subcultured before confluence was attained. After washing the cells twice with 10 ml of Hanks' balanced salt solution, cells were detached from the 100-mm culture dish by exposing them to 2 ml of a 0.8% trypsin solution in Hanks' balanced salt solution, containing 2 m*M* EDTA, at 25°C for 3 to 4 min. This reaction was stopped by the addition of 2 ml of 10% FBS-DMEM. The cell suspension was transferred with a plastic pipette to a sterile centrifuge tube and cells were collected by centrifugation at 1000 rpm (800*g*) for 5 min. Cells were resuspended in 10% FBS-DMEM at a concentration of 10^5 cells/ml and transferred to a 100-mm plastic dish (1 ml of cell suspension plus 9 ml of 10% FBS-DMEM). The cells were usually subcultured every 4 to 5 days.

Differentiation of 3T3-L1 Preadipocytes into Adipocytes. The 3T3-L1 cells spontaneously differentiate into mature adipocytes 10 to 20 days after becoming confluent. However, differentiation can be achieved in less than 1 week by culturing the cells with a mixture of 0.5 m*M* 3-isobutyl-1-methylxanthine and 0.1 μM dexamethasone in 10% FBS-DMEM for 48 hr (13) and then transferring the cells to fresh 10% FBS-DMEM containing insulin (10 μg/ml = 2 μM). After this treatment, 90–100% of the cells will dramatically increase their triglyceride content in 2–3 days, with formation of clearly visible refractive droplets observable by conventional or dark field microscopy, and both micro- and macroscopically by oil red O staining.

Coculture of NMMG and 3T3 or 3T3-L1 Cells. When different cell lines were cocultured together (NMMG and 3T3, or NMMG and 3T3-L1), both cell types were added to the same culture dish at the same time and cell number. Culture medium was changed every 48–72 hr as usual.

Interaction of NMMG and 3T3 or 3T3-L1 Cells Using Millicell Inserts. To study humoral interactions with a physical barrier between the cells to prevent direct cell-cell contact, one cell type was cultured inside a 30-mm Millicell-CM insert (Millipore) and the other cell type was cultured, as usual, on a 100-mm plastic dish. The insert was coated previously with 0.5 ml of Matrigel (Collaborative Research, Bedford, MA) according to the manufacturer's instructions. Cells were grown in the inserts until embedded within the matrix in 100-mm dishes containing 10% FBS-DMEM, and then the inserts were transferred to 100-mm dishes containing confluent cultures of the other cell type (e.g., NMMG, 3T3, or 3T3-L1 cells).

Conditioned Medium from Cultured Cells. Conditioned medium was collected from NMMG cell cultures. Cells were permitted to achieve confluence and conditioned medium was collected every 4 days. The medium was immediately filter-sterilized (0.2-μm membrane pore) and concentrated 10-fold through a 30 kDa cutoff membrane (YM30; Amicon, Beverly, MA), aliquoted, and kept frozen at −20°C until used.

Growth Factors and Triglyceride Accumulation. The following growth factors were added 2 days before the differentiation mixture (3-isobutyl-1-methylxanthine and dexamethasone) was used and replenished every 48 hr with every change of medium: epidermal growth factor (250 ng/ml), transforming growth factor [TGF-α] (100 ng/ml), insulin-like growth factor I (250 ng/ml), colony-stimulating factor (100 units/ml), basic fibroblast growth factor (10 ng/ml), platelet-derived growth factor (30 ng/ml), tumor necrosis factor (15 ng/ml), interleukin (IL)-6 (3000 units/ml), and TGF-β (0.5 and 1.0 ng/ml). Cells were grown in 6-well plates (35 mm each well) in a total volume of 2 ml of 10% FBS-DMEM.

The triglyceride content of the cells was used as the principal end point of preadipocyte differentiation. Cells were washed once with 0.15 *M* NaCl and 0.01 *M* phosphate-buffered saline at room temperature, and 1 ml of water was then added to each culture. The cells were scraped using a rubber policeman and transferred to a 15-ml plastic centrifuge tube. The cell suspension was sonicated at maximal output for 15 sec with a microtip, and 100 μl were saved for DNA determination. Triglycerides were determined using the Sigma colorimetric determination kit (Cat. no. 240; Sigma Chemical Co., St. Louis, MO) as instructed by the manufacturer.

DNA was determined in the aqueous sample (100 μl) using the diamino benzoic acid technique (14, 15).

