Differential Expression of Human Tissue Factor in Normal Mammary Epithelial Cells and in Carcinomas

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ABSTRACT

Background: Tissue factor (TF) is a glycoprotein which binds factor VIIa. The TF-VIIa complex serves as a potent initiator of the coagulation pathways. TF, an immediate early gene, may also play a role in cell growth. Expression of TF was correlated with some types of cancers.

Materials and Methods: Normal, immortalized, and tumor human mammary epithelial cells were used in the experiments. The differential display (DD) technique was used to identify genes differentially expressed in the cells. TF expression patterns were examined by Northern blot analysis, immunofluorescence staining of cultured cells, and immunohistochemical staining in human cryostat sections.

Results: In a 5-way display, an amplified polymerase chain reaction (PCR) product was found in normal and immortalized human mammary epithelial cells but not in the breast cancer cells. The PCR fragment was cloned and sequenced. The result showed that the fragment was identical to human tissue factor. Northern blot analysis showed that expression level of tissue factor mRNA remained high in growing, quiescent, and senescent normal mammary epithelial cells. Immunofluorescence staining also confirmed tissue factor expression pattern in the cell lines tested. Immunohistochemical staining showed that tissue factor was expressed in the normal luminal and myoepithelial cells of some ducts but not others. No staining was observed in invasive carcinoma cells. However, myoepithelial cell staining was seen in some residual ductal structures in invasive tumors.

Conclusions: This study shows the use of DD to reveal the loss of TF expression pattern in human breast cancer cell lines. Immunohistochemical staining results showed breast carcinoma cells expressed little TF, if any, suggesting that TF is not required for breast tumor cell invasion. The results also indicated that TF expression was independent of the proliferation status of the expressing cells. The expression pattern of TF may be a meaningful marker in the development of breast cancer.

INTRODUCTION

Tissue factor (TF) is an integral membrane glycoprotein which binds human zymogen factor VII and its active form serine protease factor VIIa (1–3). The TF-VIIa complex serves as a potent initiator of the extrinsic and intrinsic coagulation pathways (4). The observation that expression of TF, an immediate early gene, can be induced by growth factors and other cell ligands in various cell types raises the possibility that TF may also play a role in cell growth (5). Immunohistochemical studies in normal human tissues using monoclonal antibodies found that TF expressed mainly at the interface of different organs and between the internal and external environments (6). The expression pattern of TF was thus viewed as a hemostatic envelope serving to initiate the arrest of bleeding (7). In malignancy, hypercoagulatory activity was often found in cancer patients (8,9). TF was viewed as a procoagulant in certain types of cancers (10). Because of the ability of TF to initiate fibrin deposition and platelet aggregation, a potential role of TF in tumor metastasis was proposed (11,12). Immunohistochemical studies on solid tumors using polyclonal monospecific antibodies found TF-

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positive tumor cells from lung, squamous cell carcinoma of the head and neck, gastrointestinal mucosa, vaginal carcinoma, transitional cell carcinoma, pancreatic adenocarcinomas, and endometrial carcinoma. Some tumors such as breast and ovarian showed heterogeneous staining patterns. Renal cell carcinoma and prostate carcinoma, on the other hand, stained negative for TF (13). Other studies on human breast tissues described both negative (14) and heterogeneous (15) TF staining in tumor cells.

This manuscript reports a striking difference in expression pattern of TF revealed by the differential display technique using normal, immortalized, and mammary carcinoma cells in culture. The expression pattern was confirmed by Northern blot analysis and immunofluorescence staining. Immunohistochemical staining on human breast cancer tissues showed that TF was expressed in some normal ducts but not in the invasive carcinoma cells. Myoepithelial cells stained positive for TF in some ductal carcinoma in situ and in residual ductal structures of the invasive tumors. The potential importance of these findings is discussed.

