BIOCELL
2007, 31(2): 237-245

ISSN 0327 - 9545
PRINTED IN ARGENTINA

Cell-cell communication between mouse mammary epithelial cells and 3T3-L1 preadipocytes: Effect on triglyceride accumulation and cell proliferation

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Key words: triglycerides, mammary epithelium, differentiation.

ABSTRACT: Interaction between parenchyma and stroma is essential for organogenesis, morphogenesis, and differentiation. Mammary gland has being the chosen model for developmental biologist because the most striking changes in morphology and function take place after birth. We have demonstrated a regulation of triglyceride accumulation by protein factors synthesized by normal mouse mammary gland epithelial cells (NMMG), acting on a cell line, 3T3-L1, long used as a model for adipogenesis. In this paper, we demonstrate that this inhibitory effect seems to be shared by other cells of epithelial origin but not by other cell types. We found a regulation of cell proliferation when NMMG cells are cultured in the presence of conditioned media from Swiss 3T3 or 3T3-L1 cells. We found a possible point of regulation for the mammary factor on a key enzyme of the lipid metabolic pathway, the glycerol-3-phosphate dehydrogenase. The inhibitory factor seems to have an effect on this enzyme's activity and reduces it. The results presented herein contribute to the understanding of cell-cell communication in a model of a normal mammary gland.

Introduction

The predominant cell-cell interactions in the mammary gland are those between epithelial cells and adipocytes/fibroblasts. For a better understanding of adipocyte physiology, *in vitro* cell models, such as 3T3-L1 and 3T3-F442A, have been used. A model of 3T3-L1 cells can be induced to differentiate into mature adipocytes in cell culture (Qiu *et al*., 2001). Other authors demonstrated that a co-culture of mammary epithelium and 3T3-L1 adipocytes has potent growth promoting activity for mammary epithelium (Levine and Stockdale, 1985). Furthermore, mammary adipose tissue represents a significant store of lipid, which, by itself and through its derivatives, could influence the growth of mammary epithelium. Fatty acids released from adipocytes under hormonal and epithelial influence, and their derivatives, can directly influence cell proliferation and also modulate responsiveness to other growth-regulatory molecules. In response to epithelial growth and morphogenesis, substantial remodeling and cell proliferation occur within the connective and adipose tissue of the mammary gland (Hovey *et al*., 1999). We have previously shown evidence of the existence of protein factor/s that is/are produced by normal mouse mammary gland (NMMG) cells and could be involved in preadipocyte differentiation and inhibition of triglyceride accumulation by 3T3-L1 (Calvo *et al*., 1992).

Reports from other laboratories show that breast tumors are characterized by accumulation of fibroblasts

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Received on December 11, 2006. Accepted on April 9, 2007.

adjacent to malignant epithelial cells during desmoplastic reaction. Stromal fibroblasts seem to provide the structural support for cancer growth, whereas malignant cells influence the composition of the adjacent tissue. Malignant epithelial cells secrete growth factors and cytokines, such as IL-11 and TNF-α, which prevent fibroblasts differentiation into mature adipocytes in the surrounding adipose tissue (Meng *et al*., 2001). Under defined culture conditions, fibroblasts isolated from adipose tissue are capable of differentiating into mature adipocytes (Hauner *et al*., 1989). Aromatase expression is considered a marker for undifferentiated fibroblasts, and conditioned medium from breast tumor cells was found to induce this activity in adipose fibroblasts (Nichols *et al*., 1995). Another known marker of adipocyte differentiation is the glycerol-3-phosphate dehydrogenase (GPDH) activity, a key lipogenic enzyme in fat cells. It has been demonstrated that continuous exposure to TGF-β blocks differentiation; the addition of this factor at the beginning of the differentiation process results in a reduced expression of GPDH activity and cells are not able to accumulate lipid droplets (Wise and Green, 1978).

Our aim was to study factors that mediate the interaction between mammary epithelium and adipose tissue. A more profound knowledge of parenchymal-stromal interaction is essential to understanding certain pathologies, such as cancer. To facilitate the investigation of interactions between epithelial and stromal (adipose) components of the mammary gland, we used two well-characterized established cell lines, NMMG epithelial cells and 3T3-L1 preadipocytes. These cell lines have been shown to resemble the original when injected into animals (Owens, 1974; Owens *et al*., 1974).

We analyzed the interaction between both cell types by focusing on cell proliferation and differentiation and, using other cell lines for comparison, we identified a possible target for the inhibitory fraction from the NMMG cells conditioned media.

