Identification of a Protein Factor Secreted by 3T3-L1 Preadipocytes Inhibitory for the Human MCF-7 Breast Cancer Cell Line

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ABSTRACT

The 3T3-L1 cell line is a preadipocyte cell line derived from the Swiss 3T3 mouse fibroblast cell line. We have compared the effect of 3T3-L1 conditioned medium (3T3-L1 CM) and Swiss 3T3 conditioned medium (3T3 CM) on the growth of normal mouse mammary cells (NMMG) and the human MCF-7 breast carcinoma cell line. 3T3 CM increased the growth of both NMMG and MCF-7 cells by 19 ± 2% (SD) and 24 ± 3%, respectively, and increased thymidine incorporation by 74 ± 4% and 104 ± 8%, respectively. Conditioned medium from 3T3-L1 cells stimulated the growth of NMMG cells by 64 ± 2%; in contrast, 3T3-L1 CM inhibited the growth of MCF-7 cells by 36 ± 1%. In parallel with these growth studies, thymidine incorporation increased by 82 ± 6% decrease in thymidine uptake, respectively, and increased thymidine incorporation by 72 ± 5% in the MCF-7 cells.

Moreover, a similar effect was also noted in NCI H630 colon cancer cells, where 3T3-L1 CM produced a 58 ± 4% decrease in growth and a 82 ± 6% decrease in thymidine incorporation. Heating the 3T3-L1 CM at 100°C for 30 min destroyed all inhibitory activity. Several known inhibitory growth factors (fibroblast growth factor, 20 ng/ml; interleukin 6 and fibroblast growth factor had no effect. Neither transforming growth factor β nor tumor necrosis factor α activity was detectable in 3T3-L1 CM using an enzyme-linked immunosorbent assay. High-performance liquid chromatography fractionation of the 3T3-L1 CM revealed that the inhibitory activity eluted at a molecular weight of 67,000; moreover, silver staining of these eluates on a denaturing polyacrylamide gel revealed that Mr 69,000 peptide was the predominant protein band in the inhibitory fractions. Thus 3T3-L1 CM stimulates the growth of normal breast epithelial cells and inhibits the growth of MCF-7 breast cancer cells. This inhibitory activity appears to be due to a protein secreted by 3T3-L1 preadipocytes.

INTRODUCTION

The normal growth and development of the mammary gland is a complex interaction between a variety of cell types as well as a regulated hormonal environment. Normal breast epithelial cell development is coordinated under normal physiological states by multiple growth factors and hormones. These include hormones such as estrogen and progesterone, prolactin, glucocorticoids, and insulin; growth factors such as epidermal growth factor and insulin growth factor; as well as other peptides such as vitamin D and retinoids (1–3). Most of these factors are derived from endocrine glands such as the pituitary, gonadal, and adrenal glands.

Local growth-regulatory peptides are elaborated by mammary stromal cells, particularly by fibroblasts, preadipocytes, and adipocytes. These paracrine factors promote the growth, morphological development, and differentiation of mammary epithelium (4–6). The growth-stimulatory effect of adipocytes on mammary epithelium has been suggested by the observation that successful transplantation of mammary epithelial tissue depends on the presence of adipose tissue as well as epithelial cells. Several investigators have made the observation that transplanted mammary epithelium will grow only in those sites where adipose tissue is present in the host (7–10). Moreover, local growth factors produced by cells of the fat pad have been shown to be responsible for the growth of mammary tumor end buds (11). There have been several recent reports of mesenchymal cells that stimulate the growth of normal breast epithelial cells in a paracrine mode. A Mr 76,000 peptide that stimulates the growth of embryonic and adult mammary epithelial cells has been purified from embryonic mammary mesenchymal tissue (12).