MATERIALS AND METHODS

Cell Culture

All cells were cultured in DFCI-1 medium (16) to about 70% confluence for RNA preparation. Normal mammary epithelial cells, 70N, 81N, and 76N, primary tumor cell line 21NT, 21PT and metastatic tumor cell line 21MT-1 were isolated in this laboratory (16). Human papilloma virus (HPV) immortalized cell lines 1436N, 16E6E7-A2, 16E6E7-A4, 16E6E7-A5, 16A5, 18E6E7, and 18-2-1 were derived from the normal mammary epithelial cell line 76N (17). MCF-10A is a spontaneously immortalized mammary epithelial cell line (18). Metastatic breast cancer cell lines MDA-MB-157, MDA-MB-231, MDA-MB-361, MDA-MB-435, MDA-MB-436, MDA-MB-468, T-47D, BT-474, BT-549, ZR-75-1, and SK-BR-3 were obtained from the ATCC (Rockville, MD). MCF-7 was from Michigan Cancer Foundation (Detroit, MI).

RNA Preparation and Northern Blot Analysis

Purification of RNA and Northern blot analysis were performed using standard procedures adapted to mammary epithelial cells (19).

Differential Display

Removal of DNA from RNA and differential display were essentially the same as described previously (20,21) with slight modification. TF was identified through a 5-way display in which RNA samples from 70N, 16E6E7-A5, 18-2-1, MDA-MB-435, and BT549 were used to screen for expression differences in normal primary, immortalized, and tumor derived cells. At the reamplification step, the arbitrary primer concentration was increased from the recommended 0.2 μM to 0.3 μM and the T12MN primer concentration was increased from 1 to 1.5 μM to obtain sufficient polymerase chain reaction (PCR) products in one round of reamplification.

Cloning and Sequencing of cDNA

The procedure was described previously (20,22).

Reverse Transcription (RT)-PCR for TF Coding Region

RNA from 76N cells was reverse transcribed using T12MA primers from GenHunter Corp. (Brookline, MA). Tissue factor cDNA was amplified by PCR using a primer 23 base pair upstream of ATG (5′-GAT<u>GCG</u>GCCGCCAACTGGTA-3′) and an antisense primer 5 base pairs downstream of the stop codon TAA (5′-TGC<u>TC</u>TAG<u>A</u>TCCAA CAGTGC-3′). The underlined bases were changed from tissue factor sequence CTC to GCG in the upstream primer and AG and C to TC and A in the downstream primer to accommodate a NotI and XbaI site for each primer, respectively. The 936 base pair amplified fragment was used as a probe for Northern blots to confirm the expression pattern which had initially been identified by the 3′ untranslated region.

Immunofluorescence Staining

Cells were cultured in DFCI-1 in 8-well chamber slides (Nunc, Naperville, IL), rinsed twice with phosphate-buffered saline (PBS), and fixed in 100% acetone for 10 min at 4°C. After PBS wash for 5 min, the cells were incubated in a blocking solution containing 1% bovine serum albumin, fraction V (Sigma, St. Louis, MO), 5% normal goat serum (Zymed Labs, San Francisco, CA), and 0.2% Tween-20 in PBS for 1 hr. A pool of three murine monoclonal antibodies against human TF (23) was kindly provided by Dr. Nigel Mackman, the Scripps Research Institute, La-

Jolla, CA. The cells were incubated with the antibodies at 0.1 μg/ml in the blocking solution at 4°C overnight. After three washes with PBS, the cells were incubated with a 1/200 dilution of phycoerythrin-R-conjugated goat anti-mouse IgG (Zymed Labs) at room temperature for 1 hr followed by a 10-min incubation in DAPI. The slides were washed in PBS and mounted with Fluoromount-G (Fisher, Pittsburgh, PA). Photomicroscopy was performed with a Zeiss-Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

