

# Tumor Necrosis Factor $\alpha$ and Interleukin 11 Secreted by Malignant Breast Epithelial Cells Inhibit Adipocyte Differentiation by Selectively Down-Regulating CCAAT/Enhancer Binding Protein $\alpha$ and Peroxisome Proliferator-activated Receptor $\gamma$ : Mechanism of Desmoplastic Reaction<sup>1</sup>

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## ABSTRACT

The dense layer of fibroblasts that accumulate around malignant breast epithelial cells (*i.e.*, desmoplastic reaction) arises from the breast adipose tissue and provides structural and biochemical support for breast cancer. We report herein a number of epithelial-stromal interactions responsible for desmoplastic reaction in breast cancer using cultured 3T3-L1 murine fibroblasts and human adipose fibroblasts, which can be activated with a mixture of hormones to differentiate to mature adipocytes. Adipocyte differentiation was inhibited by coculturing fibroblasts with various breast cancer cell lines (T47D, MCF-7, SSC202, SSC78, and SSC30) completely or by breast cancer cell conditioned media in a dose-dependent manner; on the other hand, adipocyte differentiation was not inhibited by coculturing with normal human primary mammary epithelial cell conditioned medium. This tumor effect was eliminated using neutralizing antibodies against tumor necrosis factor (TNF)- $\alpha$  or interleukin (IL)-11. TNF- $\alpha$  and IL-11 levels were 2.5–3 times higher in T47D conditioned medium compared with control medium, and TNF- $\alpha$  transcripts were detectable in T47D but not in 3T3-L1 cells in culture, indicating that the malignant epithelial cell is the major site of cytokine production. This was confirmed *in vivo* in mastectomy specimens, where immunoreactive TNF- $\alpha$  and IL-11 were readily detectable in malignant epithelial cells but not in the majority of the surrounding fibroblasts. Adipocyte differentiation is mediated by the expression of a cascade of adipogenic transcription factors, including CCAAT/enhancer binding protein (C/EBP) $\beta$ , C/EBP $\delta$ , peroxisome proliferator-activated receptor (PPAR) $\gamma$  and C/EBP $\alpha$ . C/EBP $\alpha$  and PPAR $\gamma$  are essential for this process. We demonstrated by Northern analysis that exposure of activated 3T3-L1 cells to T47D cell conditioned medium strikingly decreased the levels of PPAR $\gamma$  and C/EBP $\alpha$  transcripts and increased the levels of C/EBP $\beta$  and C/EBP $\delta$  transcripts. In these 3T3-L1 cells, inhibition of differentiation was also confirmed by markedly suppressed levels of *aP2* mRNA, which is an adipocyte-specific gene. These *in vitro* observations were confirmed in sections of human malignant breast tumors, where immunoreactive C/EBP $\alpha$  was readily detectable in adipose fibroblasts distant to the tumor but not in intratumoral fibroblasts. Treatment of 3T3-L1 cells with T47D cell conditioned medium or TNF- $\alpha$  changed neither the numbers of cells in G<sub>0</sub>-G<sub>1</sub>, S, and G<sub>2</sub> phases nor the rate of [<sup>3</sup>H]thymidine incorporation, thus ruling out a proliferative effect of malignant cells on the surrounding fibroblasts. In summary, desmoplastic reaction primarily occurs via the action of cytokines (TNF- $\alpha$  and IL-11) secreted by the malignant epithelial

cells to inhibit differentiation of adipose fibroblasts to mature adipocytes. This tumor-induced block in adipocyte differentiation is mediated by the selective inhibition of expression of the essential adipogenic transcription factors, *i.e.*, PPAR $\gamma$  and C/EBP $\alpha$ .

## INTRODUCTION

Breast tumors are characterized by the accumulation of fibroblasts adjacent to malignant epithelial cells, which is commonly known as the desmoplastic reaction (1). In fact, the majority of breast cancers have been referred to as “scirrhous” because of their extremely hard consistency provided by large numbers of fibroblasts dispersed between malignant epithelial cells, as well as within the immediate periphery of tumors. The relationship between adipose stroma and breast cancer is unique in the sense that stromal fibroblasts seem to provide the structural support for cancer growth, whereas malignant cells greatly influence the composition of the adjacent tissue. Evidence from several laboratories indicates that this epithelial-stromal interaction also involves paracrine mechanisms that promote the development and growth of breast carcinomas (2–4). These morphologically identified intra- and peritumoral fibroblasts originate from adipose tissue and most likely represent potential preadipocytes, because fibroblasts isolated from adipose tissue are capable of differentiating to mature adipocytes under defined culture conditions (5, 6). We hypothesized that malignant epithelial cells of breast tumors secrete growth factors and cytokines to prevent the differentiation of fibroblasts to mature adipocytes in the adjacent adipose tissue. The following body of preliminary data supported this hypothesis:

(a) Tumors were found in breast quadrants with the highest P450arom<sup>4</sup> transcript levels and the highest fibroblast:adipocyte ratio (7). This parallelism between the distribution of fibroblasts and aromatase expression is not surprising, because aromatase is a marker for undifferentiated adipose fibroblasts (8, 9).

