Oncogenic epidermal growth factor receptor upregulates multiple elements of the tissue factor signalling pathway in human glioma cells

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ABSTRACT
Cancer cells frequently overexpress tissue factor (TF) and become procoagulant. This conversion may be driven by genetic transformation, including through the expression of the oncogenic epidermal growth factor receptor (EGFR) and its mutant (EGFRvIII) present in glioblastoma multiforme (GBM). Here we show that the EGFRvIII-dependent GBM cell transformation is associated with the onset of the simultaneous overexpression of TF, protease activated receptors 1 and 2 (PAR-1 and PAR-2) and ectopic synthesis of factor VII (FVII). Efficient generation of factor Xa by these cells still requires exogenous FVIIa. However, as a result of EGFRvIII-dependent transformation GBM cells become hypersensitive to TF/PAR-mediated signalling, and produce ample angiogenic factors (VEGF and IL-8) upon exposure to FVIIa and PAR-1 or PAR-2 activating peptides. Thus, oncogenes may cause complex changes in the ability of cancer cells (GBM) to interact with the coagulation system, thereby exacerbating its influence on angiogenesis and disease progression.
INTRODUCTION

Tissue factor (TF) emerges as a common denominator of multiple processes associated with cancer progression and metastasis\textsuperscript{1-3}. Interestingly, TF upregulation on the surface of cancer cells and the release of microvesicular TF into the blood\textsuperscript{4-7}, are both linked to oncogenic transformation\textsuperscript{3,5}. Notably, oncogenic epidermal growth factor receptor (EGFR), and the truncated, ligand-independent EGFR\textsubscript{vIII} mutant\textsuperscript{8}, both upregulate TF in various cancer settings, including glioblastoma multiforme (GBM)\textsuperscript{7}, the most lethal type of primary brain tumor. GBM is associated with florid angiogenesis, thrombotic complications\textsuperscript{9} and upregulation of TF\textsuperscript{10}, to which both microenvironmental and genetic influences were found to contribute\textsuperscript{7,11,12}.

TF is a 47 kDa cell-associated transmembrane protein that acts as the high affinity receptor for FVIIa\textsuperscript{13} and a triggers of both coagulation\textsuperscript{14}, and intracellular signalling\textsuperscript{2,15}. The latter aspect is mediated, at least in part, by the impact of the TF/VIIa complex on the G protein coupled, protease activated receptors (PARs), either directly (PAR-2), or via FXa (PAR-1 and PAR-2), or thrombin (PAR-1)\textsuperscript{2}.

The significance of these events in cancer is underscored by the link between TF/PAR signalling and tumour angiogenesis\textsuperscript{16,17}, invasiveness\textsuperscript{18} and progression\textsuperscript{19}, all of which are also a function of oncogenic transformation\textsuperscript{3}. It is therefore of interest to ask whether oncogenic and TF/PAR signalling pathways converge in some fashion in cancer cells, and what are the consequences? Here we show that in GBM cells the EGFR\textsubscript{vIII} oncogene simultaneously upregulates a cluster of functionally related elements of the TF/PAR pathway, including: TF, PAR-1, PAR-2 and FVII, and renders cancer cells both procoagulant and hypersensitive to TF signalling.
MATERIALS AND METHODS

Cells and Treatments

Detailed description of cells, conditions, assays and reagents is provided as Supplemental Information. Cells and were maintained in DMEM media with 10% serum. Treatments included: 10nM of FVIIa (Enzyme Research, South Bend, USA) and agonists of PAR1 (PAR1-AP: TFLLR-NH2) or PAR2 (PAR2-AP: SLIGKV-NH2), both from Bachem.

Experimental Procedures.