Immunohistochemical Staining

Freshly prepared cryostat sections from four invasive human breast carcinomas, one axillary lymph node metastases, and two normal adjacent tissues were fixed in 1% paraformaldehyde in PBS for 10 min at room temperature. Endogenous peroxidase was quenched in 0.6% H_2O_2 in methanol for 10 min. After incubation with 2% normal horse serum in PBS and 0.2% Tween 20 for 15 min, the sections were labeled with 0.5 μg/ml of mouse anti-human TF antibodies described above for 45 min. Following three 5-min washes in PBS, the sections were incubated for 10 min at room temperature with a secondary biotinylated horse anti-mouse antibody at 1/400 dilution in PBS (Vector Laboratories, Burlingame, CA). The secondary antibody was coupled to a peroxidase complex using the Vectastain Elite ABC kit (Vector Laboratories). Immunoperoxidase was detected using VIP or PAB substrate kits according to the manufacturer's instructions (Vector Laboratories). All tissue sections were counter stained with Hematoxylin.

RESULTS

In a 5-way differential display (Fig. 1), a band appeared in the 70N, in the immortal lines 16E6E7-A5 and 18-2-1 cells but not in either tumor cell line MDA-MB-435 or BT-549. The PCR product was cloned into a TA cloning vector and sequenced. The result showed that the 352 base pair fragment was identical to a sequence in the 3′ untranslated region corresponding to 1752 to 2104 of human TF cDNA (24). A 936 base pair fragment of TF coding region was obtained using RT-PCR from 76N RNA. This fragment was used for Northern blot analysis.

Figure 2A shows the results of a Northern blot using the TF cDNA probe. A 2.2-kb transcript was detected among the cell lines. Analysis of Northern blots showed that high level of TF mRNA was expressed in all three of the normal cells, all of the HPV immortalized cells, and the spontaneously immortalized cell MCF-10A. All of the 13 tumor cell lines, 21PT, 21MT, MDA-MB-157, MDA-MB-361, MDA-MB-435, MDA-MB-436, MDA-MB-468, T-47D, BT-474, BT-549, SK-BR-3, ZR-75-1, and MCF-7 (excepting MDA-MB-231) showed very little, if any, TF mRNA. The Northern hybridization results are consistent with the DD pattern. Northern blot analysis using 76N RNA also showed that expression level of tissue factor mRNA remained high in growing and senescent normal mammary epithelial cells (Fig. 2B). The fact that TF is expressed in normal, immortalized, and senescent cells as well as quiescent cells (data not shown) suggests that TF expression is independent of the proliferation status of the expressing mammary epithelial cells.

Immunofluorescence staining showed that TF was expressed in 70N, 81N, 1436N, 16E6E7-A2, and MDA-MB-231, but not in 21MT, MDA-MB-435, and MCF-7 cells, the same pattern as that seen at the transcription level (Fig. 2A).

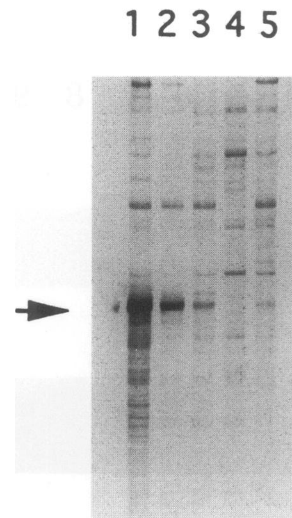

FIG. 1. Tissue factor expression detected by 5-way differential display

The PCR products displayed in each lane are: (1) 70N; (2) 16E6E7-A5; (3) 18-2-1; (4) MDA-MB-435; and (5) BT549. The position of tissue factor is indicated by an arrow.

