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Constitutive Tissue Factor Expression of Human Breast Cancer Cell Line MCF-7 is Modulated by Growth Factors

Conrad Flössel, Thomas Luther, Sybille Albrecht, Matthias Kotzsch and Martin Müller

Expression of tissue factor, the initiator of the extrinsic coagulation protease cascade, is a feature of certain malignant tumours. To study the modulation of tissue factor expression we incubated the breast cancer cell line MCF-7 with several growth factors. Epidermal growth factor (EGF), transforming growth factor α (TGFα) and interleukin-1 (IL-1) rapidly increased tissue factor expression of MCF-7 cells peaking at 6–8 h after starting point of incubation, as determined by clotting test, enzyme linked immunosorbent assay and flow cytometry. The data presented support the hypothesis that modulation of constitutive tissue factor expression in tumour cells by TGFα and IL-1 could also occur *in vivo* possibly resulting from interactions of stromal and cancer cells. The meaning for tumour biology, however, remains unclear.
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INTRODUCTION

TISSUE FACTOR (TF) is a 47 kDa transmembrane glycoprotein receptor and the essential cofactor for factors VII/VIIa, thereby triggering the cell surface assembly of coagulation protease cascade, finally leading to formation and deposition of fibrin [1–3]. The human TF gene has been cloned [4]. Its molecular biology of expression and function has been reviewed and discussed recently [5]. The constitutively expressed TF is preferentially detectable in cells corresponding to biological boundary layers (skin, epithelia of respiratory and gastrointestinal tract, cerebral cortex and adventitia of vessels) as described by Drake *et al.* [6] and Fleck *et al.* [7]. Within the vasculature, endothelia and monocytes can be induced by endotoxins or cytokines to start synthesis of TF (for review, see [5]), a phenomenon which is commonly accepted as a link between haemostasis and inflammation [5]. TF can be also detected in cells of certain malignant tumours [8, 9]. Furthermore, fibrin deposits have been localised in several tumours and metastases (10, 11], and clotting abnormalities are frequently observed in tumour patients [12, 13]. But until now, there is no clear evidence what role, if any, TF expression and activation of blood coagulation cascade proteases would play in tumour growth.

Recent studies from our laboratory have shown that human primary breast cancer tissues express TF in a highly individual manner [14]. The amount of immunohistochemically detectable TF apoprotein showed a close correspondence to the procoagulant activity (PCA) of those tumour tissues. Besides the individual variations of TF in 115 primary cancers, a highly heterogeneous distribution of TF-positive cancer cells was the predominant pattern. The latter could reflect genomic heterogeneity of the cancer cell populations, as well as different

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functional states of tumour cells, depending on regulatory factors.

The aim of the present study was, therefore, to explore the possible regulation of TF expression in the human breast cancer cell line MCF-7 by growth factors. The findings to be detailed reveal that TF in MCF-7 cells can be modulated by certain growth factors.

MATERIALS AND METHODS

Reagents

Ham's F12-medium, trypsin-EDTA solution, EDTA (disodium salt), recombinant human cytokines tumour necrosis factor (TNFα), interleukin 6 (IL-6), granulocyte/macrophage colony stimulating factor (GM-CSF), epidermal growth factor (EGF), and gentamycin were purchased from Boehringer (Mannheim, FRG). HEPES and *n*-octyl-β-D-glycopyranoside were both from Sigma Chemical Co. (St. Louis, Missouri). Dulbecco's minimal essential medium (DMEM), Tris, the recombinant cytokines platelet-derived growth factor (PDGF), transforming growth factor α and β (TGFα and β), and interferon γ (IFNγ) were obtained from Serva (Heidelberg, FRG). Phosphate-buffered saline (PBS) was from SIFIN (Berlin, FRG) and fluorescein-isothiocyanate (FITC)-conjugated rabbit anti-mouse Ig was from Dako Diagostika (Hamburg, FRG). Formaldehyde was from Merck (Darmstadt, FRG), fetal calf serum (FCS) from HyClon Laboratories (USA), and human brain thromboplastin from AWD (Dresden, FRG). *o*-Phenylenediamine (OPD) was obtained from Fluka (Switzerland). Monoclonal antibodies to human brain-derived TF (VI C12, V D8, VI C7) were generated in our laboratory [15]. Control mouse IgG1 was obtained from Becton Dickinson (Heidelberg, FRG).