TF procoagulant activity assay (TF PCA)20: Cells were treated as indicated, followed by exposure to 5nM FVIIa, 150nM FX and 5mM CaCl2 in Tris Buffered Saline (TBS). The chromogenic reaction was triggered by addition of 2mM S-2765 (Chromogenix), stopped with 20µL of 50% acetic acid, read at 405nm, and the results expressed as arbitrary units normalized to the protein content. RT-PCR analysis of gene expression: Total RNA was extracted using TRIzol (Invitrogen), transcribed into cDNA and amplified using a OneStep RT-PCR Kit (Qiagen). Aliquots of 0.5µg were used to carry out reverse transcription at 50°C for 30 minutes, followed by amplification. Western Blotting for protein detection: Cell lysates were resolved by SDS-PAGE5,7. Upon transfer, the PVDF membranes were probed with primary antibodies, including: anti human TF (1:1000; American Diagnostica 4503), β-actin (1:10,000; Invitrogen), PAR-1 (1:500; R&D Systems) and PAR-2 SAM11 (1:200; Santa Cruz). The signal was visualized using appropriate HRP-conjugated secondary antibodies and chemiluminescence (ECL; Amersham Life Sciences). FACS for immunodetection of the TF antigen: Single cell suspensions were washed with Phosphate Buffered Saline (PBS) containing 1% bovine serum albumin (BSA) and 0.1% sodium azide before staining with mouse anti-human TF antibody
For detection of angiogenic factors: Cell culture supernatants were processed and assayed as recommended by suppliers of the human VEGF and IL-8 Immunoassays (R&D Systems and BD-OptEIA)\(^7\). All experiments were independently reproduced at least three times. Mean ± SD were calculated and Student t-test used, as indicated (also see Supplemental Information).

RESULTS AND DISCUSSION

To extend our prior studies \(^7\), we examined the expression and activity of TF in a panel of GBM cell lines expressing EGFRvIII oncogene. While the parental U373 and U87 cells express low to moderate amounts of TF antigen, mRNA, and procoagulant activity, these levels increase dramatically upon the enforced expression of EGFRvIII (Fig. 1A-C). This correlates with the onset of a highly tumourigenic and angiogenic phenotype, especially in the case of U373 cells, which in the absence of EGFRvIII are non-tumorigenic in mice (\(^21\) and data not shown).

Interestingly, EGFRvIII-expressing GBM cells upregulate not only TF, but also PAR1, PAR2 and (ectopically) FVII transcripts (Fig. 2C). Thus, indolent U373 cells express TF and PAR-1 at low levels, and no detectable PAR-2 transcript, while more aggressive U87 cells are positive for TF and PAR2. However, in both cell lines the EGFRvIII-driven transformation causes a marked increase in the expression of TF, PAR1, PAR2 and FVII (Fig. 2C).

Cancer cells were previously found to ectopically produce FVII \(^22\), and our study provides one possible (oncogenic) mechanism of such a conversion. The co-expression of TF and FVII in
GBM cells also raises a possibility that such cells may be able to endogenously generate FXa (from FX), provided that FVII somehow becomes converted to FVIIa. We tested this by examining U373 (TF-low/FVII-negative) and U373vIII (TF-high/FVII-positive) cells in TF procoagulant activity assays (TF PCA), in the absence of exogenous FVIIa. Although U373vIII cells generated higher TF PCA values than their U373 counterparts, the ability of both cell lines to generate FXa under these conditions was exceedingly low (Fig. 2A, versus Figs. 1B and 2C where exogenous FVIIa was added). Hence, the efficient activation of coagulation by these cells depends on the availability of the exogenous FVIIa.

To further examine the consequences of EGFRvIII-dependent TF upregulation we verified it at the protein level (Fig. 2B), and expressed TF exogenously in U373 cells (Fig. 2C). Out of several resulting clones, some (TF U373D11 and TF U373G11) produced TF at levels similar to those of U373vIII cells, from which they did not differ in terms of their procoagulant activity in the presence of recombinant FVIIa (Fig. 2D).

Since EGFRvIII triggered not only the upregulation of TF, but also of PAR1 and PAR2 (Fig. 2B), we compared the signalling capacity of the TF/PAR pathway between parental, EGFRvIII-transformed and TF-transfected, U373-derived cell lines. The cells were stimulated with FVIIa, PAR-1 or PAR-2 activating peptides (PAR-1/2APs), and interrogated for production of angiogenic factors, such as: interleukin 8 (IL-8), and vascular endothelial growth factor (VEGF). Indeed, very low/undetectable levels of IL-8 and VEGF were found in culture supernatants of U373 cells, and their control (EV1/2 U373), or TF-expressing transfectants (TF U373D11/G11), and this did not change markedly upon stimulation. In contrast, U373vIII cells
exhibited both, constitutively high levels, and a dramatic upregulation of angiogenic factor production upon stimulation with FVIIa, PAR1-AP and PAR2-AP, albeit the responses differed between IL-8 and VEGF (Fig. 2EF).

Overall, our observations suggest that EGFRvIII, and possibly other oncogenes, may sensitize cancer cells to coagulation factor signalling, at least in part due to simultaneous upregulation of TF and PARs. This may precipitate proangiogenic and other biological consequences related to the TF/PAR pathway. Targeting this pathway may, therefore, have therapeutic value in GBM, and the levels of TF, FVII and PARs could be explored as biomarkers.