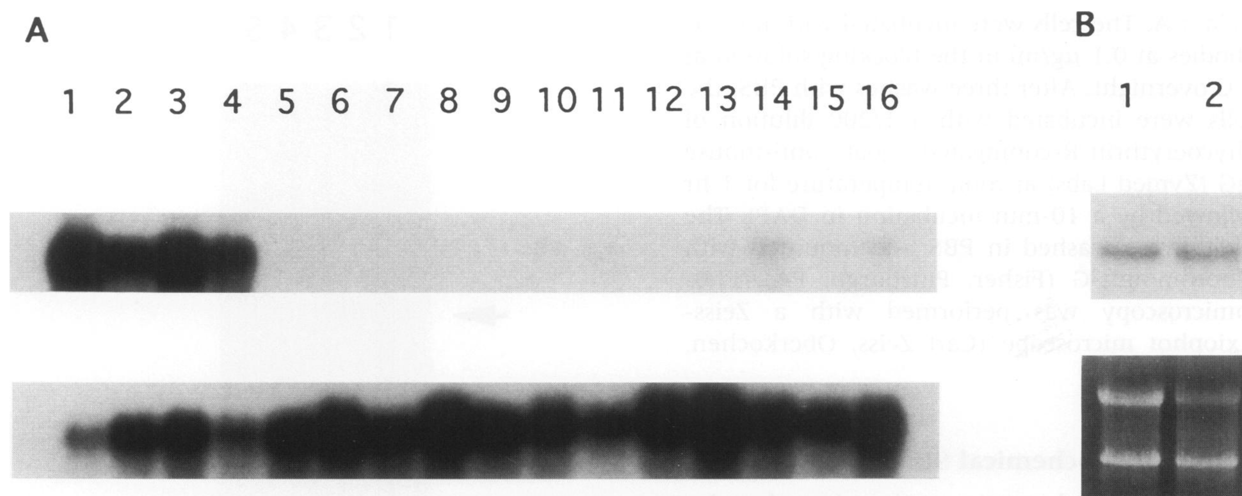

FIG. 2. A Northern hybridization result using tissue factor cDNA as probe

(A) Total RNA samples (10 μg) in each lane are: (1) 70N; (2) 76N; (3) 1436N; (4) MCF-10A; (5) MDA-MB-361; (6) MCF-7; (7) BT474; (8) ZR-75-1; (9) T47D; (10) 21PT; (11) 21MT; (12) MDA-MB-435; (13) MDA-MB-436; (14) MDA-MB-468; (15) SK-BR-3; and (16) BT549. Tissue factor transcript is shown in the top panel. The bottom panel shows 36B4 as a loading control. (B) Total RNA samples (4 μg) in Lane 1 was prepared from normal 76N cells and Lane 2 from senescent 76N cells.

Figure 3 shows the immunofluorescence staining of 81N. TF was found in the extending lamellipodia and the retracting uropod. The pattern appeared to be similar to stimulated MCF-7 cell lines in which cellular localization of TF was implicated in morphogenesis related to cell growth and differentiation (25).

Immunohistochemical staining results showed that mammary epithelial cells in some normal ducts stained positive (brown) for TF (Fig. 4A) and those in other ducts stained negative (Fig. 4C). As a control to Fig. 4A, Fig. 4B shows an adjacent section stained negative (blue) with mouse IgG. Invasive ductal carcinomas (Fig. 4 D and E), infiltrating lobular carcinoma (data not shown), and axillary lymph node metastases (Fig. 4F) were all negative. Ductal carcinoma in situ showed strong positive stain in the myoepithelial area while luminal cells showed little TF staining, if any (Fig. 4E).

FIG. 3. Immunofluorescence staining of tissue factor in normal 81N cells

DISCUSSION

Differential display (DD) has been shown to be a powerful tool to identify genes differentially expressed at the mRNA level (20,21). One of the advantages of DD is that it can be used conveniently to display and compare multiple samples simultaneously (22). The original design of DD predicted the PCR products to be amplified between a T12MN and an arbitrary upstream primer. While many of the genes identified by DD meet the expectation of the original design, TF was amplified by T12 MA only. In the case of TF, the upstream primer is T12CA and the downstream primer is T12AA. T12CA matches human TF sequence 5′-TGAAAAAAAAAAGA-3′ between position 1740 and 1753 with the second 5′ end T of the primer mismatched to G of the TF sequence. This result provides evidence that the