Cell culture

The human breast cancer cell line MCF-7 was kindly provided by Dr Theile (Central Institute of Cancer Research, Berlin-Buch, FRG). Cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air in a mixture of 45% DMEM, 45% Ham's F12 and 10% FCS with 2 mmol/l L-glutamine, non-essential aminoacids, 15 mmol/l HEPES and 60 μg/ml gentamycin. Cells were maintained in monolayer culture and passaged with trypsin-EDTA weekly.

Treatment of MCF-7 cells

For stimulation, cells were harvested from subconfluent monolayer cultures after detachment by 0.05% trypsin/0.02% EDTA (v/v) and seeded in 75 cm^2 culture flasks (ICN Biomedicals, Meckenheim, FRG) with complete medium at a concentration of 5×10^5 cells. After 3 days of culture the medium was replaced by fresh complete medium containing the cytokines and growth factors at the following final concentrations: TNFα, IFNγ, IL-6 and GM-CSF, 100 U/ml; IL-1, 20 U/ml; TGFβ, EGF and PDGF, 5 ng/ml; TGFα, 2 ng/ml. The cell culture was continued and the tumour cells were mechanically harvested at desired culture intervals with a rubber policeman, washed three times with PBS containing 5 mmol/l EDTA, and adjusted to 5×10^4 cells per ml for further use. Viability of tumour cells was 80–95% as examined by Trypan blue exclusion.

ELISA

For the detection of tissue factor in cell preparations, a sandwich enzyme linked immunosorbent assay (ELISA) was used, as described by Albrecht *et al.* [15]. Briefly, cells were pelleted by centrifugation and the supernatant was decanted. The cell pellets were frozen at −20°C, and cells were disrupted by repeated freezing and thawing. Tissue factor was solubilised by incubation with 60 μl 0.05 mol/l Tris–HCl, 0.1 mol/l NaCl, 0.1% Triton X-100, pH 7.6 containing 5 mmol/l EDTA for 30 min at 37°C.

For ELISA, microtiter plates (Nunc, Denmark) were coated with 50 μl of purified monoclonal antibody (10 μg/ml VIC12) at 4°C for 20 h. Plates were washed twice with 200 μl of wash buffer (0.02 mol/l phosphate buffer, 0.15 mol/l NaCl, 4% gelatin, 0.05% Tween 20, pH 7.6). To minimise non-specific binding, the plates were incubated for 1 h at room temperature with wash buffer. Plates were incubated for 2 h at 37°C with 50 μl of TF-containing samples. Dilutions were performed with 0.05 mmol/l Tris–HCl, 0.1 mol/l NaCl, 0.1% Triton X-100, pH 7.6 containing 5 mmol/l EDTA. Following three wash steps with wash buffer, 50 μl of peroxidase-conjugated anti-TF monoclonal antibody (Mab) (VIC7) were added and incubated for 2 h at room temperature. OPD was used as substrate. The absorbance was read at 492 nm with a SUMAL one-channel photometer (Zeiss, Jena, FRG), and converted into ng of TF by reference to a standard curve developed from immunopurified human TF-apoprotein.

Clotting test

The procoagulant activity of cell preparations was assayed by determination of the acceleration of the clotting time of recalcified citrated normal human platelet-poor plasma in an one-stage clotting test. After washing, cells were pelleted by centrifugation and the supernatant was decanted. The cell pellets were frozen at −20°C for 1–4 days. After thawing, the pellets were incubated with 30 μl of 15 mmol/l *n*-octyl-β-D-glycopyranoside for disruption and solubilized in 70 μl 25 mmol/l HEPES-saline at 37°C. In the coagulation assay, 100 μl 25 mmol/l $CaCl_2$ were added to 100 μl of citrated normal human platelet-poor plasma and 100 μl of the cell lysate. The clotting time was measured in polypropylene tubes (Greiner, Nürtingen, FRG) by means of the wire loop technique at 37°C. The time recorded was converted to milliunits (mU) per 5×10^4 cells of PCA by reference to a standard curve derived from a human brain thromboplastin standard.

For neutralisation experiments, 50 μl of cell lysate and 50 μl of anti-TF Mab (VIC7, 5 ng/ml) were mixed and incubated for 30 min at 37°C. The coagulation time was measured with prewarmed citrated human plasma. Percentage of neutralisation was calculated using cell lysate incubated without antibody as 100% coagulation (= 0% neutralisation).

