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LETTER TO THE EDITOR

Soluble factors derived from tumor mammary cell lines induce a stromal mammary adipose reversion in human and mice adipose cells. Possible role of TGF-β1 and TNF-α

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Abstract In carcinomas such as those of breast, pancreas, stomach, and colon, cancer cells support the expansion of molecular and cellular stroma in a phenomenon termed desmoplasia, which is characterized by a strong fibrotic response. In the case of breast tissue, in which stroma is mainly a fatty tissue, this response presumably occurs at the expense of the adipose cells, the most abundant stromal phenotype, generating a tumoral fibrous structure rich in fibroblast-like cells. In this study, we aimed to determine the cellular mechanisms by which factors present in the media conditioned by MDA-MB-231 and MCF-7 human breast cancer cell lines induce a reversion of adipose cells to a fibroblastic phenotype. We demonstrated that soluble factors generated by these cell lines stimulated the reversion of mammary adipose phenotype evaluated as intracellular lipid content and expression of C/EBPα and PPARγ. We also demonstrated that exogenous TGF-β1 and TNF-α exerts a similar function. The participation of both growth factors, components of media conditioned by tumoral mammary cells, on the expression and nuclear translocation of C/EBPα and PPARγ was tested in 3T3-L1 cells by interfering with the inhibitory effects of media with agents that block the TGF-β1 and TNF-α activity. These results allow us to postulate that TGF-β1 and TNF-α present in this media are in part responsible for this phenotypic reversion.

Keywords Desmoplasia · Adipogenic transcription factors · TGF-β1 · TNF-α

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Introduction

It is currently accepted that tumoral progression not only depends on the intrinsic malignancy of tumor cells but also on the surrounding microenvironment composed of stromal cells and the extracellular matrix (ECM) [1]. Moreover, it has been described that tumor cells are able to modify the adjacent stroma creating a functional structure that favors tumor progression [2].

Breast tumors belong to a group of neoplastic lesions which, under the influence of tumoral cell products, originate a fibrous structure responsible for the dense and hard consistency of the tumoral mass. This trait also constitutes a factor that increases the relative risk of tumor recurrence [3]. Either in the functional origin or in the cellular composition of this fibrotic structure, myofibroblasts are the predominant stromal phenotype which secretes abundant amount of collagen and other extracellular matrix (ECM) proteins through an active phenomenon known as "Desmoplastic Response" [4]. Some of the changes in the composition of ECM, derived from the desmoplastic response, induce changes in breast density that has been identified as a predisposing factor for breast cancer that

confers a higher risk compared with women with fatty breast [5].

The desmoplastic reaction has been attributed to fibroblast-like cells which may derive from adipose tissue in mammary tumors [6]. Fibroblast accumulation can occur due to the activity of specific growth factors produced by tumoral cells. Using 3T3-L1 cells and human adipose fibroblasts, it has been demonstrated that TNF-α and IL-11 derived from human tumor mammary cell lines inhibit adipocyte differentiation, a previous step to expansion of fibroblastic compartment without modifying fibroblast proliferation [6].

On the other hand, it has been also proposed that Platelet-derived Growth Factor (PDGF) is a paracrine factor produced by mammary cells whose primary target are stromal cells and which is considered the initiator of tumor desmoplasia [4]. Finally, it has been suggested that TGF-β1 may be one of the main factors involved in desmoplastic response. In breast cancer TGF-β1 is highly expressed, preferentially at the advancing edges of primary tumors and in lymph node metastasis, suggesting that it plays a role in interacting with neighboring stromal cells [7].

Epithelial control of mammary adipose cells is observed under physiologic conditions as well. During pregnancy and lactation, reproductive hormones induce the expansion and terminal differentiation of the mammary epithelium into secretory, milk-producing, lobular alveoli in a process that also includes the dedifferentiation of adipocytes into tiny preadipocytes [8].

CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptor-gamma (PPARs) are two families of transcription factors that play a critical role in either the onset of adipocyte differentiation or the maintenance of the fully differentiated adipocyte phenotype by transactivating adipocyte specific genes [9]. Inhibiting PPARγ activity, either in 3T3-L1 adipocytes or in the adipose tissue of mice was shown to lead to dedifferentiation or adipocyte death [10]. It has also been demonstrated that C/EBPα is a well-characterized factor that mediates the expression of genes characteristic of the terminally differentiated state and also acts as inhibitor of mitotic growth in most cell lines tested [11].

In the present work, we attempt to unravel some of the cellular mechanisms of mammary desmoplastic response by evaluating whether cell-secreted soluble factors produced by human mammary cell lines (MCF-7 and MDA-MB-231) are able to induce reversion of mature mammary adipocytes of human and mouse origin to the fibroblastic phenotype, in addition to the well-known inhibitory role on adipose differentiation. To accomplish this, we used human mammary-derived adipocyte and fully differentiated murine 3T3-L1 cells to identify the main factors secreted by the cell lines responsible for the reversion process and the impact of these factors on the expression and abundance of PPARγ and C/EBPα. Our results, obtained from cell culture studies and immunohistochemical analysis of human tumor samples, demonstrated that TGF-β1 and TNF-α of epithelial origin are in part responsible for the adipocyte reversion and that PPARγ and C/EBPα expression seem to be a molecular target of these tumoral factors.

Materials and methods

Antibodies and reagents

Antibodies anti-PPARγ and C/EBPα were obtained from Santa Cruz Biotechnology, (Santa Cruz, CA). Anti-β-actin was supplied by Sigma (St. Louis, MO), anti-TGF-β was from Chemicon International, Inc. (Temecula, CA), anti-human TNF-α (neutralizing of TNF-α) was obtained from R&D System, Inc. (Minneapolis, MN). The P144 peptide (TSLDASIIWAMMQN, encompassing aminoacids 730–743) interfering with TGF-β1 binding to its cellular receptors [12] was produced by Digna Biotech (Madrid, Spain). Recombinant TNF-α and TGF-β were purchased from R&D systems and Calbiochem (La Jolla, CA), respectively. Insulin, Dexamethasone, isobutylmethylxanthine (IBMX), Indomethacin, and Type I collagenase were purchased from Sigma Chemical Co. Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and bovine serum (BS) were purchase from Gibco^{TM} (Grand Island, NY).

Patients and normal tissue acquisition

Eight patients undergoing surgical mammary reduction at the East Metropolitan Health Service (Santiago, Chile) were recruited for the study. The experimental protocols were previously approved by institution ethics committee and all patients gave their informed consent for the procedure. All subjects were healthy and had no evidence of diabetes according to routine laboratory tests. Mammary adipose tissue biopsies were obtained at the time of surgery and immediately transported to the laboratory in sterile DMEM for processing.