Materials and Methods

Cell cultures

Swiss 3T3 (mouse fibroblastic), 3T3-L1 (mouse preadipcytes), NMMG (normal mouse mammary epithelial cells), JEG-3 (human choriocarcinoma), and HepG2 (human hepatoblastoma) cells were obtained from American Type Culture Collection (Rockville, MD, USA). HeLa (human cervical carcinoma) cells were a kind gift from Dr. Eduardo Cánepa, and C6 glioma cells (rat brain glial cells, fibroblastic) were kindly provided by Dr. Mónica Kotler. Cells were cultured in DMEM medium (GIBCO Life Technologies, Grand Island, NY, USA) containing 10% FBS (10% FBS-DMEM), pH 7.4, at 37ºC under 5% CO_2. All reagents, except when specified, were purchased from Sigma-Aldrich Co, St. Louis, MO, USA.

Differentiation of 3T3-L1 Preadipocytes into Adipocytes

The 3T3-L1 cells, a subclone derived from the Swiss 3T3 parental line, will spontaneously differentiate into mature adipocytes 10 to 20 days after becoming confluent. However, differentiation can be achieved in less than one week by culturing the cells with a mixture of 0.5 mM 3-isobutyl-1-methyl xanthine (IBMX) and 0.1 μM dexamethasone in 10% FBS-DMEM for 48 hr (Zizola *et al*., 2002) 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 that are observable by conventional or dark-field microscopy, and they were also observable microscopically by Oil Red O staining.

Conditioned Medium from Cultured Cells

Conditioned medium was collected from NMMG, 3T3, 3T3-L1, HeLa, HepG2, JEG-3, and glioma cell cultures. Cells were permitted to achieve confluence, and conditioned medium was collected every 4 days. The medium was transferred to a dialysis membrane and immediately concentrated 10-fold by using solid polyethylenglycol and the concentrate filter-sterilized (0.2 μm membrane pore), aliquoted and kept frozen at -20ºC until used.

Assay for inhibition of triglyceride accumulation

3T3-L1 cells were allowed to differentiate as mentioned previously and an appropriate amount of protein from concentrated conditioned media from the various cell lines or purified fractions of NMMG conditioned medium were added to experimental wells. This addition was done immediately after the IBMX-dexamethasone mixture was removed and exchanged for fresh insulin-containing medium. Cultures were maintained until control cells showed more than 50% differentiation. Cells were washed once with 0.01 M phosphate-

buffered saline at room temperature, and then 1 ml of water was added to each culture. The cells were scraped and sonicated, and then triglycerides were determined using the TG Color GPO/PAP AA Kit (Wiener Laboratories, Rosario, Argentina).

Column chromatography

The NMMG conditioned medium was fractionated in Sephacryl S200 High Resolution HiPrep 26/60 column (Pharmacia Biotech), equilibrated, and then eluted with 50 mM Tris-HCl buffer, pH 7.4. To determine the apparent molecular weight of the samples, a Sephacryl S 200 column was calibrated using bovine serum albumin (67,000), ovoalbumin (43,000), chymotrypsinogen A (25,000), and ribonuclease A (13,700) as standard proteins. Fractions were combined, tested for triglyceride inhibition, and then further purified using a Sepharose 12 Fast Performance Liquid Chromatography (Pharmacia Biotech) that was equilibrated and eluted with 0.01 M Phosphate saline buffer, pH 7. Eluates were evaluated for protein content at 280 nm and stored at -20ºC. When required, fractions were thawed, filter sterilized, and used in triglyceride inhibition assays with the 3T3-L1 cultures. Where indicated, fractions were pre-heated to 56ºC, 70ºC, or 100ºC for 10 min to assess thermal stability.

Proliferation curves for the different cell lines

For the proliferation analysis, two approaches were used: 1) cells were plated at a starting number of 10^5 cells/100 mm plastic dish, and, at the appropriate times, attached cells were trypsinized and counted using a Neubauer chamber, or; 2) tritiated thymidine incorporation was determined. For tritiated thymidine incorporation, 2 μCi of [^{3}H]thymidine (5 Ci/mmol) was added to each culture plate, and the reaction was stopped by the addition of 100 μl of a 20 mM unlabelled thymidine (Sigma-Aldrich., St. Louis, MO) solution. The medium was aspirated, and the cells were washed twice with 1 ml of Hank's solution that contained 2 mM unlabelled thymidine. After the second wash, cells were harvested with trypsin solution (2 ml of a 0.08% trypsin solution in Hank's medium) and transferred to a glass tube. After centrifugation and washing of the pellet, we added 1 ml of 5% trichloroacetic acid that contained unlabelled thymidine. The pellet was collected by centrifugation and dissolved in 0.5 ml of 1 N NaHO. Fifty microliters of the solution were taken for protein determination and the rest for radioactivity measurement by liquid scintillation counting. Depending on the assay, tritiated thymidine remained present for the whole experiment or was added 3 h prior to cell harvest.