Flow cytometric analysis of tissue factor expression

A cocktail of two monoclonal anti-TF antibodies (VD8 and VIC7, IgG1) was utilised for flow cytometric analysis of TF expression on tumour cells. After washing, cells were resuspended in cold PBS, fixed for at least 30 min by adding 1 ml of cold 1% formaldehyde buffer and then washed twice. The fixed cells were seeded in polypropylene tubes (8 × 38 mm, Polyplast Halberstadt, FRG) and incubated for 30 min at 4°C with anti-TF Mab-cocktail. As negative control served control mouse IgG1. After washing, cells were stained with FITC-conjugated rabbit anti-mouse Ig for 10 min at room temperature. The stained cells were washed twice, resuspended in PBS and analysed by flow cytometry using a FACScan (Becton Dickinson, Heidelberg, FRG).

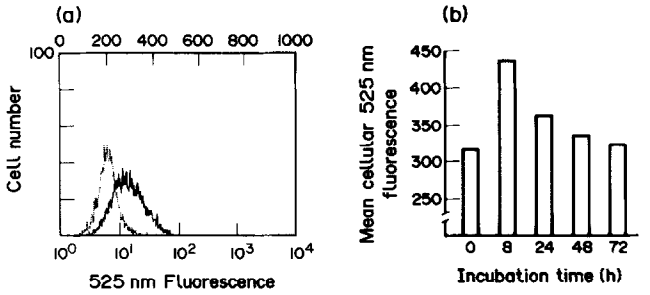

Fig. 1. (a) 525 nm fluorescence histogram of MCF-7 cells stained with anti-TF/rabbit anti-mouse^{FITC} (——) in comparison with the negative antibody control (----). (b) Representative kinetics of TF expression of EGF (5 ng/ml) stimulated MCF-7 cells over 72 h analysed by flow cytometry (determination of mean cellular 525 nm fluorescence after anti-TF/rabbit anti-mouse^{FITC} staining, negative control level over 0–72 h (215 ± 10).

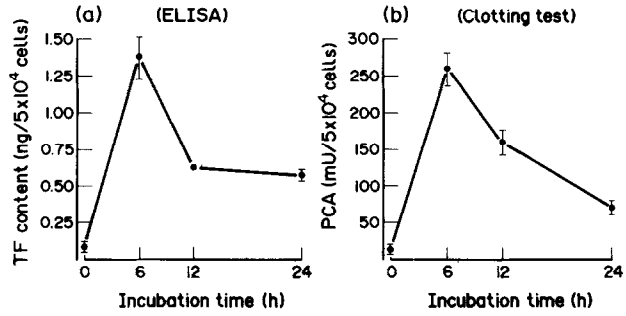

Fig. 2. Kinetics of TF expression analysed by a sandwich ELISA (a) and PCA analysed by clotting test (b) of cell lysates from MCF-7 cultures with EGF (5 ng/ml) over 24 h. Results are presented as mean (SD) of three experiments.

Statistical analysis

Results were analysed for statistical significance by Student's *t*-test. *P* values less than 0.05 were considered to indicate statistically significance differences.

RESULTS

Constitutive TF expression

In the presence of serum without additional growth factors and cytokines MCF-7 cells harvested from subconfluent monolayer cultures showed both constitutive PCA by clotting test (19.2 ± 3.4 mU/5 × 10^4 cells) and molecular TF expression by flow cytometric analysis (Fig. 1a). PCA was TF as determined with factor VII- and factor X-deficient plasma (data not shown).

Modulation of TF activity

In a series of experiments we incubated the MCF-7 cells in the presence of various cytokines and growth factors over 20 h as indicated in Materials and Methods and measured the PCA, to determine if any modulation of clotting activity took place during this time. From the factors tested in three to five separate experiments IL-1, TGFα and EGF enhanced PCA of tumour cells up to 2-fold (IL-1: 166 ± 45% PCA, $P \leq 0.03$; TGFα: 196 ± 37% PCA, $P \leq 0.03$; EGF: 212 ± 34% PCA, $P \leq 0.02$; control = 100% PCA). TNFα, IFNγ, IL-6, TGFβ, PDGF and GM-CSF had no significant modulatory effects on PCA of MCF-7 cells.