Adipocyte isolation and three-dimensional collagen gel culture system

Samples of mammary adipose tissue were minced finely and digested in a collagenase solution (0.25 mg/ml Type I collagenase, 3% bovine serum albumin in DMEM) with shaking at 37°C for 1 h. The resultant digested material

was then centrifuged at 1,500*g* for 5 min and the supernatant was washed three times with 3% BSA in DMEM. Under these experimental conditions, a floating layer that contains only very low density cells (that we identify as unilocular mature adipocytes) was generated. This cellular fraction was extensively washed three times with HBSS. The remaining more dense phenotypes present in the breast sample, sediment in the cellular pellet.

A collagen gel solution was prepared as previously described [13]. Briefly, 8 volumes of type I rat tail collagen was mixed with 1 volume of tenfold concentrated DMEM and 1 volume of reconstruction buffer (2.2 g of NaHCO3 and 4.77 g of HEPES in 100 ml of 0.05 N NaOH at 4°C) to obtain a homogeneous solution. The collagen solution was mixed with the purified mature adipocytes at room temperature and 1 ml of collagen gel solution containing nearly 10^5 cells was placed in a 35 mm culture dish. The culture dishes were immediately warmed to 37°C to allow the gel to form and were covered with 2 ml of DMEM overnight. Afterward, cultures were treated with increasing concentrations of conditioned media (CM) from mammary tumoral cells lines MCF-7 and MDA-MB-231 for 10 days. Collagen was obtained from rat tail tendon as previously described [14].

Mammary cell lines and preparation of conditioned media

Mammary tumoral cells lines MCF-7 and MDA-MB-231 cells were purchased from ATCC (Manassas, VA) and were grown in a phenol red-free DMEM/F12 enriched with 10% FCS. To prepare conditioned media, approximately 10^5 cells/cm^2 of MCF-7 and MDA-MB-231 were cultured in standard conditions and further incubated for 48 h in serum-free DMEM. After this, media were collected and centrifuged for 5 min at 2,000 rpm to clarify.

Oil red O staining

The intracellular lipid content was evaluated with the lipophilic dye Oil Red O [15]. To do so, media was removed and cells in culture were fixed and dehydrated for 5 min with 2 ml of 100% isopropanol. Samples were then stained for 1 h with 2 ml of a saturated solution of Oil Red O in 60% (v/v) isopropanol and then washed twice with distilled water for 15 min.

ELISA assay

To determine TGF-β and TNF-α concentrations in mammary tumoral cell conditioned media, TGF-β (559119) BD Bioscience (San Diego, CA) and TNF-α (T916008) US Biological (Swampscott, MA) ELISA kits were used. The samples (in quadruplicate) were processed strictly following the supplier's instructions. At the end of the procedure, absorbance was immediately determined at a wavelength of 450 nm for TGF-β and 405 nm for TNF-α, with corrections at 570 and 650 nm, respectively. TGF-β and TNF-α concentrations were calculated using standard curves prepared for each individual determination.

Immunohistochemistry

Immunostaining for TGF-β, TNF-α, PPARγ, and C/EBPα was performed on 5 μm sections of formalin fixed, paraffin-embedded human mammary tissue biopsies. Tissue sections were deparaffinized in xylene and hydrated in a graded sequence of ethanol solutions. For antigen retrieval, sections were pretreated in a microwave in citrate buffer (pH 6.0). After cooling, nonspecific binding was blocked with diluted serum (4% normal goat serum) followed by incubation with antibodies against TGF-β (1:300), TNF-α (1:150), PPARγ (1:50), and C/EBPα (1:2,000) at room temperature in a humidified chamber. Negative controls were analyzed on adjacent sections incubated without primary antibody. After incubation with the primary antibody, sections were washed with PBS and subsequently treated using the corresponding biotinylated secondary antibody from Ultravision ONE kit, Thermo Fisher Scientific (TL-015-HDJ, Fremont, CA) according to the manufacturer's protocol. Peroxidase activity was visualized using the 3,3′-diaminobenzidine and sections were counterstained with haematoxylin. Assessment of staining intensity and distribution was made using the semiquantitative histologic score (HSCORE) system as described by Budwit-Novotny et al. [16]. This score was calculated in four control and nine tumor samples using the following equation: HSCORE $= \Sigma Pi$ $(i + 1)$, where $i =$ intensity of staining with a value of 1, 2, or 3, (weak, moderate or strong, respectively) and Pi the percentage of stained mammary adipose cells for each intensity, varying from 0 to 100%. This HSCORE has been previously reported [17, 18].

Adipocyte 3T3-L1 differentiation

3T3-L1 cells were purchased from ATCC (Manassas, VA) and cultured in DMEM media supplemented with 10% BS, 100 μg/ml streptomycin and 100 units/ml of penicillin in a humidified atmosphere with 5% CO_2 at 37°C. Two days after reaching confluence, the induction of adipocyte differentiation was started by culturing for 2 days with adipogenic medium containing 1 μM Dexamethasone, 0.5 mM IBMX, and 400 nM insulin

solution in DMEM 10% FBS. For the following 8 days, cells were incubated with DMEM 10% FBS containing 400 nM insulin alone. During this period, media was replaced every 2 days.

Western blots

Mammary mature adipocyte cells or differentiated adipocyte 3T3-L1 cells were lysed with a lysis buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM MgCl2, 2 mM EGTA, 1% Triton X-100, 10% glycerol, 2 mM PMSF, 2 μg/ml pepstatin, 2 μg/ml leupeptin and 1 mM sodium orthovanadate) at 4°C. Equal amounts of proteins from different treatments were resolved by SDS–PAGE and analyzed by immunoblotting with antibodies anti-PPARγ, C/EBPα using the ECL chemiluminescence detection kit (Amersham, Arlington Heights, FL).

Immunofluorescence

3T3-L1 cells were plated on cover slips in 24-well plates and differentiated as described above. After the treatment with mammary cell CMs and inhibitors (neutralizing Anti-TNF-α and P144), cells were washed in PBS with 1 mM Ca^{+2}, fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.25% Triton X-100 for 10 min at RT. Samples were blocked with 10% goat serum in PBS for 1 h and then co-incubated at 4°C overnight with the primary antibodies in blocking solution: monoclonal anti-PPARγ (1:50) and polyclonal anti-C/EBPα (1:100). The cover slips were washed in PBS and incubated with secondary antibodies (all from Molecular Probes) diluted 1:100 in blocking solution: Alexa 488-anti mouse, Alexa 546-anti rabbit at room temperature for 2 h. Cover slips were washed in PBS and water. Finally, samples were mounted in fluorescence mounting media (Dako).