Results

Figure 1 shows the results from the coculture of NMMG and 3T3-L1 preadipocytes. The two cell types were plated together simultaneously, and when the culture was confluent, differentiation of the 3T3-L1 cells was induced by stimulation with 3-isobutyl-1-methylxanthine, dexamethasone, and insulin. The same treatment was applied to individual control cultures of NMMG and 3T3-L1 cells. While the preadipocytes responded with a large accumulation of triglycerides, the NMMG cells accumulated very little (data not shown). The combination of both cell types resulted in a marked inhibition of total triglyceride accumulation. This was also confirmed by direct microscopic examination for lipid droplets and by oil red O staining, observed macro- or microscopically.

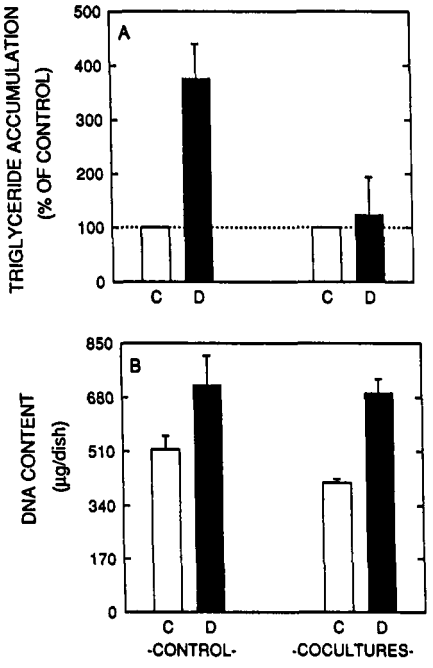

Figure 1. Triglyceride accumulation in 3T3-L1 or NMMG cells cultured alone and when cocultured together. An equal number of cells was plated. After cultures reached confluence, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, and insulin were added. Triglycerides and DNA were determined on each culture dish. (A) Control: triglycerides in individual NMMG cultures, and triglycerides in individual 3T3-L1 cultures were determined, expressed per mg DNA, and added to obtain the 100% value. Cocultures: triglycerides in cultures containing both cell types. Open bars: no IBMX, dexamethasone, or insulin added. Solid bars: plus IBMX, dexamethasone, and insulin. (B) Control: DNA was determined in the individual NMMG and 3T3-L1 cultures, and added to get the 100% for comparison. Cocultures: DNA was determined in the cultures containing both cell types growing together. Results are the mean of triplicate cultures ± SE.

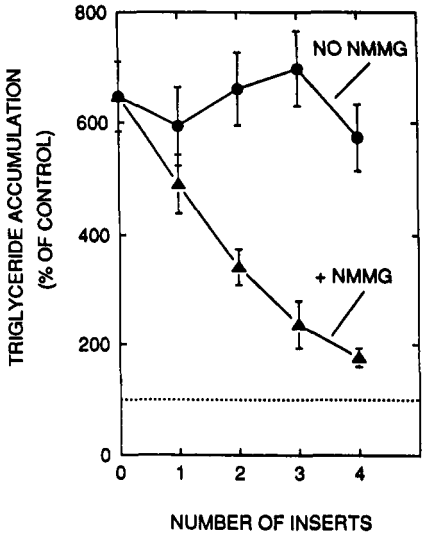

Figure 2. Triglyceride content of 3T3-L1 cells cultured beneath Matrigel-coated Millicell-CM inserts containing NMMG cells. After 3 days of culture, one, two, three, or four of these inserts were placed over confluent 3T3-L1 cells in 100-mm dishes. Inserts without NMMG cells but coated with Matrigel served as controls. 3T3-L1 cells without inserts were included as positive controls for differentiation. After 8 days of culture, the 3T3-L1 cells were exposed to 3-isobutyl-1-methylxanthine and dexamethasone for 48 hr and insulin thereafter. The inserts were then removed and the triglycerides in the 3T3-L1 cells were measured. Each point represents the mean ± SE of triplicate cultures.

Direct coculture experiments may reflect metabolic, humoral, or paracrine effects, but direct cell-cell interaction (e.g., gap junctions), contact-inhibition, substrate availability, or other factors may also be involved. Although the DNA contents were additive (Fig. 1B), the relative numbers of both cell types were difficult to quantify.