Determination of glycerol-3-phosphate dehydrogenase (GPDH) activity

The monolayer of 3T3-L1 cells was washed with PBS. To each well (24-well plate), 200 μl of distilled water were added and cells were sonicated for 50 sec. Only 40 μl of cell suspension were used for the determination of enzyme activity (between 1 and 100 μg of protein). The reaction mixture consisted of 500 μl of TEA buffer (200 mM triethanolamine, 5 mM EDTA, pH 7.5), 10 μl 2-mercaptoethanol, 25 μl of NADH (5.6 mM in distilled water), 12.5 μl of dihydroxyacetone phosphate (DHAP, 16.8 mM in distilled water), and sample or water up to a final volume of 1 ml. Activity was determined by measuring the change in absorbance at 340 nm, at 30-second intervals, for 5 min at 25ºC. Protein content of the sample was determined using the Bradford reagent.

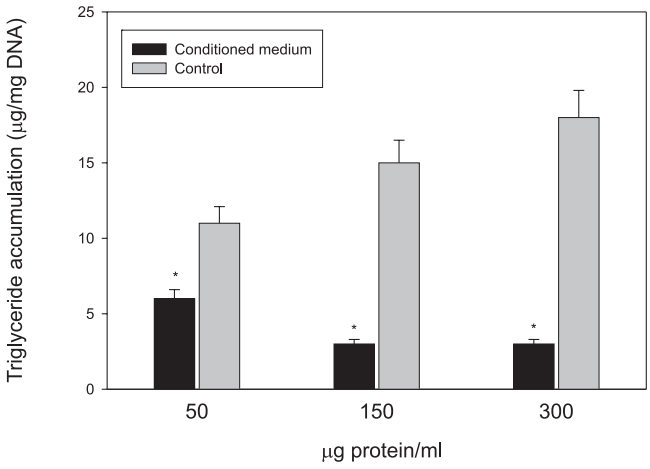

FIGURE 1. Triglyceride accumulation in 3T3-L1 cells treated with different protein concentrations from NMMG conditioned medium (black bars) or with vehicle (gray bars). Experimental conditions were as described in Materials and Methods. *p<0.01 compared to corresponding vehicle bars. Each bar represent the mean ± S.E. of triplicate experiments.

Statistics

Results are expressed as mean ± S.D. Statistical analysis was performed by one-way analysis of variance, followed by post-anova analysis (Student-Newman-Keules, Microcal Origin, Microcal Software Inc., Northamptom Maryland and GraphPad Instat 3, GraphPad Software Inc.).

Results

In a previous report (Calvo *et al*., 1992), we showed the effect of NMMG conditioned medium on triglyceride accumulation in 3T3-L1 preadipocytes, where a protein concentration of 300 μg/ml of NMMG conditioned medium was enough to inhibit almost 85% of triglyceride accumulation in 3T3-L1 cells. When a dose-inhibition curve was performed (Fig. 1), significant inhibition was already observed at 50 μg protein/ml. Conditioned media from tumoral cells

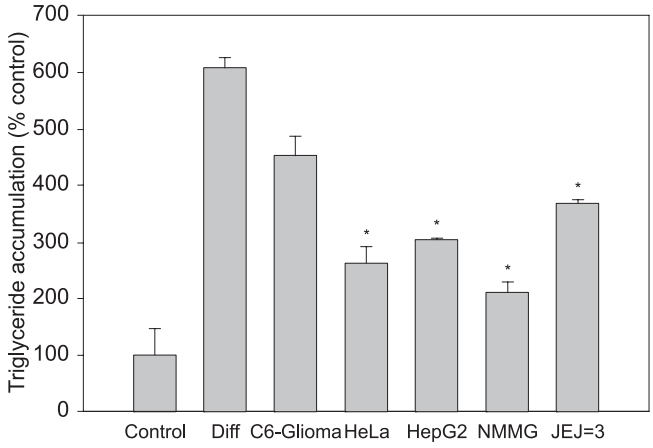

FIGURE 2. Triglyceride accumulation in 3T3-L1 cells, cultured in the presence of conditioned media from cells from epithelial or non-epithelial origin. Experimental conditions were as explained in the Materials and Methods sections. Each bar represents the mean ± S.E. of triplicate experiments. Significant differences ($p<0.05$) were observed for each medium compared to the differentiated cells with no addition, except for the C6 glioma cells, where no significant difference was obtained.