Levine and Stockdale (13, 14) have demonstrated that the 3T3-L1 cell line, a subclone of Swiss 3T3 cells, has the morphological and biochemical characteristics of adipocytes and can promote the growth and hormone-dependent differentiation of normal mammary epithelium. In addition, Enami et al. (15) have described a growth-stimulatory effect on normal and neoplastic mammary epithelial cells using conditioned medium obtained from mouse mammary fibroblasts. Mammary epithelial cells incubated in contact with 3T3-L1 adipocytes have also been shown to produce casein several hours after the addition of prolactin; in the absence of 3T3-L1 cells no casein is produced (16). These effects appear to be partially mediated by growth factors and by the deposition of extracellular materials that promote the growth and differentiation of mammary epithelium; however, the identification and characterization of these growth factors produced by 3T3-L1 adipocytes has not yet been accomplished.

To date there have been no reports of growth factors secreted by 3T3 or 3T3-L1 cells that inhibit the growth of either normal or malignant mammary cells. The objective of this work was to investigate the differential effect of 3T3-L1 preadipocytes on the growth regulation of normal and malignant mammary epithelial cells.

MATERIALS AND METHODS

Cells. The characteristics of the 3T3-L1 preadipocytes, Swiss 3T3 fibroblasts, NMMG2 epithelial cells, human MCF-7 breast carcinoma cells, and NCI H630 colon carcinoma cells have been previously described (17–20). These cells were obtained from the American Type Tissue Culture Collection (Rockville, MD). Lipids that are present in commercial preparations of phenol red have been shown to have estrogenic activity (21). To prevent possible steroid-like artifacts from these compounds, the cell lines were maintained in DMEM without phenol red. The medium also contained 10% FBS, penicillin (1000 units/ml), streptomycin (1 mg/ml) (Gibco, Inc., Grand Island, NY), and 2 mM glutamine. All cells were grown in 75-cm² plastic culture flasks (Falcon Labware, Oxnard, CA) at 37°C in a humidified 5% CO² atmosphere. The cells were subcultured every 5–7 days.

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2 The abbreviations used are: NMMG, normal mouse mammary gland; CM, conditioned medium; ELISA, enzyme-linked immunosorbent assay; HPLC, high-performance liquid chromatography; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; FGF, fibroblast growth factor; TGF, transforming growth factor; TNF, tumor necrosis factor; IL-6, interleukin 6; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IFN, interferon.
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Ninety-six-well immunonl plates were purchased from Dynatech (Chantilly, VA), and alkaline phosphatase-labeled immunoaffinity-purified goat anti-rabbit immunoglobulins were purchased from Kirckgaard and Perry Laboratories (Gaithersburg, MD). The [3H]thymidine (specific activity, 15 Ci/mmol) was obtained from New England Nuclear (Boston, MA). FGF, TGF-β, TNF-α, and interleukin 6 (IL6) were obtained from Genzyme (Boston, MA). Antibodies to TGF-β and TNF-α were obtained from R&D Systems (Minneapolis, MN). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or the NIH stores.

Preparation of 3T3-L1 Conditioned Medium. 3T3-L1 cells (preadipocytes) were plated at a density of 5 × 10^4/cm^2 onto tissue culture flasks (Falcon) and incubated at 37°C in DMEM plus 10% FBS. Two days after the cells reached confluency (the point where cells merge together), 10% of cells appeared rounded and enlarged and began to accumulate lipid droplets. At this time the cells were washed with Hanks' balanced salt solution two to three times, and DMEM was added without serum to produce 3T3-L1 serum-free conditioned media. After 2 days, the serum-free conditioned medium was removed and filtered through a 0.2-μm filter and stored at 4°C. The conditioned medium was concentrated 10-fold through an Omegacell ultrafiltration membrane (Pharmacia, Piscataway, NJ), with a 30,000 molecular weight cutoff, at 4°C under positive pressure using nitrogen. As a control, Swiss 3T3 fibroblast-conditioned medium was produced in an identical fashion.