(b) In the cancer-free human breast, the highest fibroblast:adipocyte ratios and P450arom transcript levels were found in the lateral and upper region (10). This distribution roughly correlates with the most common sites where infiltrating duct carcinomas develop.

(c) Breast tumor conditioned medium was found to induce aromatase activity in adipose fibroblasts (11). This tumor-induced effect can be inhibited and titrated by addition of an anti-IL-11 antibody.<sup>5</sup> When aromatase expression is viewed as a fibroblast marker, these results suggested that malignant epithelial cells secrete factors, such as IL-11, to prevent differentiation of fibroblasts. Additionally, estradiol

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<sup>4</sup> The abbreviations used are: P450arom, aromatase P450; IL, interleukin; C/EBP, CCAAT/enhancer binding protein; PPAR, peroxisome proliferator-activated receptor; TNF, tumor necrosis factor; DEX, dexamethasone; MIX, 1-methyl-3-isobutyl-xanthine; TCM, T47D cell conditioned medium; NCM, normal mammary epithelial cell conditioned medium.

<sup>5</sup> J. E. Nichols, S. E. Bulun, and E. R. Simpson, unpublished observations.

pretreatment of cancer cells potentiated aromatase induction in a dose-dependent fashion, which is suggestive of a paracrine loop (11). Moreover, we demonstrated that certain adipogenesis inhibitors such as IL-11 are secreted by the T47D breast cancer cell line (12). Again, estradiol stimulated IL-11 expression in T47D cells in a dose-dependent fashion (12).

(d) IL-11 and TNF $\alpha$  stimulation of the fibroblast marker aromatase in adipose fibroblasts can be interpreted as inhibition of adipocyte differentiation, because aromatase expression in adipose tissue primarily resides in fibroblasts but not in mature adipocytes (8, 9, 13, 14).

There may be multiple potential mechanisms responsible for accumulation of adipose fibroblasts within the tumor and in adjacent stroma. It is possible that fibroblasts proliferate in response to tumor-derived growth factors. Although treatment of murine 3T3-L1 fibroblasts in culture with serum, insulin, insulin-like growth factor I and epidermal growth factor initially causes proliferation, cell replication rapidly stops under these conditions, and fibroblasts eventually differentiate into mature adipocytes (15–18). Thus, it follows that there have to be other effective mechanisms for peritumoral fibroblast accumulation. Inhibition of differentiation or dedifferentiation of pre-existing adipocytes may provide these critical mechanisms responsible for extremely high fibroblast:adipocyte ratios in the stroma surrounding cancer cells. This process may be under the control of cytokines secreted by the malignant epithelial cells.

The cellular and molecular mechanisms responsible for the differentiation of stromal fibroblasts into mature adipocytes have been well characterized (19–24). During mammalian development, embryonic mesoderm gives rise to several highly specialized cell types, including adipocytes. Differentiation of adipocytes from multipotential fibroblastic precursors is primarily controlled by two tissue-specific transcription factors: the C/EBP $\alpha$  and PPAR $\gamma$ . During the process of differentiation, C/EBP $\beta$  (and possibly C/EBP $\delta$ ) are initially expressed and convert multipotential mesenchymal precursor cells into preadipocytes (19). These “determined” preadipocytes are able to respond subsequently to potent adipogenic inducers such as PPAR $\gamma$ . The PPAR isoform PPAR $\gamma$  (21) is a member of the ligand-activated transcription factor family that heterodimerizes with retinoid X receptor  $\alpha$  and binds to the promoters of adipocyte-specific genes. A third adipocyte-enriched transcription factor, C/EBP $\alpha$ , has been shown to promote terminal adipocyte differentiation (20). When expressed together, PPAR $\gamma$  and C/EBP $\alpha$  act synergistically to powerfully promote adipocyte differentiation in fibroblastic cells, regardless of tissue of origin (22, 24). For practical purposes, we will refer to undifferentiated mesenchymal precursors and determined preadipocytes in human adipose tissue as adipose fibroblasts, because both cell types appear as fibroblasts morphologically. Fibroblasts isolated from adipose tissue differentiate into adipocytes when cultured in a defined medium (5, 6). On the other hand, certain substances, such as TNF- $\alpha$ , are not only capable of inhibiting adipocyte differentiation but also of reversing it by suppressing the expression of PPAR $\gamma$  (25, 26). Most of the work in this field has been performed using rodent fibroblasts and has been related to obesity and diabetes. Possible roles of malignant epithelial cells in paracrine regulation of these transcription factors and on adipocyte differentiation have not been studied to date. We report herein a number of epithelial-stromal interactions in the breast cancer, which represent cellular and molecular mechanisms responsible for the development and maintenance of desmoplastic reaction.