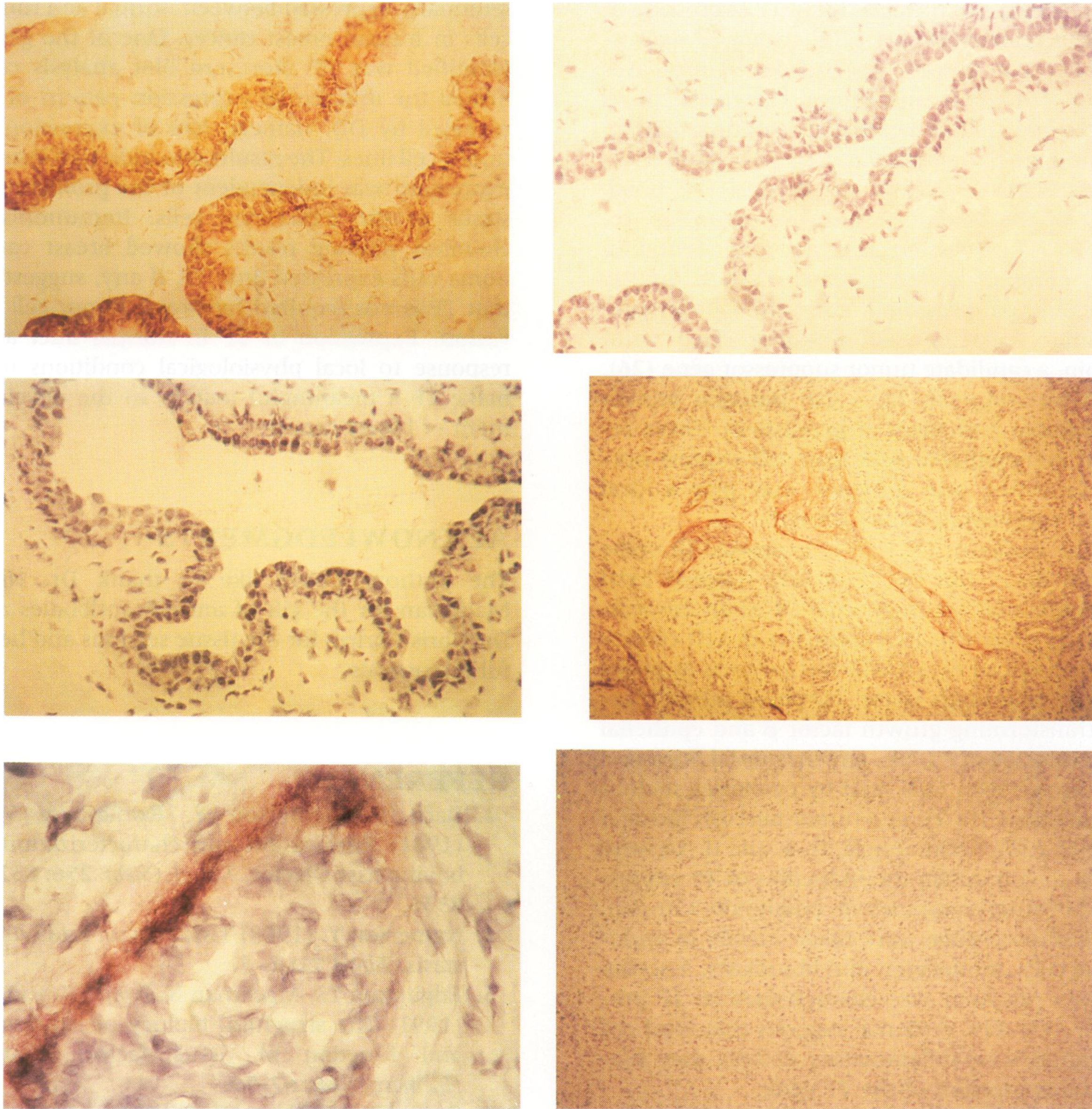

FIG. 4. Immunohistochemical staining of tissue factor in human normal and cancer breast tissues