Cell Growth Experiments. NMMG cells, MCF-7 cells, and NCI H630 cells were plated at a density of 5 × 10^4 cells/35-mm well and incubated at 37°C in 1.8 ml of DMEM plus 10% FBS. After 24 h the cultures were washed and refed with fresh medium supplemented with 200 μl 10x concentrated 3T3-L1 or 3T3 conditioned medium (1:1 final dilution). Control cells were supplemented with 200 μl of DMEM. All growth assays were performed in triplicate. At 24, 48, 72, and 96 h the cells were detached by addition of 0.25% trypsin and 0.2% EDTA for 5 min. Detached cells were collected and counted using a hemocytometer.

[3H]Thymidine Incorporation. NMMG cells, MCF-7 cells, and NCI H630 cells were plated at a density of 5 × 10^4 cells/well in 6-well plates (Costar, Cambridge, MA) in 2 ml of DMEM supplemented with 10% FBS and 2 μg/ml tunicamycin. After 24 h the cultures were washed and refed with fresh medium supplemented with 200 μl of 3T3-L1 or 3T3 concentrated conditioned medium (1:1 final dilution) or 200 μl of DMEM in control plates. After 48 h [3H]thymidine incorporation was measured by incubating the cells in the exponential phase of growth with 1 μCi/ml of [3H]thymidine in DMEM for 3 h. Following the [3H]thymidine pulse the medium was removed, and the cells were then washed two times with Hanks' balanced salt solution and two times with cold 5% trichloroacetic acid and solubilized in 2 ml of 0.5 N sodium hydroxide. Total counts incorporated were determined in a Beckman liquid scintillation counter.

Growth Factor Studies. NMMG cell, MCF-7 cell, and NCI H630 cell conditioned medium on the proliferation of normal and malignant mammary epithelial cells. A final dilution of 1:1 conditioned medium from 3T3-L1 or 3T3-L1 conditioned medium inhibited the growth of malignant MCF-7 breast cells by 36 ± 1% (Fig. 1). Incubation of cells with 3T3-L1 conditioned medium showed no inhibitory effect on the growth of NMMG cells (Fig. 2). In contrast, 3T3-L1 conditioned medium inhibited the growth of malignant MCF-7 breast cells by 36 ± 1% (Fig. 1).

HPLC Fractionation of the 3T3-L1 CM. A 0.2-ml aliquot of a 10x concentrated sample of conditioned medium was applied to a HPLC size exclusion column (Hydropore-S-Sec 4.6 × 250 mm; Ranin Instruments, Woodburn, MA) having a usable molecular weight range up to 1000 kDa. The column was developed using a flow rate of 0.1 ml/min of 0.1 M KH2PO4, pH 7.0, under isocratic conditions. One hundred-μl fractions were collected for 30 min. The molecular weight of the protein of interest was determined by comparison of the retention time of the inhibitory activity with the protein standards. Each fraction was tested for the inhibitory activity of MCF-7 breast carcinoma cells using both the growth-inhibition assay and the inhibition of thymidine incorporation.

SDS-PAGE Analysis of HPLC Fractions. Fifty μl of the HPLC column eluates were resolved on a 15% SDS-PAGE according to the method of Laemmli (25). The gel was subsequently silver stained using the commercially available kit (Bio-Rad Laboratories, Richmond, VA). Protein concentrations were determined using the Bio-Rad protein assay kit (26).