## MATERIALS AND METHODS

**Tissue Acquisition.** Breast adipose tissue was obtained from five patients undergoing reduction mammoplasty. These tissues were immediately pro-

cessed for primary cultures of adipose fibroblasts. Breast cancer and surrounding adipose tissue samples were obtained from 25 mastectomy specimens for immunohistological detection of C/EBPs ( $\alpha$ ,  $\beta$ , and  $\delta$ ), IL-11, and TNF- $\alpha$ . These studies were conducted following protocols approved by the Institutional Review Boards of the University of Texas Southwestern Medical Center and Tohoku University School of Medicine.

**Detection of Transcripts of Adipocyte-specific Genes and Cytokines by RNA Blot Analysis.** Total RNA was isolated from fibroblasts/adipocytes in culture, electrophoretically fractionated (10  $\mu$ g), and transferred to a charged membrane. Duplicate measurements of absorbance (260  $\mu$ m) were performed to equalize loading, which was confirmed by visual inspection of 18S and 28S RNA stained with ethidium bromide. Northern blots were hybridized with cDNA probes labeled by random priming using [<sup>32</sup>P]dCTP. cDNA templates for adipocyte P2 (aP2), C/EBP $\delta$ , C/EBP $\beta$ , PPAR $\gamma$ , C/EBP $\alpha$ , and TNF- $\alpha$  were kindly provided by Drs. Steve McKnight, Gokhan Hotamisligil, Carol Mendelson, Bruce Beutler, and Gretchen Darlington.

**Cell Cultures and Differentiation of Fibroblasts to Adipocytes.** We routinely performed primary cultures of human adipose fibroblasts as described previously (8). Differentiation of human adipose fibroblasts to mature adipocytes was performed following a modified protocol originally outlined by Hauner *et al.* (5). Breast adipose tissue, obtained from women at the time of reduction mammoplasty, was processed by mincing, washing, digestion (with collagenase), and centrifugation steps. The floating mature adipocytes were aspirated, and the sedimented fibroblast fraction was resuspended in DMEM with 10% FCS as described previously (8). Nucleus-containing cells were inoculated at a density of 50,000/cm<sup>2</sup> into six-well plates. Cultures were grown for a 24-h period in DMEM with 10% FCS. Cells were then placed in a chemically defined phenol red-free and serum-free medium consisting of DMEM/F12 (1:1, v/v), 15 mM NaHCO<sub>3</sub>, 15 mM HEPES, 33  $\mu$ M biotin, 17  $\mu$ M pantothenate, 0.67  $\mu$ M human insulin, 0.2 mM triiodothyronine, 0.5 mM DEX, and antibiotics for 21 days. Within 15–21 days, cells achieve maximum differentiation. Cells were regarded as differentiated by morphological criteria when, after acquiring a round shape, the cytoplasm was completely filled with multiple lipid droplets as assessed by Oil Red O staining. The proportion of differentiated cells is estimated by direct counting under the microscope of total and differentiated cells, using a micrometer.

The 3T3-L1 fibroblasts were grown in DMEM with 10% FCS. T47D cells were initially grown in RPMI 1640 with 10% FCS containing 0.02 mM HEPES, whereas MCF-7 cells were grown in MEM with 10% FCS until confluent. SSC202, SC78, and SSC30 breast cancer cells (kindly provided by Dr. Adi Gazdar) were grown to confluence in DMEM with 10% FCS. Primary human mammary epithelial cells purchased from Clonetics, Inc. (Walkersville, MD) were grown to confluence in the MEGM medium supplied by the manufacturer (Clonetics). Cell conditioned medium from T47D and human mammary epithelial cells were collected for coculture experiments in the following fashion. Confluent cells were maintained in DMEM for 12 h for washout. Then, cells were incubated in DMEM for 24 h to allow accumulation of factors secreted by these cells. These conditioned media were subsequently used for coculture experiments. All cells were incubated at 37°C in 5% CO<sub>2</sub>. To induce the adipogenic differentiation of 3T3-L1 fibroblasts within 2 days of reaching confluence, these cells were treated with DEX (0.25  $\mu$ M), MIX (0.5 mM), and insulin (1  $\mu$ g/ml) for 2 days and then maintained in DMEM with 10% FCS for 6 additional days. Cells containing multiple fat droplets were scored as differentiated by phase contrast microscope after staining with Oil Red O. All culture media were phenol red free.

Neutralizing antibodies against human IL-11 (Ab-218-NA), TNF- $\alpha$  (Ab-210-NA), IL-2, and normal goat IgG (AB-108-C) were purchased from R&D Systems, Inc. (Minneapolis, MN). These were added to the conditioned medium at a final concentration of 40 ng/ml.