A section of normal breast tissue was stained positive (brown) in ductal epithelial cells with monoclonal anti-tissue factor antibodies (A). Adjacent sections were stained with mouse IgG as negative controls (B). (C) A normal mammary duct stained negative (blue) for tissue factor with the anti-tissue factor antibodies. An invasive ductal carcinoma section was stained with anti-tissue factor antibodies (D and E). (E) Enlarged from the area indicated by an arrow in Panel D. Anti-tissue factor antibodies stained negative in axillary lymph node metastases (F). (A, B, and C) Photographed at 500× magnification; (D) at 1250×; (E and F) at 125×.

number of PCR products amplified from each T12MN and an arbitrary primer may reach a maximum of 22 instead of 4 combinations.

The in vivo results from immunohistochemical staining confirm the evidence from DD and Northern hybridizations that TF is expressed in normal epithelial cells but not in invasive primary tumors or metastases. Our results are also consistent with previous reports (14,15). Taken together, these observations suggest that the metastatic potential of primary breast carcinoma cells does not depend on TF expression. Whether TF expression on breast cancer cells can actually deter their invasion through the lymphatic and vascular systems in vivo (i.e., act as a tumor suppressor) is not known.

In contrast to the absence of TF expression in invasive primary cancer cells, strong TF expression was seen in some residual ductal structures surrounded by invasive tumor. Figure 4D shows an example of myoepithelial immunostaining, defining the border of a residual duct. Immunostaining with TF clearly demarcates the break down of the basement structure as in Fig. 4E. Similar TF expression patterns were found in many areas of invasive cancer tissues. Similar expression patterns were also found in primary mammary carcinomas immunostained with maspin, a candidate tumor suppressor gene (26), and with connexin 26, a gap junction protein (Tsukamoto and Sager, unpublished). These residual myoepithelial cells may still express normal functions, and therefore may play a local role in suppressing tumor growth.

The evidence that TF is expressed in some normal ducts and not in others suggests local environmental heterogeneity. TF is an immediate early gene (5). Its expression in cell culture can be induced by growth factors such as platelet-derived growth factor, fibroblast growth factor, transforming growth factor β and epithelial growth factor (5,27,28), and by steroid hormone such as estrogen (29), and by cytokines such as interleukin-1 (IL-1), and tumor necrosis factor-α (TNF-α) (28). Retinoic acid, IL-4, and IL-10 were found to counteract the induction of TF expression by other cytokines or endotoxin (30–32). These observations were made mostly in endothelial cells, monocytes, and fibroblasts. A serum response element was characterized in TF promoter (33). The evidence suggests that local cell proliferation or inflammatory activity may trigger the expression of the TF gene.

To test this hypothesis with mammary epithelial cells, we examined the effects of serum starvation, IL-1, and TNF-α on TF expression in cell culture. Northern blot analysis showed that human breast tumor cell lines, 21NT, MCF-7, and MDA-MB-435 did not respond to the treatments (data not shown). This result suggests that TF expression in these cell lines may be different from that in other cell types. It has been reported that TF expression in different cell types can react to the same stimulus differently (34). Understanding the regulatory mechanisms underlying TF expression in normal breast and in premalignant stages may help explain the heterogeneous expression patterns we and others have observed.

In summary, this manuscript reports the use of a 5-way DD technique to identify genes differentially expressed between normal and tumor cells in human breast cancer. One of the genes identified was TF. Northern blot analysis confirmed the differential expression pattern of TF revealed by DD, namely, loss of expression in tumor cell lines. The results also indicated that TF expression was independent of the proliferation status of the expressing cells. Immunohistochemical staining results showed breast carcinoma cells expressed little TF, if any, suggesting that TF is not required for breast tumor cell invasion. Expression of TF in normal duct as a response to local physiological conditions may make TF a meaningful marker in the development of breast cancer.

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