Adipocyte size image analysis

Sections of 5 μm of paraffin-embedded human mammary tissue obtained from adjacent regions of both normal and tumoral samples were stained with haematoxylin and eosin to evaluate adipocyte size as described by Chen et al. [19], with some modifications. Briefly, images of the histology sections were obtained in a jpg format at 10× magnification and were converted into a binary format (8-bit) with Adobe PhotoShop 5.0 (Adobe Systems, San Jose, CA) and Image J (National Institutes of Health, http://rsbweb.nih.gov/ij/). The binary images were compared with the original images to ensure an accurate conversion. For the determination of the total number and cross-sectional areas of adipocytes, the commands Image/Adjust/Threshold and then Analyze/Analyze Particules were used. Adipocyte cross-sectional areas were expressed as pixel number, and the results saved as an Excel document for analysis.

Results

Media conditioned by human mammary tumoral cells revert to mammary adipose phenotype

To test whether soluble factors produced by human tumoral mammary cells affect the lipid content of mammary adipose tissue, we seeded recently dispersed human mammary fat cells into a semisolid collagen solution and cultured them in the presence or absence of media conditioned (MC) by the weakly invasive MCF-7 and the strongly invasive MDA-MB-231 mammary cells.

After 10 days, cells were stained with Red Oil O and the proportion of fibroblast-like cells was scored. Figure 1A shows a representative image of human adipose mammary cells exposed to 50% MC by MDA-MB-231. As displayed in this image, a high proportion of mammary fatty cells lost their lipid content and acquired a fibroblast shape. Figure 1B shows the quantitative analysis of these experiments using a range of concentrations of MC derived from MDA-MB-231 and MCF-7 cells. This data indicated that MC derived from MDA-MB-231 cells was more effective in reverting the adipose phenotype to spindle-shaped cells (Fig. 1B).

It has been proposed that TGF-β1 and TNF-α, two soluble factors produced in a high proportion by tumoral cells, also play a role in the control of cellular lipid content [6, 20]. To test whether these factors induce lipid loss in our system, we assessed their effectiveness in the reversion of adipose phenotype and found that both factors are capable of generating a conversion of adipose cells into fibroblasts (Fig. 1C).

TGF-β1 and TNF-α are differentially expressed in human mammary cell lines

Next, we analyzed the presence of TGF-β1 and TNF-α in MC by MCF-7 and MDA-MB-231 cells using an ELISA assay. Figure 2 shows that while both cell lines produced a fairly similar amount of TNF-α, the more invasive MDA-MB-231 cells produced five times more TGF-β1 than the less invasive MCF-7 line.

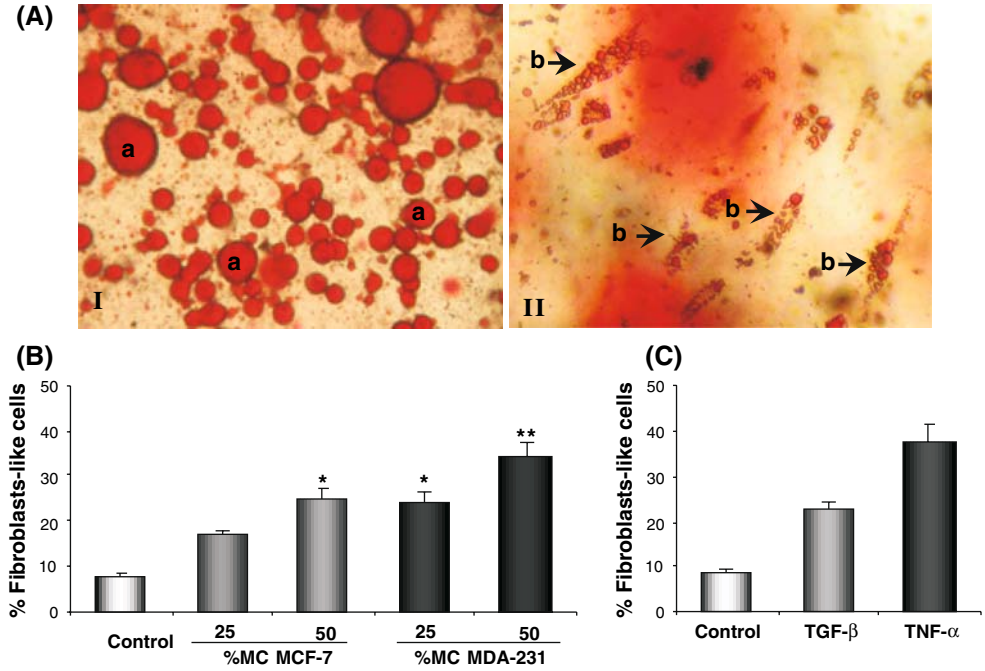

Fig. 1 Adipocyte reversion in three-dimensional collagen gel culture. **A** 10^5 human mature mammary adipocytes were cultured in 1.5 ml semisolid collagen gel for 10 days in the absence (*I*) or presence (*II*) of 50% medium conditioned by MDA-MB-231 cells. Cells were stained with oil red O that identified lipid content in mature spherical adipocytes (*a*) and elongated cells with a fibroblast phenotype (*b*). **B** Quantification of fibroblast-like cells in cultures treated with increasing proportions of media conditioned by mammary tumoral cells MCF-7 and MDA-MB-231. **C** Quantification of fibroblast-like cells in collagen matrix cultures treated for 10 days with 10 ng/ml of TGF-β and TNF-α. *Bars* represent mean ± SE from five independent experiments. A Kruskal–Wallis one-way ANOVA followed by a Dunn's post hoc analysis was used to determine significant differences from control. * $P < 0.05$; ** $P < 0.001$