RESULTS

We first investigated the effect of the 3T3-L1 and Swiss 3T3 conditioned medium on the proliferation of normal and malignant mammary epithelial cells. A final dilution of 1:1 conditioned medium from 3T3-L1 preadipocytes stimulated the growth of NMMG cells by 64 ± 2% (SD). In contrast, 3T3-L1 conditioned medium inhibited the growth of malignant MCF-7 breast cells by 36 ± 1% (Fig. 1). Incubation of cells with [3H]thymidine demonstrated a 20 ± 4% increase in thymidine incorporation in NMMG cells, whereas thymidine incorporation was decreased by 72 ± 5% in MCF-7 cells using 3T3-L1 conditioned medium (Fig. 2). The effect of 3T3-L1 conditioned media was also examined in NCI H630 human colon carcinoma cells. In NCI H630 cells, a 1:1 dilution of 3T3-L1 conditioned media inhibited growth by 58 ± 4% and decreased thymidine incorporation by 82 ± 6% (Fig. 3). The effect of conditioned medium from Swiss 3T3 fibroblast cells was also studied. In comparison to medium from 3T3-L1 cells, a 1:1 dilution of conditioned medium from Swiss 3T3 cells increased both NMMG and 5 and 77 ± 4% and decreased thymidine incorporation by 82 ± 6% (Fig. 3). The effect of 3T3-L1 conditioned media had no significant effect on NCI H630 cells (Fig. 3).

The effect of 3T3-L1 conditioned medium on the growth of MCF-7 cells was studied at various time points (24, 48, 72, or 96 h). Using a 1:1 final dilution of 3T3-L1 conditioned media, 15% growth inhibition of the MCF-7 cells was apparent at 24 h (Fig. 4). By 96 h a 55% decrease in growth was observed. In contrast, 3T3-L1 conditioned medium showed no inhibitory...
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Control 3T3-L1 CM 3T3 CM

MCF-7 cells

10

6

4

2

0

Cell Number x 10^-4

Cell Number x 10^-4

Thymidine Incorporation (% Control)

Thymidine Incorporation (% Control)

NMMG cells

MCF-7 cells

Fig. 1. The effect of 3T3-L1 CM (200 µl, 1:1 final dilution) or 3T3 CM (200 µl, 1:1 final dilution) on the growth of NMMG and MCF-7 cells. Cells were plated in equivalent numbers and incubated at 37°C with 200 µl 3T3-L1 or 3T3 concentrated conditioned medium (1:1 final dilution) or, in control cells, 200 µl DMEM. Cells were detached and counted after 48 h. These results represent the mean ± SD of at least five separate experiments.

Fig. 2. The effect of 3T3-L1 CM (200 µl) or 3T3 CM (200 µl) on the incorporation of thymidine into NMMG and MCF-7 cells. Cells were plated in equivalent numbers and incubated at 37°C with 200 µl 3T3-L1 or 3T3 CM (1:1 final dilution) or, in control cells, 200 µl of DMEM. After 48 h, [3H]thymidine incorporation was measured by incubating the cells in the exponential phase of growth with 1 µCi/ml [3H]thymidine in DMEM for 3 h. These results represent the mean ± SD of at least five separate experiments.

effect on NMMG cells, and by 24 h a growth-stimulatory effect was apparent (data not shown).

To determine if MCF-7 cell proliferation was dependent on the amount of 3T3-L1 conditioned media added to the culture, increasing amounts of the 3T3-L1 conditioned media were added to MCF-7 cells for 48 h. Ten µl of 3T3-L1 media (1:20 final dilution) caused a 16% decrease in thymidine incorporation, while 200 µl (1:1 final dilution) produced a 75% inhibition in thymidine incorporation (Fig. 5). Thus the 3T3-L1 CM inhibited the proliferation of MCF-7 cells in a dose-dependent fashion. To demonstrate that a heat-labile factor(s) contained within the 3T3-L1 medium was responsible for the inhibitory effect, 3T3-L1 conditioned media were heated at 60°C and 100°C for 30 min. The heat-treated 3T3-L1 conditioned media were added to MCF-7 cells in the logarithmic phase of growth for 48 h. Heating at 60°C partially inactivated the inhibitory effect by 40%, while heating to 100°C destroyed all inhibitory activity (Fig. 6). In addition, when the 3T3-L1 conditioned medium was passed through an ultrafiltration membrane with a 30,000 molecular weight cutoff, the retained solution contained the inhibitory activity while the ultrafiltrate did not. These experiments suggest that the inhibitory factor(s) was a protein with a molecular weight in excess of 30,000.