As an alternative to the use of breast cancer cell conditioned media, 3T3-L1 and human adipose fibroblasts were cocultured with breast cancer cells using 35-mm, six-well plates. 3T3-L1 cells or adipose fibroblasts were plated on the bottom wells. Breast cancer cells were seeded on the permeable membrane (0.45- $\mu$ m) tissue culture inserts. 3T3-L1 cells were cultured in DMEM with 10% FCS, after they reached confluence for 2 days. At this time, inserts containing cancer cells were introduced, and the medium was changed to the appropriate differentiation medium for 48 h. Then the medium was switched back to DMEM plus 10% FCS for 6 days. In the case of human adipose fibroblasts, cocultures were maintained for 15–20 days in the differentiating

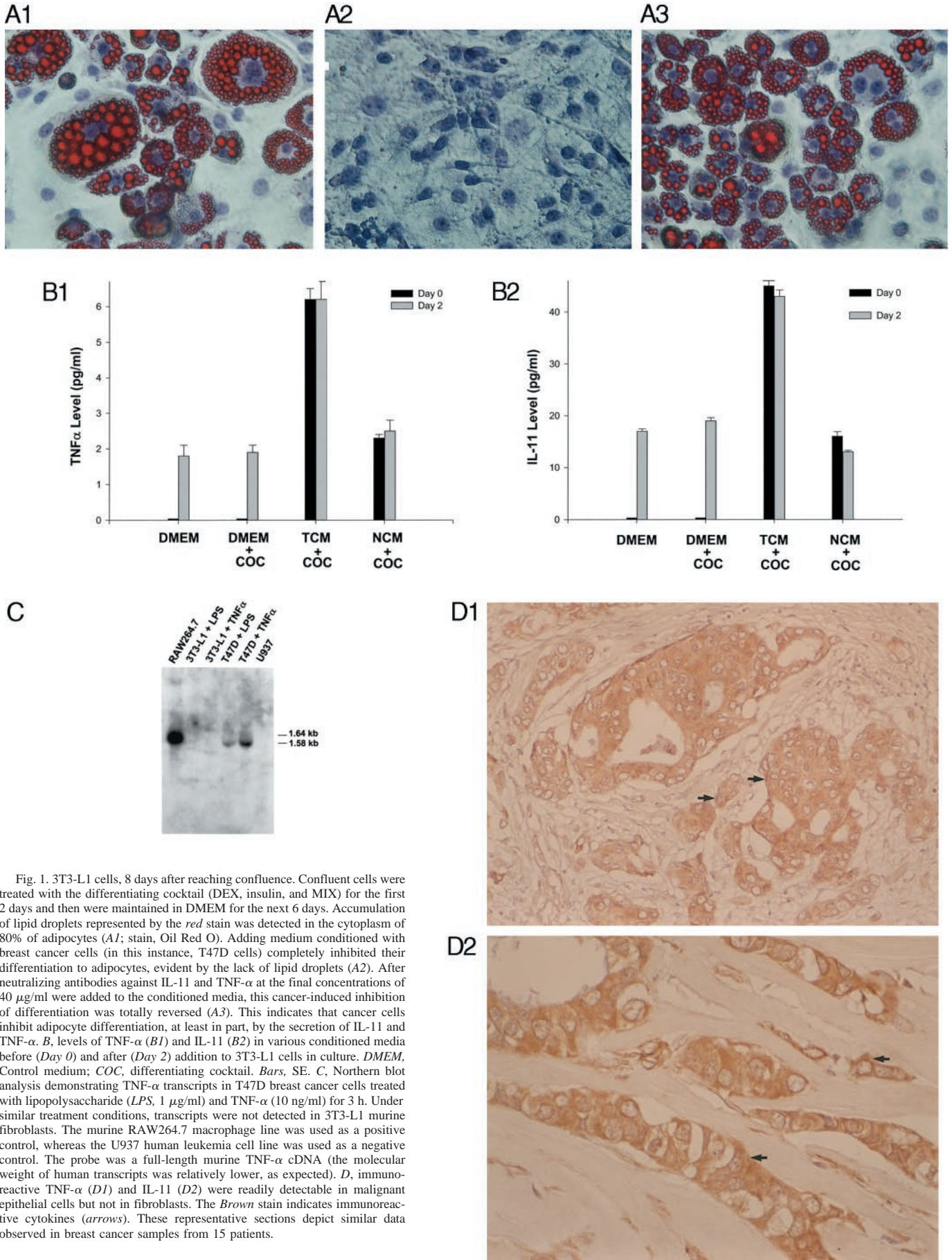


Fig. 1. 3T3-L1 cells, 8 days after reaching confluence. Confluent cells were treated with the differentiating cocktail (DEX, insulin, and MIX) for the first 2 days and then were maintained in DMEM for the next 6 days. Accumulation of lipid droplets represented by the red stain was detected in the cytoplasm of 80% of adipocytes (A1; stain, Oil Red O). Adding medium conditioned with breast cancer cells (in this instance, T47D cells) completely inhibited their differentiation to adipocytes, evident by the lack of lipid droplets (A2). After neutralizing antibodies against IL-11 and TNF- $\alpha$  at the final concentrations of 40  $\mu$ g/ml were added to the conditioned media, this cancer-induced inhibition of differentiation was totally reversed (A3). This indicates that cancer cells inhibit adipocyte differentiation, at least in part, by the secretion of IL-11 and TNF- $\alpha$ . B, levels of TNF- $\alpha$  (B1) and IL-11 (B2) in various conditioned media before (Day 0) and after (Day 2) addition to 3T3-L1 cells in culture. DMEM, Control medium; COC, differentiating cocktail. Bars, SE. C, Northern blot analysis demonstrating TNF- $\alpha$  transcripts in T47D breast cancer cells treated with lipopolysaccharide (LPS, 1  $\mu$ g/ml) and TNF- $\alpha$  (10 ng/ml) for 3 h. Under similar treatment conditions, transcripts were not detected in 3T3-L1 murine fibroblasts. The murine RAW264.7 macrophage line was used as a positive control, whereas the U937 human leukemia cell line was used as a negative control. The probe was a full-length murine TNF- $\alpha$  cDNA (the molecular weight of human transcripts was relatively lower, as expected). D, immunoreactive TNF- $\alpha$  (D1) and IL-11 (D2) were readily detectable in malignant epithelial cells but not in fibroblasts. The Brown stain indicates immunoreactive cytokines (arrows). These representative sections depict similar data observed in breast cancer samples from 15 patients.