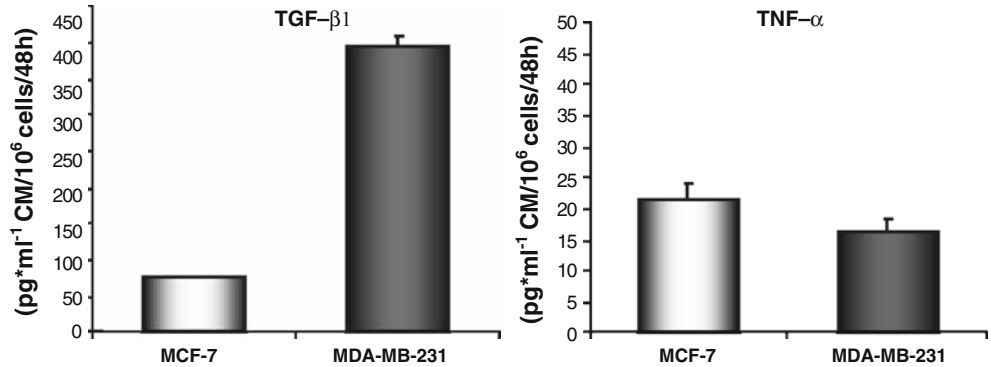

Fig. 2 Quantification of TGF-β and TNF-α in mammary cancer cell conditioned media. Samples of media conditioned by mammary tumoral cell lines MCF-7 and MD-MDA-231 were collected and immediately quantified by an ELISA immunoassay for TGF-β and TNF-α as described under "Materials and methods". Data represent the mean ± SE

TGF-β1 and TNF-α are expressed in human mammary tumors

To evaluate the cellular origin and distribution of TGF-β1 and TNF-α in mammary tumor samples, we analyzed by immunohistotochemistry the presence of these factors in samples of human ductal infiltrant mammary tumors. As Fig. 3 shows, both factors were expressed only by tumoral cells located at the invasive front without a relevant expression in the stromal adipose cells.

TGF-β1 and TNF-α inhibit the expression of transcriptional factors involved in the maintenance of adipose phenotype

The maintenance of the adipose phenotype largely depends on the expression of specific transcription factors such as C/EBPα and PPARγ, which are involved in the maintenance of adipose homeostasis [21]. To assess whether the expression of these transcriptional factors are sensitive to TGF-β1 and TNF-α, we analyzed by western blotting, the expression of

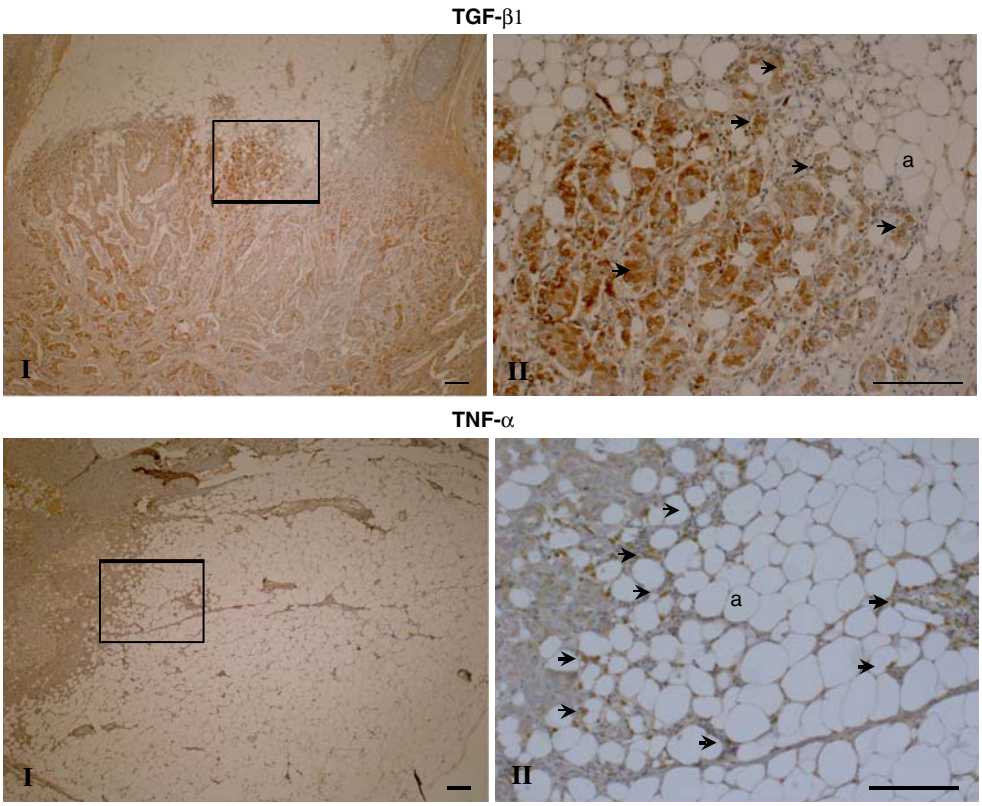

Fig. 3 Immunohistochemistry for TGF-β and TNF-α in human breast tumors. Representative microphotographs of immunohistochemistry for TGF-β and TNF-α including the edge of the tumoral epithelia. *Arrowheads* indicate positive tumoral cells for TGF-β and TNF-α invading the adipose stroma (*a*). Magnifications: (*I*) = ×4; (*II*) = ×20; *Bar* 100 μm

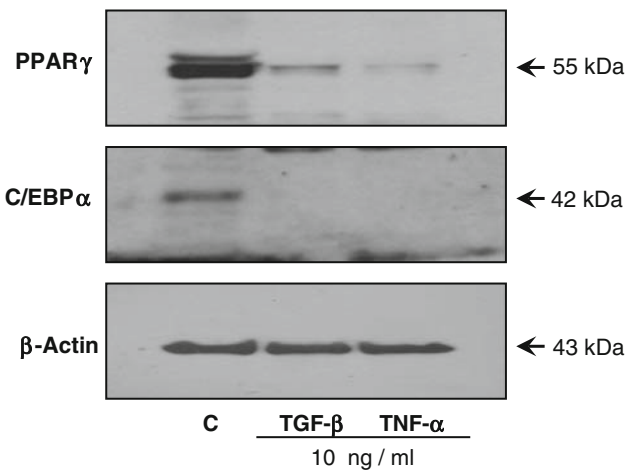

Fig. 4 TGF-β and TNF-α inhibit the expression of PPARγ and C/EBPα transcription factors in human mammary adipocytes. Mature adipocytes were obtained from reductive mammary surgery and cultured in serum-free D-MEM in the absence or presence of 10 ng/ml of TGF-β or TNF-α for 48 h. After this period, the expression of transcription factors was determined by western blot as indicated in "Material and methods"

C/EBPα and PPARγ in adipose differentiated mammary cells cultured in the presence of 10 ng/ml of both factors in serum-free media. As Fig. 4 shows, either TGF-β1 or TNF-α significantly reduced the expression of both transcriptional factors, suggesting that in the presence of TGF-β1 or TNF-α adipose cells may lose their fatty phenotype.