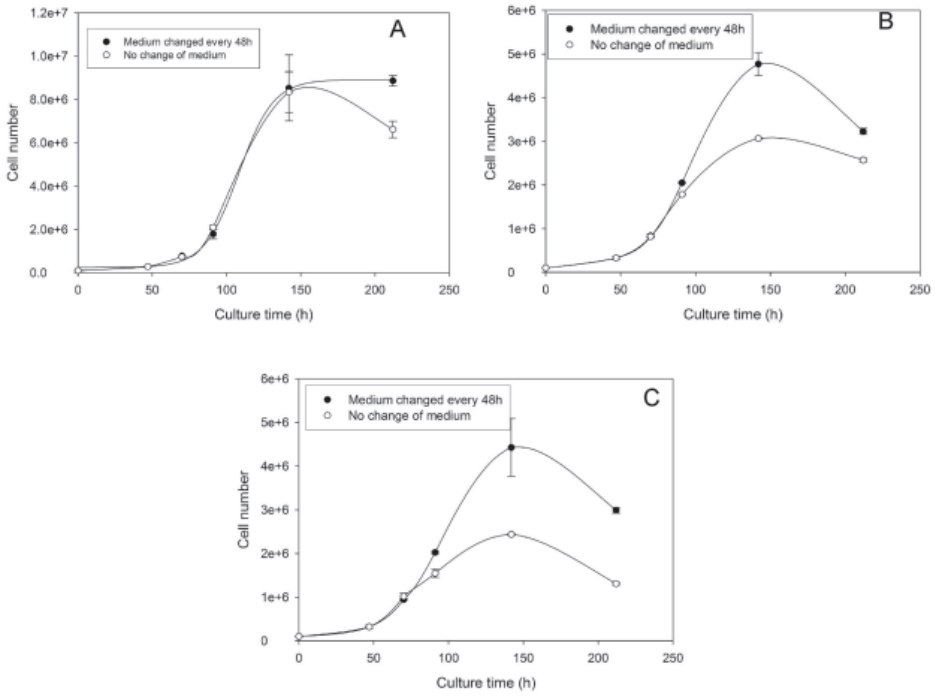

FIGURE 3. Panel A: Time course for NMMG cells proliferation in two different culture conditions: closed symbols show the proliferation rate when culture medium was changed every 48 h and open symbols show data obtained without medium change for the whole duration of the experiment. No significant differences were observed up to 144 h, decreasing afterwards ($p< 0.02$). Panel B: Time course for Swiss 3T3 cells proliferation in two different culture conditions: closed symbols show the proliferation rate when culture medium was changed every 48 h and open symbols show data obtained without medium change for the whole duration of the experiment. No significant differences were observed up to 96 h, significantly decreasing afterwards ($p< 0.05$). Panel C: Time course for 3T3-L1 cells proliferation in two different culture conditions: closed symbols show the proliferation rate when culture medium was changed every 48 h and open symbols show data obtained without medium change for the whole duration of the experiment. No significant differences were observed up to 96 h, decreasing afterwards ($p< 0.05$). Each point represent the mean ± S.E. of triplicate experiments.

of mammary epithelial origin, T47D, produced similar inhibition at a much lower protein concentration (23 μg/ml) (data not shown).

To test the specificity of this effect, other cells from epithelial origin, not related to mammary tissue, such as HeLa, JEG-3, and HepG2, were tested and also proved inhibitory for triglyceride accumulation, although at a reduced level when compared to NMMG cells (Fig. 2). A gliomal non-epithelial cell line that was also tested was non-effective in reducing triglyceride content. Nevertheless, when compared to NMMG cells, all the other cell lines were much less effective in inhibiting the triglyceride accumulation by 3T3-L1 cells.

Next, we tested the ability of the conditioned media from NMMG, 3T3, and 3T3-L1 cells to regulate the proliferation of each cell type under study.

Figure 3 (Panels A, B and C) shows the growth curve of NMMG, 3T3, and 3T3-L1 cells in two conditions: with medium being changed every 48 h and without any change of medium for the whole duration of the experiment. This was necessary to assure that the conditioned media that we were using was not depleted of essential nutrients before the 4-day period of collection.

It can be observed that up to 4 days (96 h) there was no difference in the growth rate for any of the cells studied. Thus, we consider that it was safe to use the conditioned medium after a 4-day culture period.