AUTHORSHIP CONTRIBUTIONS

NM and DG performed experiments, NM, DG and JR wrote the manuscript

CONFLICT OF INTEREST

Authors declare no conflict of interest

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References


FIGURE LEGENDS

Figure 1. Upregulation of tissue factor (TF), factor VII, PAR1 and PAR2 in glioma cell lines expressing EGFRvIII oncogene. A. TF antigen expression on the surface of two different GBM cell lines (U373, U87) and their sublines transfected with EGFRvIII (FACS). B. TF procoagulant activity assay (TF PCA) indicating the increased ability of EGFRvIII-transfected GBM cells to generate factor Xa (calibrated to the standard rabbit brain thromboplastin, RBT). Control (%) - refers to the value obtained for U373vIII cells, which was the most procoagulant cell line in this panel (U373 and U373vIII ** p < 0.005) and (U87 and U87vIII *p < 0.05) N=4. C. The impact of EGFRvIII on simultaneous expression of TF, PAR1, PAR2 and FVII mRNA in two different GBM cell lines U373 and U87 (RT-PCR). For details see text, Matherials and Methods and Supplemental Information.

Figure 2. Functional impact of the EGFRvIII oncogene on the ability of the TF pathway to regulate procoagulant and angiogenic phenotype of glioblastoma cells. A. Differential, but low level FXa generating activity exhibited by U373 and U373vIII cells in the absence of exogenous FVIIa (compare panel D). In this setting U373vIII cells exhibit greater ability to activate FX, possibly in relation to their production of endogenous FVII. B. Simultaneous upregulation of TF, PAR-1, and PAR-2 proteins, as a function of EGFRvIII-dependent cellular transformation of U373 glioblastoma cells (Western blot; beta-actin is used as loading control). These changes correspond to those observed at the mRNA level, as shown in Fig. 1C. C. FACS analysis documenting the results of the enforced expression of the exogenous human TF in parental U373 cells (human TF sequence was introduced using pcDNA3.1hygro vector). Viable TF U373D11
and TF U373G11 cells, and cells transfected with empty vector (EV1 U373 and EV2 U373) were
stained for surface TF antigen. Two out of several similar clones in each category are shown. D.
Manifestation of a robust TF-dependent procoagulant activity (TF-PCA – generation of FXa) by
EGFRvIII-transformed U373vIII cells expressing endogenous TF. Their EGFRvIII non-
expressing U373 counterparts transfected with TF (TF U373D11, TF U373G11) exhibit a
comparable levels of TF PCA. In contrast, parental U373 cell line and several control
transfectants (EV1 U373, EV2 U373) display a negligible procoagulant activity. Unlike in the
case of data presented in panel A, this assay was conducted in the presence of the exogenous
recombinant FVIIa (** - p < 0.005, N=4). E. VEGF release upon addition of FVIIa or PAR
agonistic peptides (PAR1-AP and PAR2-AP) to EGFRvIII, TF and control U373-derived cells
(VEGF detection in conditioned medium was detected by ELISA; dashed line – the limit of the
assay sensitivity, as defined by the supplier; dark bars – cells treated as indicated by “+”, light
bars - cells untreated, mostly very low values). Only in EGFRvIII-transformed U373vIII cells,
but not in their parental (U373), TF transfected (TF U373D11, TF U373G11), or mock
transfected (EV1 U373, EV2 U373) counterparts an appreciable increase in VEGF secretion was
detected (24 hours stimulation with FVIIa 10nM or PAR-1/2APs 100µM each * p< 0.05, **
p<0.005; N=2). F. IL-8 upregulation in glioma cell lines upon activation of the TF/PAR
pathway (test carried out using human IL-8 ELISA; designations as in panel E). Appreciable
increase in IL-8 production was observed upon exposure to FVIIa, PAR1-AP or PAR2-AP only
in the case of EGFRvIII-transformed cells, but not in cells expressing TF in the absence of
EGFRvIII (TF U373D11, TF U373G11), or in cells with low levels of TF and PARs (U373, EV1
U373, EV2 U373). All experimental conditions are as in panel E (responses to treatments were
compared to corresponding the untreated cells, * p< 0.05, ** p<0.005) N=6). For details see text, Materials and Methods and Supplemental Information.
A  
**TF – surface expression**

B  
**TF – procoagulant activity**

C  
TF, EGFR, EGFRvIII, FVII, PAR1, PAR2, GAPDH
Magnus et al Figure 2
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