Peritumoral adipose tissue from infiltrant mammary tumors exhibit a lower expression of both C/EBPα and PPARγ

To test if factors produced by mammary epithelial cells affect the expression of C/EBPα and PPARγ in human samples, we analyzed by immunohistochemistry the expression of both transcriptional factors in two different conditions: in samples of infiltrant tumors and in healthy control samples. In both cases, the expression of nuclear C/EBPα and PPARγ was evaluated in adipose cells located within the vicinity of normal and tumoral epithelia. As Fig. 5 shows, adipose cells in contact with normal epithelia expressed a higher amount of C/EBPα and PPARγ compared to adipose cells in contact with tumoral epithelia.

Adipose cells in tumor samples exhibit a smaller size than their normal counterpart

Since our initial observations strongly suggested that factors produced by carcinoma cells can alter the mammary

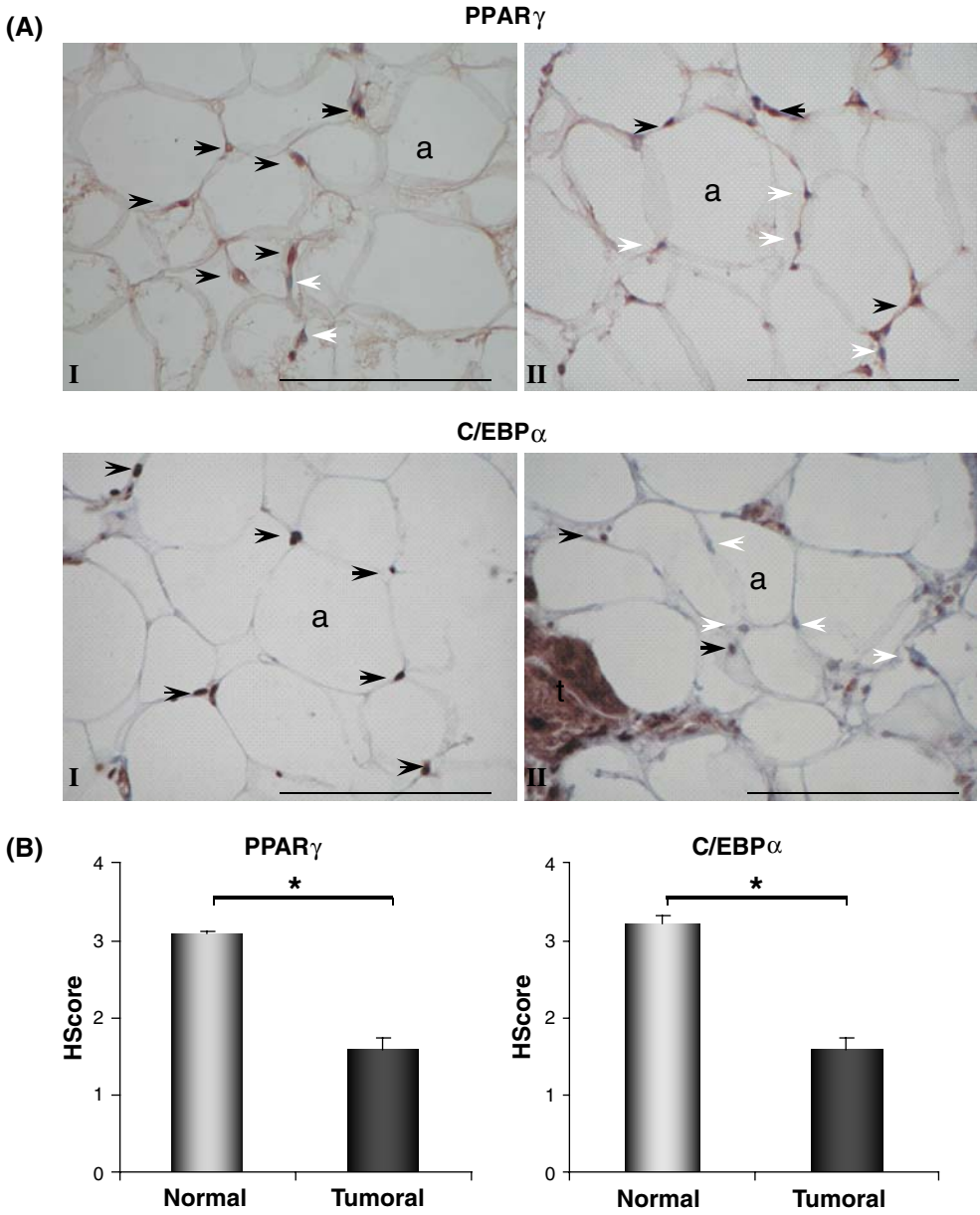

Fig. 5 Immunohistochemistry for PPARγ and C/EBPα in normal and tumoral mammary samples. **A** Representative microphotographs of immunohistochemistry for nuclear PPARγ and C/EBPα in adipose stromal tissue adjacent to normal (*I*) and tumoral (*II*) mammary tissues. Positive and negative immunoreactive cells are indicated by *black* or *white arrowheads*, respectively. Magnification ×40; *Bar* 100 μm. **B** H-SCORE for staining of PPARγ and C/EBPα in normal and tumoral adipocytes from mammary samples. Assessment of staining intensity and distribution was made using the semiquantitative histological score calculated as described in "Material and methods". (*a*) adipose cells; (*t*) tumor. A Mann–Whitney test analysis was used to determine significant differences. * $P < 0.05$

phenotype, we reasoned that as a final outcome of this phenomenon, adipose cells within the vicinity of tumors should contain fewer lipids than their normal counterparts. Figure 6b represents the cumulative frequency of cell surface in cross-sectional areas in both types of tissue samples. Curves show that values representing the lower surface area were more frequent in tumoral samples. For example, sixty percent of adipose cells derived from normal tissue display a size with a higher limit of 150 UR. On the contrary, the same proportion of cells from the tumoral sample shows a size below 45 UR. Moreover, on average, tumoral cells also display a smaller size than their counterparts in the normal sample (Fig. 6c).