To demonstrate that inhibition of triglyceride accumulation was not an artifact of the coculture system, both cell types were cocultured separated by a permeable membrane that would allow the passage of the soluble molecules but prevent direct physical contact between the two different cell types. Figure 2 shows the triglyceride accumulation by 3T3-L1 cells in the presence of inserts containing NMMG cells. A dose-response inhibition with increasing number of inserts (1, 2, 3, or 4) was observed. This implied the soluble nature of the inhibitory activity, and indicated that no direct contact between the two cell types was necessary for

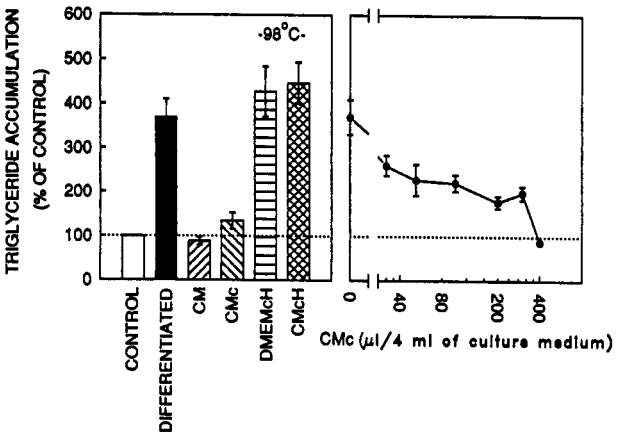

Figure 3. Triglyceride content in 3T3-L1 cells cultured in the presence of conditioned medium from confluent cultures of NMMG cells. (Left) Open bar: control cells, in 10% FBS-DMEM; solid bar: differentiated cells (after 3-isobutyl-1-methylxanthine, dexamethasone, and insulin for 4 days, always in 10% FBS-DMEM); stippled bars: CM, 4 ml of conditioned medium containing 10% FBS; CMc, 400 µl of the 10-fold concentrated conditioned medium plus 10% FBS-DMEM to keep volume constant; DMEMc, 400 µl of 10% FBS-DMEM, 10-fold concentrated plus the regular 10% FBS-DMEM to keep volume constant (this control was added to discard a direct effect of the concentrated culture medium). (Right) Dose-response curve for concentrated conditioned medium from NMMG cells in a total volume of 4 ml of 10% FBS-DMEM.

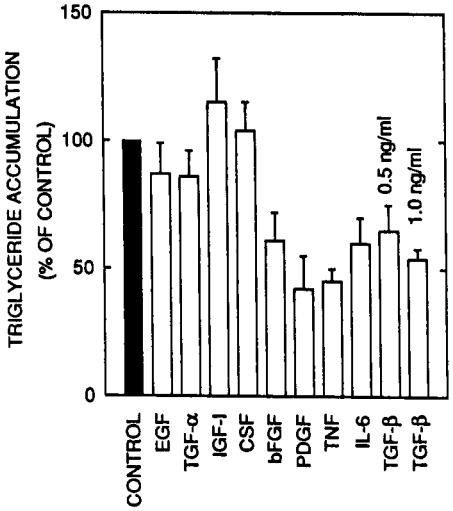

Figure 4. Triglyceride content in 3T3-L1 cells cultured in the presence of different growth factors and cytokines. Epidermal growth factor ([EGF] 250 ng/ml), TGF-α (100 ng/ml), insulin-like growth factor ([IGF-I] 250 ng/ml), colony-stimulating factor ([CSF] 100 units/ml), basic fibroblast growth factor ([bFGF] 10 ng/ml), platelet-derived growth factor ([PDGF] 30 ng/ml), tumor necrosis factor ([TNF] 15 ng/ml), IL-6 (3000 units/ml), and TGF-β (0.5 and 1.0 ng/ml), were added to confluent 3T3-L1 cells 2 days before the addition of 3-isobutyl-1-methylxanthine and dexamethasone. The factors were replenished every 48 hr, whenever the culture medium was changed. The results are expressed as percentage of control; the control corresponds to 3T3-L1 cells differentiated in the presence of 3-isobutyl-methylxanthine, dexamethasone, and insulin, in the absence of any other factor. Bars represent mean ± SE of triplicate cultures.

this inhibition to occur (confirmed by light microscopy).

Figure 3 shows the effect of conditioned medium from NMMG cells on the triglyceride accumulation by 3T3-L1 cells. The conditioned medium is able to reproduce the inhibitory activity observed previously by cocultures and use of Millicell-CM inserts. Moreover, when the conditioned medium was concentrated 10-fold by filtration through a membrane of 30-kDa cutoff, the inhibition was still observed and a dose-response curve could be obtained with increasing volumes of the concentrated conditioned medium (Fig. 3).

When the medium was incubated for 30 min at 40, 60, and 98°C, the activity was lost at the highest temperature and the results were variable at 60°C. No loss of activity was observed at 40°C. This suggests the protein nature of the molecule(s) involved in this inhibition.