We examined the effect of several known inhibitory growth factors on NMMG and MCF-7 cell growth to determine if the 3T3-L1 inhibitory activity could be due to a previously characterized growth factor. These included FGF, TNF-α, IL-6, and TGF-β at concentrations 2- to 4-fold above those known to produce 90% inhibition of breast cancer cell growth, i.e., 20 ng/ml of FGF, 15 ng/ml of TNF-α, 1 ng/ml of TGF-β, and 1000 units/ml IL-6. Neither IL-6 nor FGF inhibited thymidine incorporation into MCF-7 or NMMG cells (Fig. 7). In contrast, TNF-α and TGF-β caused a 97 ± 2% and 67 ± 6% inhibition of thymidine uptake into MCF-7 cells, respectively. TGF-β also decreased thymidine uptake into NMMG cells by 98 ± 1%, whereas TNF-α had no effect on NMMG cells. To determine whether 3T3-L1 conditioned medium contained any detectable TNF-α or TGF-β, ELISA assays with sensitivities of 15 pg/ml were performed using 100 µl of 1:10 concentrated 3T3-L1 conditioned media. No TGF-β or TNF-α activity could be detected, suggesting that neither of these factors was present in serum-free 3T3-L1 conditioned medium. Moreover, monoclonal antibodies to TNF-α (30 ng/ml), γ-interferon (100 units/ml), and interleukin 1 (100 units/ml) did not reverse the inhibition of MCF-7 cells by the 3T3-L1 conditioned medium (data not shown).

Characterization of the Inhibitory Factor. The 3T3-L1 conditioned medium was collected and concentrated 10-fold, and a
inhibited thymidine incorporation from 53% to 67% (Fig. 8). Fraction 26 contained the peak inhibitory activity for both thymidine incorporation and cell growth. No inhibition was obtained in fractions other than 26–28. Fraction 26 eluted at a molecular weight of 67,000 (Fig. 8). Sodium dodecyl sulfopolyacrylamide gel electrophoresis followed by silver staining of the eluted material revealed that a Mr 69,000 protein was the predominant band in fractions 26–28. A minor band was also seen at Mr 67,000 (Fig. 8).

**DISCUSSION**

The 3T3-L1 cell line is a preadipocyte cell line derived from the Swiss 3T3 mouse fibroblast cell line. When 3T3-L1 cells enter the resting state, they accumulate triglycerides and acquire the signet ring appearance of adipose cells and ultimately lose the ability to revert to the growing state (17). In this study we have demonstrated the differential effects of 3T3-L1 preadipocytes on the proliferation of both normal and malignant mammary epithelial cells. We have shown that 3T3-L1 cells not only stimulated the growth of normal breast epithelial cells but inhibited the growth of human MCF-7 malignant breast cells (Fig. 1). Consistent with the growth inhibition effect, we have also demonstrated that 3T3-L1 conditioned media produced a decrease in the thymidine incorporation in MCF-7 cells (Fig. 2). In contrast, the parent Swiss 3T3 fibroblast cell line did not display this inhibitory activity but stimulated the growth of both normal and malignant breast epithelial cells and increased thymidine incorporation into both cell types. Thus it would appear that the 3T3-L1 preadipocyte subclone of Swiss 3T3 cells is capable of producing this differential effect on normal and malignant cells and in particular can produce inhibition of breast and colon cancer cell growth.

The 3T3-L1 conditioned media inhibited the proliferation of the MCF-7 cells in a dose-dependent fashion, and this inhibitory activity was partially destroyed by heating the 3T3-L1 conditioned medium to 60°C and completely destroyed by heating to 100°C for 30 min. The preservation of the inhibitory activity following ultrafiltration using a 30,000 molecular
results indicate that 3T3-L1 cells appear to secrete a protein that is inhibitory for the human MCF-7 breast cancer cell line. Furthermore, the protein appears to be monomeric in that it migrates at an apparent molecular weight of approximately 65,000–69,000 in both native (molecular sizing column) and denatured (SDS-PAGE) states.