Figure 4 (Panels A, B and C) shows the effect of the conditioned media from one cell type on the rate of proliferation of the other two cell lines under study. It is clear that only the combination of conditioned media from the fibroblastic cell lines (3T3 and 3T3-L1) was

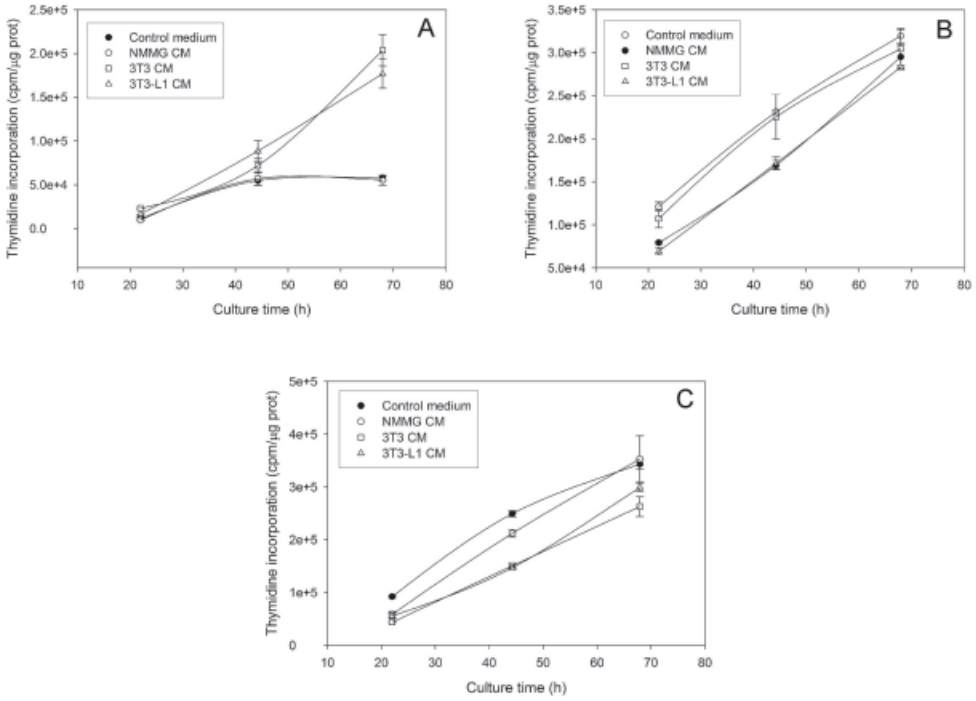

FIGURE 4. Panel A: Time course for NMMG cells in the presence of conditioned medium from NMMG, Swiss 3T3 or 3T3-L1 cells. Tritiated thymidine was added at the beginning of the culture period and remained present throughout the whole experiment. Cultures were stopped at the designated times and cells processed as indicated in Materials and Methods section. Each point represents the mean ± S.E. of triplicate experiments. Significant differences were observed for the Swiss 3T3 and 3T3-L1 conditioned media, when compared to no addition or NMMG conditioned media, at the last time analyzed ($p < 0.001$). Panel B: Time course for Swiss 3T3 cells in the presence of conditioned medium from NMMG, Swiss 3T3 or 3T3-L1 cells. Tritiated thymidine was added at the beginning of the culture period and remained present throughout the whole experiment. Cultures were stopped at the designated times and cells processed as indicated in Materials and Methods section. Each point represents the mean ± S.E. of triplicate experiments. No significant differences were observed for any of the conditioned media. Panel C: Time course for 3T3-L1 cells in the presence of conditioned medium from NMMG, Swiss 3T3 or 3T3-L1 cells. Tritiated thymidine was added at the beginning of the culture period and remained present throughout the whole experiment. Cultures were stopped at the designated times and cells processed as indicated in Materials and Methods section. Each point represents the mean ± S.E. of triplicate experiments. No significant differences were observed for any of the conditioned media.

able to enhance the proliferation of the NMMG cells (Fig. 4, Panel A). Virtually no effect was observed for the conditioned medium from the epithelial cells on the growth of 3T3 and 3T-L1 cells.

To study the effect of the conditioned medium on triglyceride accumulation, we next tested the ability of each of the cell lines to regulate this metabolism. Figure 5 shows that only the conditioned medium from the epithelial cells was able to reduce triglyceride accumulation. Moreover, the media collected from each of the fibroblastic cell lines stimulated triglyceride accumulation.

With the aim of characterizing the macromolecules present in NMMG conditioned medium, we used a Sephacryl 200 S column. We collected five fractions with a molecular weight near or lower than 67000. Because conditioned medium was obtained from NMMG cells cultured with 10% fetal bovine serum, fractions 1 and 2 showed several peptide bands when SDS-acrylamide gel electrophoresis was performed (data not shown), with an important contaminating band of bovine serum albumin. Only some fractions collected from the Sephacryl column inhibited triglyceride accumulation in 3T3-L1 cells, with fraction 1 producing a significant inhibition, almost 70% compared with differentiated cells, $p < 0.001$ (Fig. 6). This result correlated with microscope morphological observation (not shown).