medium, because human cells required prolonged exposure to this medium in contrast to 3T3-L1 murine fibroblasts. At the end of coculture experiments, human adipose fibroblasts or 3T3-L1 cells in the bottom plate were evaluated for differentiation to mature adipocytes and for proliferative indices (rate of [<sup>3</sup>H]thymidine incorporation and flow cytometry) or were harvested for RNA isolation.

**Immunohistochemistry.** Antihuman IL-11, TNF- $\alpha$ , C/EBP $\alpha$ , C/EBP $\beta$ , and C/EBP $\delta$  antibodies were purchased from R&D Systems, Inc. The immunohistochemical procedures were performed, as described previously, on 2.5- $\mu$ m-thick sections mounted on poly-L-lysine-coated slides using the biotin-streptavidin amplified technique with a histone immunostaining kit (Nichirei, Tokyo, Japan). Briefly, this staining procedure was performed as follows: (a) routine deparaffinization; (b) inactivation of endogenous peroxidase activity with 0.3% H<sub>2</sub>O<sub>2</sub> in methanol for 20 min at 23°C; (c) blocking with 1% goat serum for 45 min at 23°C; (d) incubation with the primary antibody at 4°C for 18 h; (e) incubation with biotinylated goat antirabbit antibody for 30 min at 23°C; (f) incubation with peroxidase-conjugated streptavidin for 30 min at 23°C; (g) colorimetric reaction with a solution containing 0.05% Tris-HCl (pH 7.6), 0.66 M 3,3'-diaminobenzidine, and 2 M H<sub>2</sub>O<sub>2</sub>; and (h) counterstaining with 1% methyl green.

**ELISA Assay.** For the determination of IL-11 and TNF- $\alpha$  concentrations in breast cancer cell conditioned media, IL-11 and TNF- $\alpha$  ELISA immunoassay kits (R&D Systems, Inc.) were used according to the instructions supplemented by the vendor. Briefly, samples of media were collected immediately before and 2 days after the addition of these media to 3T3-L1 cells. Samples (100  $\mu$ l) were assayed in triplicate. After 2–16 h of incubation at room temperature, each well was aspirated. IL-11 or TNF- $\alpha$  conjugates (200  $\mu$ l) were added to the wells and incubated for 2 h at room temperature. Each well was aspirated. The substrate solution (200  $\mu$ l) was then added to each well and incubated for 30 min. After adding 50  $\mu$ l of the stop solution to each well, the absorbance was immediately determined at a wavelength of 450 nm. IL-11 or TNF- $\alpha$  concentrations were calculated by creating standard curves and plotting the mean absorbance for each standard on the Y axis against the concentration on the X axis.