Media conditioned by mammary cell lines reverse the expression of C/EBPα and PPARγ in differentiated 3T3-L1 cells

Human adipose cells isolated from mammary tissues frequently suffer lysis when exposed to MC from mammary

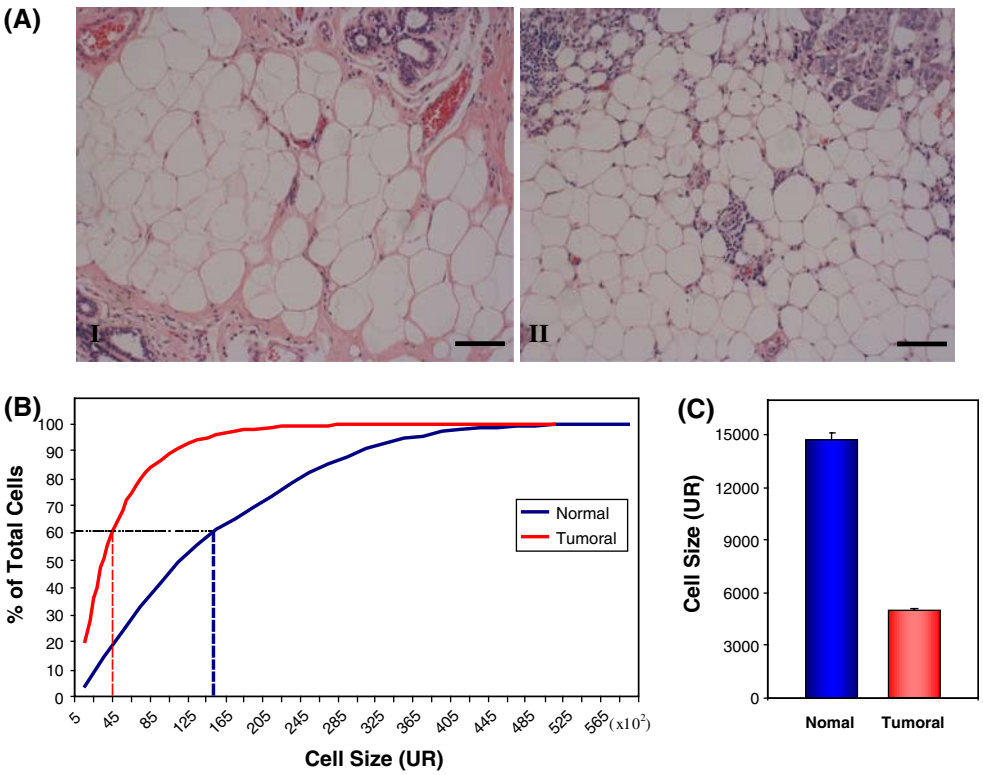

Fig. 6 Determination of adipocyte cells size in histological sections of normal and tumoral mammary tissue. **a** Hematoxylin/eosin stained sections of a normal (*I*) and infiltrating tumor mammary tissue (*II*) showing the area located in the vicinity of epithelia. Magnification: ×20, *bar* 50 μm. **b** Cumulative frequency of mammary adipose cells surface area in normal and tumoral tissue. Cell surface area was expressed in relative units according to "Material and methods". *Dotted line* shows that a 60% of adipose cells from tumoral sample have a size below 45 UR. In normal tissue this proportion of cells reaches a size around 150 UR. **c** Mean surface area of adipocytes derived from normal and tumoral tissue evaluated as in (**b**)

cancer cell lines while cultured in cell suspension. To avoid this consequence, we assessed the potential of MCs MCF-7 and MDA-MB-231 cells to reverse the adipose phenotype in 3T3-L1 murine adipose cells, a standardized cellular system frequently used to study the adipose differentiation process.

Figure 7a shows that after 10-days culturing with an adipogenic media, 3T3-L1 cells strongly expressed either PPARγ or C/EBPα at the protein level. Further treatment for 5 days with increasing concentrations of MC from MCF-7 and MDA-MB-231 cells decreased the expression of these transcriptional factors in a dose-dependent manner.

To test if this reduction of expression of transcriptional factors was associated with their functional nuclear location, we utilized a similar set of adipose cells to perform an immunofluorescence experiment. Figure 7b shows that MC from human mammary cell lines strongly reduces the nuclear expression of PPARγ C/EBPα. In both types of experiments, MC from MDA-MB-231 cells exert a clearly more potent effect on the expression of both transcriptional factors.

Inhibitors of TGF-β1 and TNF-α block the effects of CM by breast tumoral cell lines

To identify whether the presence of TGF-β1 and TNF-α in MC from breast tumoral cells is responsible for the diminished expression of adipose transcriptional factors, we inhibited the action of both factors using a blocking antibody against TNF-α and a betaglycan-derived soluble peptide that inhibits the binding of TGF-β1 to its receptor [12]. In a similar manner to previous experiments, we assayed the inhibitory capacity of these two agents by measuring the expression of PPARγ and C/EBPα with western blotting and immunofluorescence. Figure 8a shows that the addition of both inhibitors to cell culture contained MC by MDA-MB-231 cells, effectively counteracted the inhibitory effect of conditioned media enhancing the expression of PPARγ C/EBPα. On the other hand, Fig. 8b shows by immunofluorescence that the expression and nuclear location of PPARγ and C/EBPα, initially inhibited by the breast cell line CM, partially recovered their adipose characteristic pattern. These results strongly suggest that TGF-β1 and TNF-α are, in fact, two components of the CM that are able to modify the adipose phenotype in 3T3-L1 cells.

Discussion

Invasion into neighboring tissue is a crucial event for cancer progression and a prerequisite to metastasis [22]. During epithelial carcinogenesis, an active molecular cross-talk is established among cancer and stromal cells. As a result, functional changes produced in stroma may contribute to cancer invasion [23]. In human breast cancer,