Fraction 1 was re-chromatographed on the FPLC column, and 6 peaks were obtained and tested for inhibition of triglyceride accumulation (Fig. 7). Fraction A

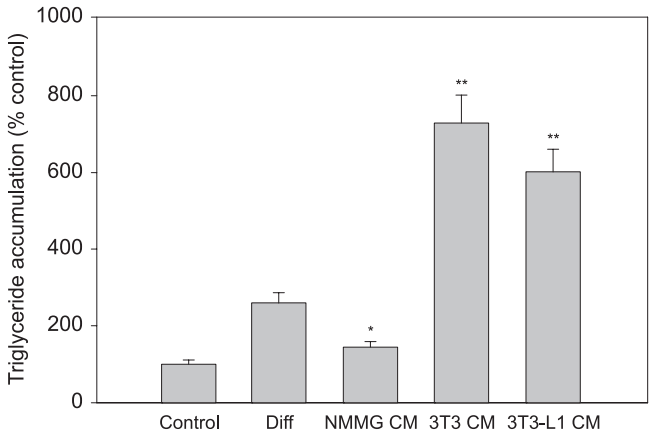

FIGURE 5. Triglyceride accumulation in 3T3-L1 cells, differentiated in the presence of conditioned media from NMMG, Swiss 3T3 or 3T3-L1 cells. Each bar represents the mean ± S.E. of triplicate experiments. Inhibition was observed for the NMMG conditioned medium ($p< 0.05$, compared to differentiated cells) and stimulations for the other two conditioned media ($p< 0.001$, compared to differentiated cells).

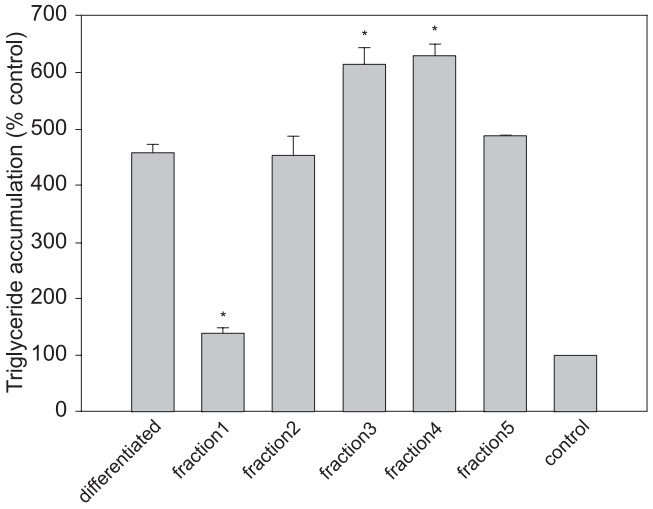

FIGURE 6. Effect of the different Sephacryl fractions on triglyceride accumulation in 3T3 –L1 cells. The cells were seeded in 10% FBS – DMEM and, when 70% of confluence was reached the differentiated treatment was performed. An aliquot of column fractions were added after the differentiating medium was removed. Experimental conditions were as described in Materials and Methods. *$p< 0.001$ compared to control differentiated value. Each bar represents the mean ± S.E. of triplicate experiments.

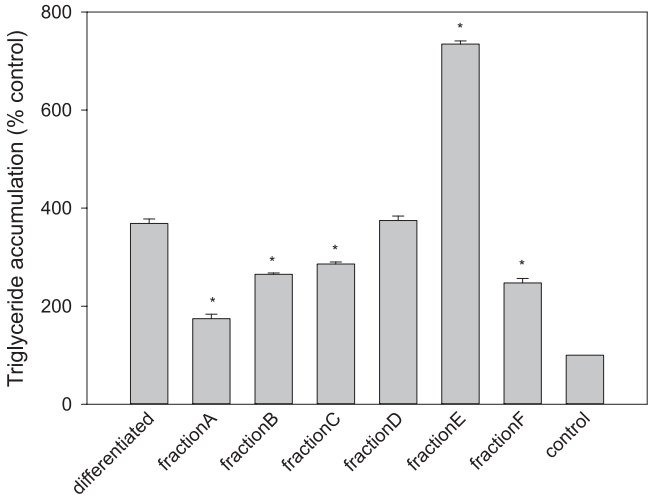

FIGURE 7. Triglyceride accumulation in 3T3 –L1 cells cultured in the absence of presence of FPLC fractions. The cells were seeded in 10% FBS – DMEM and, when 70% of confluence was reached, the differentiation treatment was performed. Where indicated, fractions from the FPLC column were included in the culture medium Experimental conditions were as described in Materials and Methods. *$p< 0.001$ compared to control differentiated value. Each bar represents the mean ± S.E. of triplicate experiments.

produced a significant inhibition (50% of triglyceride accumulation compared with differentiated cells, $p<0.001$), while fractions B, C, and F produced a significantly lower inhibition. Contrarily, fraction E showed an induction effect in triglyceride accumulation by 3T3-L1 cells, with almost a 100% increase in lipid concentration compared to differentiated cells ($p< 0.001$).