## RESULTS

**Effects of Tumor Cells on the Differentiation of Fibroblasts to Adipocytes.** Confluent 3T3-L1 cells differentiate to mature adipocytes within 4 or 6 days after a 48-h treatment with a mixture including insulin, DEX, and MIX. At this stage, the cells appear rounded and possess numerous large cytosolic lipid spheres, as revealed by Oil Red O staining (Fig. 1A1). The effect of the T47D breast cancer cell line on the adipogenic differentiation of the 3T3-L1 cells was evaluated with cocultures or T47D cell conditioned medium. Medium conditioned with T47D breast cancer cells completely inhibited the differentiation of 3T3-L1 cells (Fig. 1A2). Upon addition of neutralizing antibodies to human IL-11 and TNF- $\alpha$ , this effect was totally reversed. In other words, neutralizing both of these cytokines reversed the inhibitory effect of breast cancer cells on adipocyte differentiation (Fig. 1A3). This reversal was partial when either antibody was used separately and was dose dependent (data not shown). Antihuman IL-2 neutralizing antibodies or the normal goat IgG antibody did not reverse the inhibition, indicating that the effects of anti-IL-11 and TNF- $\alpha$  neutralizing antibodies were specific.

Cocultures of T47D cancer cells also inhibited the differentiation of human adipose fibroblasts completely. Other breast cancer cell lines (MCF-7, SSC202, SSC78, and SSC30) also inhibited the differentiation of both 3T3-L1 murine fibroblasts and human adipose fibroblasts. Moreover, the liver cancer cell line HepG2 and the choriocarcinoma cell line JEG3 also inhibited the differentiation of these cells to adipocytes (data not shown). Because all malignant epithelial cells tested inhibited differentiation, normal primary mammary epithelial cell conditioned medium was used to further determine the specificity of inhibition of differentiation. Normal mammary epithelial cells did not inhibit the differentiation of 3T3-L1 cells (data not shown), which

demonstrated that the inhibition on differentiation is specific for malignant cells.

**Cellular Localization of the Cytokines Inhibitory for Adipocyte Differentiation in the Breast Cancer.** Previous experiments have suggested that IL-11 and TNF- $\alpha$  mediated the inhibition of adipocyte differentiation by cancer cells. Thus, we determined the cellular origin of these cytokines in breast cancer:

(a) Both TNF- $\alpha$  and IL-11 concentrations in TCM were 2.5–3 times those found in media conditioned with control cells. The levels of these cytokines did not increase during the incubation of 3T3-L1 cells, indicating that 60–75% of IL-11 and TNF- $\alpha$  in the medium arises from T47D cells (Fig. 1, B1 and B2).

(b) We demonstrated TNF- $\alpha$  transcripts in T47D breast cells but not in 3T3-L1 cells, using Northern blotting (Fig. 1C).

(c) We determined the *in vivo* cellular distribution of immunoreactive IL-11 and TNF- $\alpha$  in 15 mastectomy specimens. These two cytokines were primarily expressed in all malignant epithelial cells. Less than 25% of fibroblasts contained immunoreactive IL-11 or TNF- $\alpha$  with considerably less staining intensity (Fig. 1D).

**Expression of PPAR $\gamma$ , C/EBP $\alpha$ , and aP2 in 3T3-L1 Cells Cocultured with Breast Cancer Cells.** We determined the levels of transcripts of C/EBP $\alpha$ , C/EBP $\beta$ , C/EBP $\delta$ , PPAR $\gamma$ , and aP2 by Northern analysis in cocktail-treated 3T3-L1 cells exposed to conditioned media from T47D breast cancer cells (TCM) and HEMC normal breast epithelial cells (NCM). Fig. 2 depicts these results. Treatment with TCM strikingly decreased the transcript levels of C/EBP $\alpha$  and PPAR $\gamma$  in 3T3-L1 cells, whereas it significantly potentiated the effects of the differentiation mixture to induce the expression of C/EBP $\beta$  and C/EBP $\delta$  during days 4 and 6 after the initiation of treatment. As expected, TCM also inhibited the expression of aP2, which is a marker for adipocyte differentiation (Fig. 2B). Upon the addition of a neutralizing antibody against TNF- $\alpha$ , the effects of TCM on C/EBPs and PPAR $\gamma$  expression were reversed completely (Fig. 2C), whereas NCM did not change the expression pattern of C/EBPs and PPAR $\gamma$ . This demonstrated that the inhibition caused by TCM on the induction of C/EBP $\alpha$  and PPAR $\gamma$  was specific for malignant epithelial cells. These results were further confirmed by performing human breast tumor immunohistochemistry.

We determined the number and staining intensity of immunoreactive cells for C/EBP $\alpha$ , C/EBP $\beta$ , and C/EBP $\delta$  in fibroblasts mixed with malignant epithelial cells (intratumoral), within fat 1 cm from the tumor and within fat 2–4 cm from the tumor. An H-scoring system was used to determine the number of immunoreactive fibroblasts, and this is illustrated in Fig. 3. C/EBP $\alpha$  was not detectable in intratumoral fibroblasts located proximal malignant epithelial cells, but it was readily detectable in fibroblasts/adipocytes in fat tissue biopsies distal to the tumor, whereas immunoreactive C/EBP $\beta$  and C/EBP $\delta$  were present in intratumoral fibroblasts proximal to malignant cells (Fig. 3). These results are in agreement with those of the Northern analysis.