In agreement with our results, other investigators have found that both 3T3 fibroblasts and 3T3-L1 preadipocytes promote the proliferation of normal mammary gland cells (13). These effects appear to be partially mediated by the deposition of extracellular materials that promote the attachment and growth of mammary epithelial cells. Levine and Stockdale (13) have shown that adipocytes have a greater stimulatory effect on cell growth than fibroblasts. This appears to be the result of qualitative and quantitative differences in the elaboration of soluble factors that have potent mitogenic activity for normal mammary epithelium. This effect may also reflect differences in the cellular interaction between mammary epithelial and adipocyte cells. Other investigators have demonstrated the importance of breast epithelial–mesenchymal cell interactions, particularly with the fatty stroma in both embryonic and adult mammary

weight cutoff membrane along with the lability of the inhibitory activity with heating suggests that the factor is a protein at least Mr 30,000 in apparent size.

Fractionation of the 3T3-L1 conditioned medium on a molecular sizing column showed that the peak of inhibitory activity eluted at a molecular weight of 67,000. This finding was consistent with the retention of inhibitory activity following the concentration of the 3T3-L1 medium using a Mr 30,000 ultrafiltration membrane. To further investigate the nature of this factor, several fractions from the HPLC column were resolved on a denaturing 15% SDS polyacrylamide gel. Silver staining of the gel demonstrated that in fractions with the greatest inhibitory effects, the major band was a Mr 69,000 protein band with other minor bands at Mr 65,000 and Mr 67,000 (Fig. 8). These
cell growth and development (5, 6, 27, 28). While several laboratories have reported the identification and partial isolation of stimulatory mammary growth factors secreted by fibroblasts, preadipocytes, adipocytes, and mesenchymal cells, there have been no previous reports of inhibitory growth factors produced by these cells.

Several growth factors that inhibit the growth of normal and malignant mammary epithelial cells have been well described. Tumor necrosis factor α, interleukin 6, transforming growth factor β, and the interferons have all been shown to inhibit the growth of MCF-7 cells (2, 24–32). We found no inhibitory effect on the MCF-7 cells by either FGF or IL-6. In agreement with previous studies, we found that TNF-α was inhibitory only for the malignant breast cells (22, 23). We tested the 3T3-L1 conditioned medium for TNF-α and TGF-β activity using an ELISA method, and no activity for either peptide could be detected. Furthermore, the pattern of heat sensitivity suggested that the inhibitory activity was a protein and unlikely to be TGF-β, since its inhibitory activity is heat-stable (24–29). Finally, the molecular weights of TNF-α or TGF-β (17,000/35,000 and 25,000) markedly differ from the size of the inhibitory 3T3-L1 protein. Thus neither TNF-α or TGF-β would appear to be the cause of the inhibitory activity. Antibodies to IL-1 and IFN-γ did not reverse the 3T3-L1 inhibitory activity, and the molecular weight of IL-1 (17,500), IFN-γ (35,000), and IFN-α (17,000) differ substantially from that of the inhibitory 3T3-L1 protein (33). In addition to these inhibitory factors, a novel peptide, mammastatin (Mr 47,000 and 65,000), which is inhibitory to breast cancer cells has recently been described (34). A monoclonal antibody to mammastatin (provided by Dr. P. Ervin, Department of Hematology/Oncology, University of Michigan) demonstrated no reactivity with 3T3-L1 conditioned media and did not reverse the inhibitory effect (data not shown).

We conclude that 3T3-L1 preadipocyte conditioned medium not only stimulates the growth of normal mammary epithelial cells but inhibits the growth of human MCF-7 breast cancer cells and NCI H630 colon cancer cells. This inhibitory effect appears to be due to a Mr ~65,000–69,000 protein secreted by 3T3-L1 preadipocyte cells that differs from other known growth factors secreted by stromal cells. Further work will be necessary to fully characterize the nature of this inhibitory factor.

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