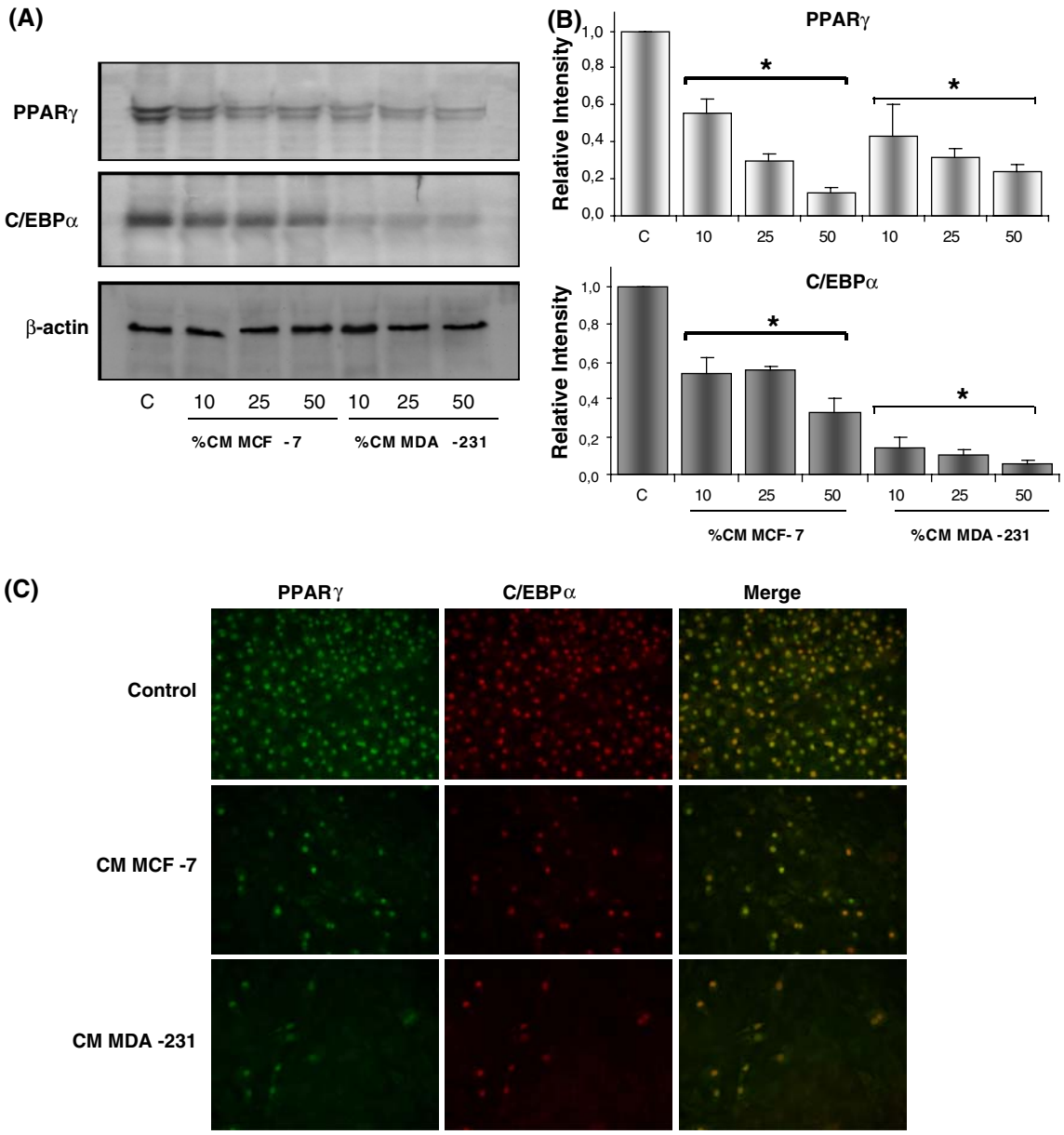

Fig. 7 Media conditioned by tumoral mammary cells inhibit the expression of PPARγ and C/EBPα in differentiated 3T3-L1 adipocytes. **a** PPARγ and C/EBPα expression was evaluated by western-blot in 3T3-L1 cells cultured during 10 days in adipogenic medium followed by the exposure for 5 days to increasing concentrations of conditioned media derived from mammary tumoral cells lines MCF-7 and MDA-MB-231. Representative blots obtained from three different experiments. **b** Quantitative analysis of PPARγ and C/EBPα expression normalized against β-actin as a loading control. Data are presented as fold change over control. *Bars* represents mean ± SE. * $P < 0.05$. **c** Shows an immunofluorescence for nuclear PPARγ (*green*) and C/EBPα (*red*) in cells cultured in the presence of CM derived from mammary cells. (Color figure online)

the main stromal change observed during cancer progression is a fibrotic response that has been identified as a predictor of a poor prognosis [3].

In the present work, we proposed that part of this fibrotic response is attributed to a dedifferentiation phenomenon. Under the effect of soluble factors produced by carcinoma cells, mammary adipocytes, the main component of the breast stroma, revert their phenotype to one in which fibroblast-like cells predominate. Our results show that mature mammary human adipocytes cultured in the presence of a media conditioned by tumoral mammary cell lines (MCF-7 and MDA-MB-231) reverted to a fibroblast-like phenotype in which fat accumulation is markedly diminished. This finding is expected to represent a dedifferentiation phenomenon because in these experimental conditions (culture in semisolid conditions) cell proliferation does not occur in normal cells. Our data also demonstrated that TNF-α and TGF-β1 produced by mammary