**Effects of T47D Breast Cancer Cells on the Proliferation of 3T3-L1 Fibroblasts.** We determined whether T47D cancer cells affected the proliferation indices of 3T3-L1 fibroblasts, when these two cell types were cocultured. We did not observe any differences in the DNA histograms of 3T3-L1 cells incubated in the absence or presence of T47D cells using flow cytometry (data not shown). Neither did we see an effect of T47D breast cancer cells on the [<sup>3</sup>H]thymidine incorporation into 3T3-L1 cells (data not shown). Thus, we conclude that breast cancer cells induce accumulation of fibroblasts in the tumor tissue by the inhibition of differentiation of these cells to mature adipocytes but not by promotion of their proliferation.

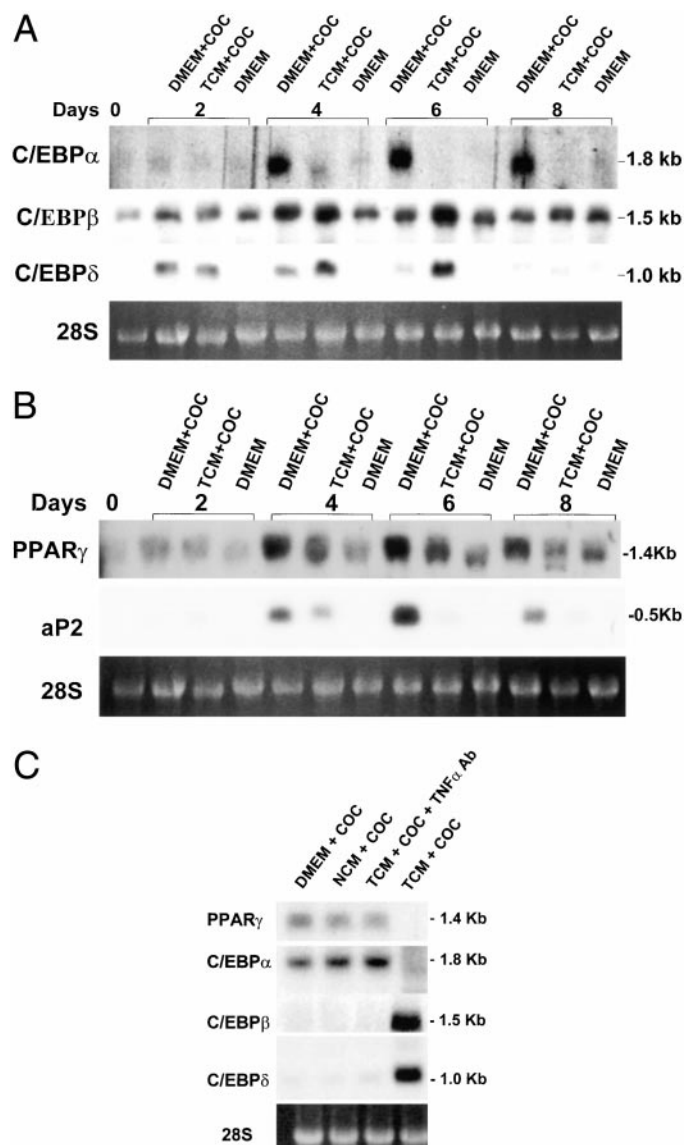


Fig. 2. Northern blot showing the levels of transcripts of C/EBP $\alpha$ , C/EBP $\beta$ , C/EBP $\delta$  (A), PPAR $\gamma$ , and aP2 (B) in 10  $\mu$ g of total RNA from 3T3-L1 cells incubated under conditions explained in the legend of Fig. 1B. The 28S RNA fraction was included to demonstrate the presence of comparable amounts of RNA in each lane. DMEM, control medium; CO $C$ , differentiating cocktail. T47D cells (TCM) inhibited expression of C/EBP $\alpha$  and PPAR $\gamma$  and enhanced expression C/EBP $\beta$  or C/EBP $\delta$  in 3T3-L1 cells after exposure to the differentiating cocktail (CO $C$ ). T47D cells (TCM) also suppressed the expression of aP2, a marker of differentiated adipocytes. C, on day 6, NCM did not change the expression patterns of C/EBPs and PPAR $\gamma$ . Furthermore, the addition of an anti-TNF $\alpha$  neutralizing antibody completely abolished the influence of TCM on C/EBPs and PPAR $\gamma$ , indicating the essential role of TNF- $\alpha$  in mediating the effects of TCM. Please note that Northern blots in A–C represent separate experiments, and exposure times of autoradiographs were different.