When GPDH activity (a key step in lipid metabolism) was tested, conditioned medium from NMMG cells was successful in inhibiting triglyceride accumulation (Fig. 8) as well as GDPH activity (Fig. 9). When the thermal stability of the media was tested, the factor/s from the medium was/were thermostable (Figs. 8 and 9). Interestingly enough, temperature seemed to potentiate the inhibitory effect of the factor/s, which was/were more potent at 56ºC and returning to the original potency at 100ºC. The inhibition of triglyceride accumulation was lost at 100ºC. However, at this same temperature, GPDH activity was not inhibited, which indicates that possibly more than one factor is acting on the enzyme and the lipid storage.

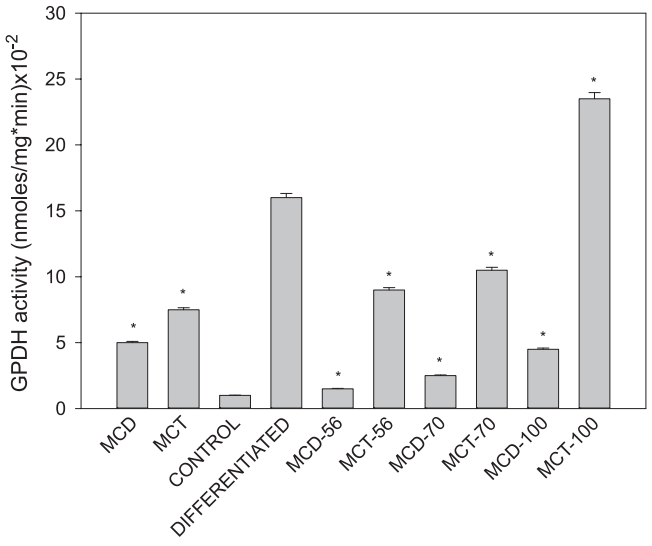

FIGURE 8. GPDH activity in control and inhibited 3T3-L1 cells. 3T3-L1 cells were cultured in the presence or absence of NMMG conditioned medium. As indicated, in some cases the medium was heated before treatments. Experimental conditions were as described in Material and Methods. Figure shows the effect of heating on the inhibition (MCD= NMMG conditioned medium, MCT= control medium treated in the same manner as the conditioned medium.
Temperatures used were: 56ºC, 70ºC and 100ºC for 10 min). *p<0.01 compared to control differentiated value. Each bar represents the mean ± S.E. of triplicate experiments.

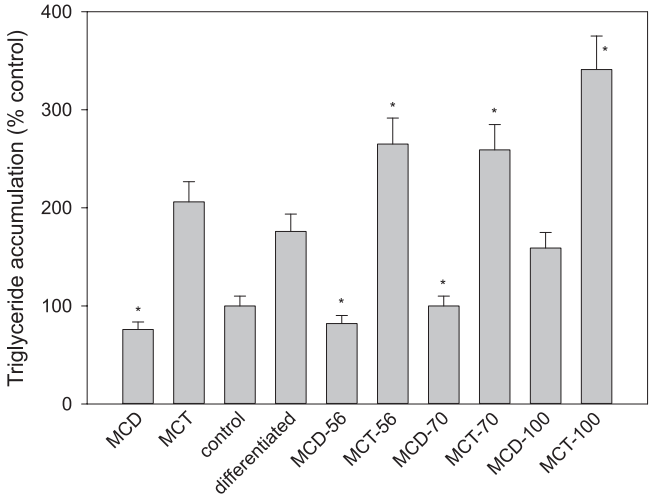

FIGURE 9. Triglyceride accumulation in control and inhibited 3T3 –L1 cells. 3T3-L1 cells were cultured in the presence or absence of NMMG conditioned medium. As indicated, in some cases the medium was heated before treatments. Experimental conditions were as described in Material and Methods. Figure shows the effect of heating on the inhibition (MCD= NMMG conditioned medium, MCT= control medium treated in the same manner as the conditioned medium. Temperatures used were: 56ºC, 70ºC and 100ºC for 10 min). *p<0.01 compared to control differentiated value. Each bar represents the mean ± S.E. of triplicate experiments.