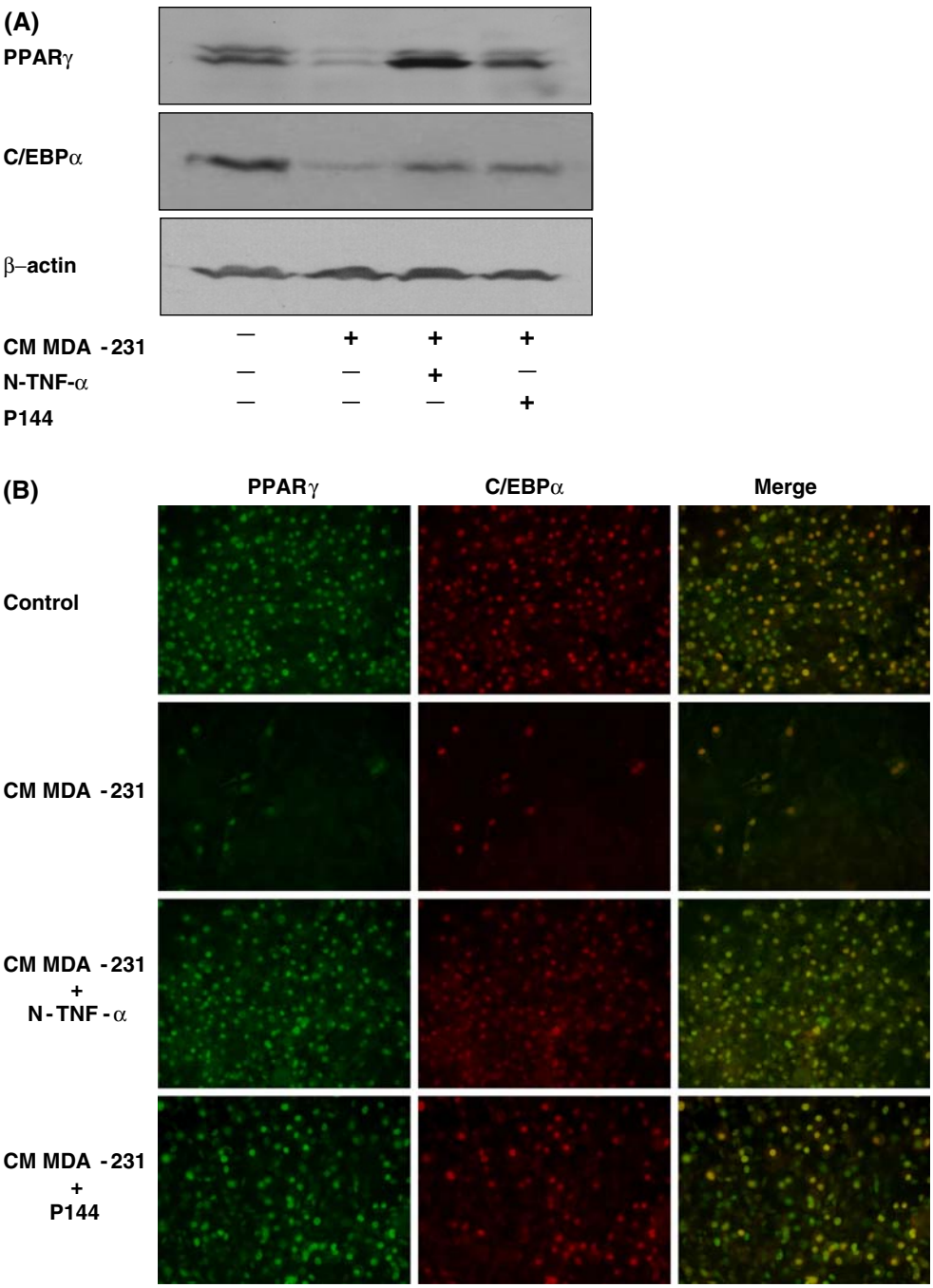

Fig. 8 TGF-β and TNF-α present in media conditioned by MDA-MB-231 cells are responsible for the inhibition of expression of PPARγ and C/EBPα in differentiated 3T3-L1 adipocytes. **a** Western-blot of PPARγ and C/EBPα from 3T3-L1 adipose cells prepared as in Fig. 7 and exposed for 5 days to a 50% medium conditioned by MDA-MB-231 cells in the presence of either 0.6 μg/ml of blocking antibodies against TNF-α or a 100 μg/ml of P-144. **b** Immunofluorescence for both transcription factors in cells treated in the same conditions as in (**a**). PPARγ (*green*) and C/EBPα (*red*). Magnification ×40. (Color figure online)

cell lines are two candidates potentially responsible for this phenotypic change. Both factors were produced by these cell lines and were expressed in the human breast tumoral samples evaluated in this study.

To evaluate whether the intrinsic invasive capacity of mammary cell lines was correlated with their potential to revert to the adipose phenotype, we compared this activity in MCF-7 cells, a weakly invasive cell line, and in MDA-MB-231, a highly invasive cell line. From our results, two important conclusions arose. First, media conditioned by MDA-MB-231 cells behave more actively than media from MCF-7 cells in terms of reversion of the adipose phenotype. Second, while the TNF-α content in media conditioned by both cell lines was similar, the concentration of TGF-β1 in the invasive cell lines is five times higher than that of MCF-7. This data allow us to suggest that some of the differential effects in the conversion to a fibroblast phenotype found with MDA-MB-231 conditioned medium are mainly due to the autocrine production of TGF-β1.

TGF-β1 is a well-described cancer cell-derived growth factor, abundantly expressed in tumoral cells, that directly stimulates the transdifferentiation of stromal fibroblasts into myofibroblasts and strongly stimulates desmoplasia in a model of human pancreatic carcinoma [24, 25]. The participation of TGF-β1 in fat accumulation has been previously demonstrated in an in vivo model in which the overexpression of TGF-β1 induces a strong fibrosis in some tissues and also a severe reduction in body fat [26]. Our results show that TGF-β1 not only induces a reduced fat content in mammary adipose cells, but also a marked decreased expression of PPARγ and C/EBPα, two of the most important adipogenic transcriptional factors. In fact, it has been proposed that, within the concept of microenvironmental niche, the balance between TGF-β1 and PPARγ defines the fate of the adipose or myofibroblasts differentiation pathway [27]. Accordingly, in samples of human mammary tumors, stromal cells located within the vicinity of the epithelial edge that express a higher amount of TGF-β1 and TNF-α (Fig. 2) also display a lesser proportion of stain for nuclear PPARγ and C/EBPα (Fig. 5). Our results also showed that around the tumoral cells, which represent the main source of antiadipogenic growth factors, also a reduction in the size of the neighbor adipose cells is observed (Fig. 6). A reduction in fat mass is associated with a decrease in the size of adipocytes [28]. We suggest that diminution of size of adipose cells in tumoral samples is the consequence of an external (tumoral) stimulus that impairs the expression of PPARγ and C/EBPα which, in turn, diminishes the adipose accretion in these cells.

The confirmation that TGF-β1 and TNF-α, present in the media conditioned by mammary cells, are responsible for the adipose phenotypical reversion comes from experiments in which the activity of both growth factors present in MC from MDA-MB-231 cells was interfered by a blocking antibody (TNF-α) and a peptide that inhibits the binding of TGF-β1 to its receptor. Under these experimental conditions the expression and the nuclear location of PPARγ and C/EBPα were restored to the initial conditions in the absence of conditioned media.

From our data, one may extrapolate that mammary epithelial cells are able to promote an adipose dedifferentiation process that generates a stiffer desmoplastic stroma which is richer in fibroblast-like cells. As a result, these cells are able to provoke important changes in ECM composition, integrity, and topology [29]. These conditions generate a new extracellular microenvironment responsible for a new mechanosignalling pattern among stromal and epithelial cells that is very likely to induce a new transcriptional pattern in favor of tumoral progression. Previous data from our laboratory shows that the same conditions that inhibit the expression of PPARγ and C/EBPα transcription factors in mouse adipose cells also activates RhoA, a small GTPase responsible for the actin cytoskeleton assembly and which is functionally expressed in the fibroblast phenotype (data not shown).

Taken together, the results presented in this paper propose that adipose reversion, an important feature in the mechanism of mammary desmoplasia response, is mediated by TGF-β1 and TNF-α, which are factors produced by tumoral cells. These findings will increase our understanding of the cellular mechanisms involved in the epithelial-stroma interactions which are key events in the determination of tumoral cells to invade, and represent a probable new target for future therapies.

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