## DISCUSSION

Peri- and intratumoral fibroblasts provide structural support to tumor tissue, and secretory products of fibroblasts may promote tumor growth. We herein demonstrated that malignant breast epithelial cells actively participate in the process of accumulation of stromal fibroblasts in and around the tumor tissue (*i.e.*, desmoplastic reaction). Secretory products of cancer cells prevent the differentiation of fibroblasts to adipocytes and, in fact, may even reverse adipocyte differentiation. We also demonstrated that tumor-derived cytokines act on adjacent adipose stroma by down-regulating the expression of adipogenic factors such as the C/EBP $\alpha$  and PPAR $\gamma$ . This study also provides evidence that breast cancer cells (or their secretory products)

do not induce proliferation of fibroblasts. Thus, inhibition of differentiation seems to be the major mechanism responsible for desmoplastic reaction.

We chose to study IL-11 and TNF- $\alpha$  as the malignant epithelial cell-derived factors, because the potent antiadipogenic effects of these two cytokines have been well established (27–29). In our hands, the combined effects of IL-11 and TNF- $\alpha$  were sufficient to inhibit adipocyte differentiation completely. Most importantly, both cytokines are expressed in abundant levels in malignant epithelial cells. The effects of these cytokines are specific for malignant cells, because other cytokines or benign breast epithelial cells did not inhibit adipocyte differentiation.

Differentiation of fibroblastic cells to adipocytes appears to be primarily controlled by a cascade of adipogenic factors. C/EBP $\beta$  and C/EBP $\delta$  appear to mediate the earlier phase of the differentiation program. PPAR $\gamma$ , a nuclear hormone receptor, is expressed next in the differentiation process and becomes adipogenic after binding to its synthetic (BRL49653; Ref. 26) and natural (15-deoxy- $\Delta^{12,14}$ -prostaglandin J $_2$ ) ligands (23). PPAR $\gamma$  binds to its response elements in the promoters of its target genes. To recognize a PPAR $\gamma$  response element, PPAR $\gamma$  must form a heterodimer with retinoid X receptor  $\alpha$ , a basic-leucine zipper transcription factor. On the other hand, C/EBP $\alpha$  is not expressed until relatively late in the differentiation process. It binds to and transactivates the promoters of a number of adipocyte-specific genes. Thus, breast cancer cells seem to exert their inhibitory effect on relatively later stages of the differentiating process, *i.e.*, inhibition of the expression of PPAR $\gamma$  and C/EBP $\alpha$ . This is consistent with the isolated effects of TNF- $\alpha$ , which has been shown to inhibit the expression of PPAR $\gamma$  and C/EBP $\alpha$  in 3T3-L1 cells and fetal brown adipocytes (27, 28).

This report lays the groundwork for the mechanism of desmoplastic reaction as an epithelial-stromal interaction in the breast cancer. Further studies are required to identify other key molecules in the cancer-mediated inhibition of adipocyte differentiation. Two candidate substances are CHOP, a transcription factor that acts as a negative dominant regulator of adipocyte differentiation, and Pref-1, which is a transmembrane protein with epidermal growth factor-like motifs and another negative regulator of adipocyte differentiation (30). The determination of the roles of these candidate substances will further increase our understanding of the epithelial-stromal interactions in the

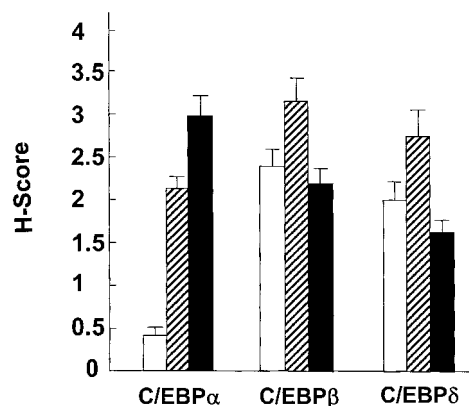


Fig. 3. Expression patterns of C/EBPs in mastectomy specimens from 10 patients with breast cancer. Immunoreactive proteins were scored in the three biopsies (intratumoral; fat, 1 cm to tumor; and fat, 2–4 cm to tumor; from each of the 10 mastectomy specimens). H-scoring was used in the following manner: 0, 0–5% fibroblasts are positive; 1, 6–25% fibroblasts are positive; 2, 26–50% fibroblasts are positive; 3, 51–75% fibroblasts are positive; and 4, 76–100% fibroblasts are positive. Immunoreactive C/EBP $\alpha$  is not detected in the fibroblasts within the breast tumor but is readily detectable in the fibroblasts/adipocytes distant to the tumor. Immunoreactive C/EBP $\beta$  and C/EBP $\delta$  are readily detectable in all of the three biopsy sites.  $\square$ , intratumoral;  $\square$  (hatched), adipose tissue (1 cm);  $\blacksquare$ , adipose tissue (2–4 cm). Bars, SE.

breast cancer. Finally, we recently showed that cancer cell-induced up-regulation of C/EBP $\beta$  mediates aromatase overexpression in adipose fibroblasts (31).

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