Several factors are known to inhibit triglyceride accumulation by 3T3-L1 cells. These include basic fibroblast growth factor (16), TGF-β (17), platelet-derived growth factor (16), tumor necrosis factor (17), IL-1 (18), and IL-6 (our observations). Epidermal growth factor, TGF-α, colony-stimulating factor, and insulin-like growth factor I had no effect on the triglyceride accumulation. In contrast, platelet-derived growth factor, basic fibroblast growth factor, bFGF, IL-6, and tumor necrosis factor were all inhibitory of the triglyceride accumulation by 3T3-L1 cells in our culture system (Fig. 4).

Discussion

The original goal of the present investigation was to look for factors synthesized by mammary epithelium that regulate differentiation and de-differentiation of the adjacent adipose tissue.

The results demonstrate the presence of factors produced by NMMG cells that inhibit the triglyceride accumulation by 3T3-L1 preadipocytes.

The factor(s) responsible for this inhibition was secreted by the epithelial cells into the extracellular medium. Conditioned medium from the mammary epithelial cells contained inhibitory activity that was retained by a membrane with a nominal 30-kDa cutoff. Heating at 98°C for 30 min completely destroyed the inhibitory activity. These results strongly suggest the presence of a secreted protein factor that inhibits triglyceride accumulation in preadipocytes. The presence of 10% fetal bovine serum in the culture medium precluded the protease sensitivity study that we attempted with trypsin. Serum-free conditions should be set up for the purpose of this study, as well as to facilitate the characterization and isolation of the inhibitor.

Several molecules have been shown to inhibit triglyceride accumulation as well as the expression of enzymes specifically related to lipid metabolism. Tu-

mor necrosis factor (19), interleukin 1 (20, 21) and interleukin 6 (our observations), transforming growth factor-β (17), basic fibroblast growth factor, and platelet-derived growth factor (16) are all molecules that inhibit the normal expression of the adipocyte genotype and phenotype. All these peptides inhibited triglyceride accumulation in 3T3-L1 preadipocytes in our culture system. In contrast, several other peptides, including epidermal growth factor, insulin growth factor I, transforming growth factor-α, and colony-stimulating factor, had no inhibitory effect.

Some of these substances might correspond to our putative triglyceride inhibitory factor (TGIF), although indirect results might allow the exclusion of many of them. For example, basic fibroblast growth factor, platelet-derived growth factor, and transforming growth factor-β stimulate the [^{3}H]glucosamine and [^{35}S]sulfate incorporation into hyaluronic acid, glycoproteins, and/or proteoglycans synthesized by 3T3-L1 cells (data not shown, to be published elsewhere). The conditioned medium from NMMG cells does not modify this incorporation when compared with control cells. Tumor necrosis factor produces cellular lysis, as evidenced by the presence of multiple vacuolated 3T3-L1 cells (Ref. 18 and our observations), whereas TGIF does not modify the cell morphology (our observations). Although these observations are strong enough to assume nonidentity, further studies are needed to rule out the possibility of TGIF being one of the previously mentioned molecules. These studies should include purification by affinity and size column chromatography, ammonium sulfate precipitation, use of specific antibodies, etc.

Although most of the growth factors can be ruled out because they are smaller than 30 kDa, one must be aware that the membrane cutoff is not an absolute measurement of molecular weight. Also, the possibility of the factor being bound to a carrier protein cannot be ruled out.

Our model system requires the triglyceride-inhibiting factor to be specifically produced by the mammary epithelium and act, possibly in a paracrine fashion, on the preadipose components of the stroma. This will require the screening of other normal epithelial cell lines or primary cultures from different tissue origin.

Considerable interest has also evolved in studying the role of the extracellular matrix components as regulators of organogenesis, morphogenesis, and differentiation (22). Our laboratory is presently investigating the changes in the hyaluronic acid and proteoglycan components of the extracellular matrix in 3T3-L1 cells during their differentiation (4) in order to further explore the connections between the stromal and the parenchymal components in the mammary gland. We will also consider the possible role of the proteoglycans as "local concentrators" of active molecules such as growth factors (23).

After purification and characterization of the inhibitory activity, the evaluation of its physiological importance in the regulation of the *in vivo* function of the mammary gland needs to be done. To accomplish this, a change to a primary culture system using animals in different developmental stages (immature, pubertal, adult, pregnant, and lactating) will be required to try to identify the presence of the TGIF activity.

Although the extrapolation of results obtained with cells in culture to the *in vivo* situation is not always direct, the results presented herein are thought provoking and could lead to interesting directions in the study of the regulation of the mammary gland function and development.

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