Kinetics of TF modulation

To study the time-course of growth factor-stimulated TF synthesis, tumour cells were cultured with EGF (methods) over a period from 0 to a maximum of 72 h and TF expression was analysed by PCA, ELISA technique and flow cytometry (Figs 1b, 2). There was a rapid increase in TF expression during the first hours, reaching the maximum at 6–8 h, declining after 12 h and meeting the value of the starting point after 48 h. Neutralisation of PCA with anti-TF Mab was 93 ± 5%. Interestingly, functional TF expression (PCA), and immunoreactive TF content in cell lysates (ELISA) showed a close correspondence. On the other hand, increase of TF expression at the cell surface measured by flow cytometry was less, when compared with the total increase of immunoreactive TF protein in cell lysates (Figs 1b, 2a). This could be explained assuming that only a limited amount of *de novo* synthesised TF becomes accessible for antibodies at the cell surface.

DISCUSSION

The data presented here have shown constitutive TF expression in MCF-7 cells to be up-regulated by EGF/TGFα and also by IL-1. The modulation of constitutive TF expression in other systems has been, so far, only initially inveestigated. In COS-7 cells, expressing high levels of TF in culture, a novel serum response element has been found in the TF promoter region [16]. In general, the TF gene has been classified as an immediate early gene responsive to serum, purified growth factors or certain hormones (review in [5]). Exposure of monocytes to LPS [17] or endothelial cells to TNFα [18] rapidly induces transcription of TF gene. In murine AKR-2B fibroblasts, TGFRβ_1 is a strong inducer and PDGF, EGF and insulin are weak inducers of transcriptional TF gene activation [19]. All these data suggest that TF may participate in biological processes other than hemostasis including cell proliferation, inflammatory responses, wound healing and the effector limb of the immune system [5].

Since constitutive TF expression of MCF-7 breast cancer cells can be modulated by mononuclear cell products as, for example, TGFα and IL-1, it seems possible that similar modulations could occur *in vivo*, resulting from interactions of stromal and cancer cells. The cell-bound assembly of a thrombin generating protease cascade can evoke other signals, which mediate activation of a variety of cell types and functions [20]. However, the possible meaning for tumour biology remains a subject of speculation.

In our laboratory experiments are in the process of investigating possible interactions of TF-triggered tumour cell surface assembly of proteases and extracellular matrix with regard to tumour cell proliferation, differentiation and invasion.

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Modulation of Tumour Marker CA-125 Expression in Cultured Ovarian Carcinoma Cells

C. Marth, A.G.Zeimet, G. Böck and G. Daxenbichler

The aim of this study was to elucidate whether proliferation of ovarian carcinoma cells may affect the biosynthesis and release of CA-125. In a cell culture model the tumour marker CA-125 expression in cytosol, surface membrane, and release into culture medium was studied in six human carcinoma cell lines. Cell cycle analysis of propidium iodide stained nuclei was performed using a fluorescent activated cell sorter. The turnover of CA-125 is very rapid, within 24 h the equivalent amount found in each cell was also released in the supernatant culture medium. A good relation between cytosolic, membrane, and released CA-125 was observed. CA-125 expression was associated predominantly with the G_0/G_1 phase of the cell cycle and was dependent on cell density. The results presented here demonstrate that factors associated with tumour cell proliferation could influence the CA-125 serum level in ovarian cancer patients.
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INTRODUCTION

THE CA-125 TUMOUR marker, an antigenic determinant which is recognised by the monoclonal antibody OC125 is associated with high molecular weight glycoproteins [1]. These antigens are expressed by more than 80% of non-mucinous epithelial ovarian tumours as well as by other tissues of Müllerian origin [2, 3]. Due to its clinical value in pre-operative diagnosis and monitoring of ovarian cancer, CA-125 is the leading tumour marker in this disease. The function of the glycoproteins carrying CA-125 is unknown and, because of their complex nature, only little information about the physical and immunological properties of these antigens is available. It is important to know the factors regulating the expression of CA-125 to be able to further evaluate the definite role of CA-125 in monitoring patients. It is well known that CA-125 serum levels are closely related to tumour mass. The level of the tumour marker is elevated in only a small proportion of patients with microscopic disease, but in more than 70% of patients presenting with a tumour mass greater than 1 cm [4, 5]. A persistently rising serum CA-125 concentration is commonly associated with progression of the disease and is frequently observed several months prior to clinical evidence of progression. Apart from the number of tumour cells, other factors possibly influencing the level of CA-125 are still unknown. The aim of our study was to elucidate whether proliferation of tumour cells may affect the biosynthesis and release of CA-125. Moreover, we were interested in the kinetics of CA-125 turnover and its interactions with expression-inducing agents, such as interferons [6].

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