Discussion

Previous results from our laboratory demonstrates that the interaction between normal mammary cells and preadipocytes *in vitro* produces a strong inhibition of triglyceride accumulation by 3T3-L1 (Calvo *et al*., 1992). The 3T3-L1 cells are not adipogenic in subconfluent cultures.

Upon confluence, these cells become committed preadipocytes. Treatment of confluent cultures with a differentiation-inducing mixture (dexamethasone, insulin, and methyl-isobutylxanthine) induces the expression of proteins associated with mature adipocytes and the accumulation of lipids. The triglyceride content of the cells was used as the principal end point of preadipocyte differentiation. In this present paper, we continued with the purification of factor/s produced by NMMG cells, which inhibit the triglyceride accumulation by 3T3-L1 adipocytes, and showed that these factors also had an important effect on glycerol phosphate dehydrogenase enzyme activity. To our knowledge, this is the first observation of a factor synthesized by mammary epithelial cells with an effect on GPDH activity.

The inhibitory effect on triglyceride accumulation

was not a unique feature of NMMG epithelial cells, but is shared by other epithelial cell lines, such as T47D, HeLa, JEG-3, and Hep G2. Since adipose tissue is a generalized stromal component, present in diverse glands and organs, it seems reasonable to expect a communication between different cellular components of those systems. Every differentiation process includes a "talk" between the differentiating cell and its surroundings. Therefore, it is important to study the molecular events that take place during this exchange of information. Interestingly, this inhibitory effect was not observed with conditioned media from cells of non-epithelial origin, such as the C6 glioma, Swiss 3T3, or the same 3T3-L1 cells, thus reinforcing the idea of a very specific regulation between epithelial and stromal cells.

This interaction is not solely observed during differentiation, considering that cell proliferation was also regulated when NMMG cells were cultured in the presence of conditioned media from fibroblasts and not the opposite combination. When observing the triglyceride accumulation, these media were not only non-effective in reducing lipid accumulation but, instead, resulted in an important stimulation of this process. This stimulation, could represent an autocrine regulation of these cell types.

Recently, other authors reported that malignant epithelial cells secrete factors such as IL-1 and TNFα to prevent the differentiation of fibroblasts into mature adipocytes in the adjacent adipose tissue. The potent antiadipogenic effects of these two cytokines have been well established. However, Meng *et al*. (2001) claim that antiadipogenic effects were specific for malignant cells because other cytokines or benign breast epithelial cells did not inhibit adipocyte differentiation.

Here, we demonstrated that conditioned medium from normal mammary epithelial cells was able to inhibit triglyceride accumulation and adipocyte differentiation, probably by acting on early steps in the differentiation program.

Insulin is the hormone responsible for inducing mitotic clonal expansion by acting through the IGF-I receptor in 3T3-L1 cells during the induction of differentiation, with IGF receptor levels decreasing in mature adipocytes compared to cells before the initiation of the differentiation process (Zizola *et al*., 2002). Differentiated cells present a decreased expression of both erbB2 and EGFR regardless of whether the cells were hormonally or spontaneously differentiated. Because erbB2 and EGFR expression are down modulated as differentiation progresses, it is conceivable that a mechanism of switching from a mitogenic to a differentiating signaling pathway may be involved, through regulation of the expression of these growth factor receptors (Pagano and Calvo, 2003). Although treatment of murine 3T3-L1 fibroblasts in culture with serum or insulin initially causes proliferation, cell replication rapidly stops under these conditions, and fibroblasts eventually differentiate into mature adipocytes; inhibition of differentiation may provide these critical mechanisms that are responsible for this process, which may be under the control of cytokines secreted by the surrounding epithelial cells (Deb *et al*., 2004). The decreased GPDH activity, in correlation with the inhibitory effect on triglyceride accumulation, as observed with the factor/s, is a possible indication of a genomic, rather than an acute, lipolytic effect. The differences observed in the thermal stability of the factor/s when the inhibition of the GPDH activity or the triglyceride accumulation was assessed indicate that possibly more than one factor is acting differentially on each end point. When temperature seems to potentiate the effect on enzyme activity, it also seems to decrease the effect on lipid accumulation. The use of these two end points, combined, should be important to the differentiation of the factors involved.

To further analyze the genomic versus non-genomic effect, research will require the study of mRNA for this and other marker enzymes from the lipid metabolic pathway, through PCR and Northern blot analysis, as well as Western blot determinations.

Experiments are in progress towards the final purification and partial sequencing of the protein factor/s to elucidate its molecular identity and, thus, further investigate its physiological role in the mammary gland.

Acknowledgements

We thank Dr. Laura Matkovic for her helpful assistance with the FPLC purification of the column fractions.

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