Biochemical and Biophysical Research Communications 454 (2014) 262–268

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

The contribution of tumor and host tissue factor expression to oncogene-driven gliomagenesis

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ARTICLE INFO

Article history:
Received 6 October 2014
Available online 23 October 2014

Keywords:
Cancer
Tissue factor
Oncogene
Brain tumor
EGFR
RAS

ABSTRACT

Glioblastoma multiforme (GBM) is an aggressive form of glial brain tumors, associated with angiogenesis, thrombosis, and upregulation of tissue factor (TF), the key cellular trigger of coagulation and signaling. Since TF is upregulated by oncogenic mutations occurring in different subsets of human brain tumors we investigated whether TF contributes to tumourigenesis driven by oncogenic activation of EGFR (EGFRvIII) and RAS pathways in the brain. Here we show that TF expression correlates with poor prognosis in glioma, but not in GBM. *In situ*, the TF protein expression is heterogeneously expressed in adult and pediatric gliomas. GBM cells harboring EGFRvIII (U373vIII) grow aggressively as xenografts in SCID mice and their progression is delayed by administration of monoclonal antibodies blocking coagulant (CNTO 859) and signaling (10H10) effects of TF *in vivo*. Mice in which *TF* gene is disrupted in the neuroectodermal lineage exhibit delayed progression of spontaneous brain tumors driven by oncogenic *N-ras* and *SV40* large T antigen (*SV40LT*) expressed under the control of sleeping beauty transposase. Reduced host TF levels in low-TF/SCID hypomorphic mice mitigated growth of glioma subcutaneously but not in the brain. Thus, we suggest that tumor-associated TF may serve as therapeutic target in the context of oncogene-driven disease progression in a subset of glioma.

1. Introduction

Astrocytic malignancies (gliomas) comprise a diverse cluster of primary brain tumors, of which glioblastoma multiforme (GBM) is especially aggressive [1]. GBM is also amongst the most vascular and procoagulant human malignancies [1–3] where microvascular vaso-occlusive thrombi occur regularly within tumor masses, and patients are at high risk of peripheral venous thromboembolism (VTE) [2,4]. These exclusively intracranial and lethal tumors have recently been subdivided into at least four molecular forms, including: proneural, neural, classical and mesenchymal GBM. Each of these subtypes is associated with a unique mutational and gene expression signatures, which are indicative of divergent pathogenetic mechanisms [5,6]. This diversity also includes distinctive profiles of genes related to coagulation and fibrinolytic systems (coagulome), many of which are expressed by cancer cells ectopically (e.g., FVII) [7]. In this context, the key receptor triggering the coagulation cascade, tissue factor (TF) is especially highly expressed in the classical subtype of GBM, which is also characterized by the upregulation of the oncogenic epidermal growth factor receptor (EGFR) and the expression of its transforming mutant (EGFRvIII), along with activation of the RAS signaling pathway [7,8]. This is consistent with findings suggesting that the EGFR/RAS pathways, regulate TF expression in cancer cells, including in GBM [9–12].

Abbreviations: EGFR, epidermal growth factor receptor; EGFRvIII, EGFR variant III; GBM, glioblastoma multiforme; PAI-1, plasminogen activator inhibitor 1; PAR (1–4), protease activated receptor (1–4); RAS, rat sarcoma oncogene; SCID, severe combined immunodeficiency (in mice); TF, tissue factor; VTE, venous thromboembolism.

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http://dx.doi.org/10.1016/j.bbrc.2014.10.041
0006-291X/

TF serves as the key cell-associated co-activator/receptor for soluble coagulation factor VII/VIIa. The TF/VIIa complex activates factor X to Xa, and thereby promotes generation of thrombin (IIa), fibrin deposition and activation of platelets [13]. These effects also trigger cellular signaling responses chiefly mediated by coagulation protease activated receptors 1 (PAR-1/thrombin receptor) and 2 (PAR-2) and the resulting in expression of several genes involved in proliferative, migratory, pro-inflammatory and angiogenic phenotypes of cancer and stromal cells [13–15].

Although activation of the coagulation system has traditionally been viewed as an 'unspecific' side effect of cancer, deregulation of TF by GBM-related oncogenic pathways, especially EGFR, suggest a more tumor-specific mechanism. However, the consequences of TF expression and activation during progression of glial brain tumors remains poorly studied, and so are their therapeutic implications.

Here we document the elevated, but heterogeneous, expression of TF mRNA and immunoreactivity in a panel of human gliomas. We suggest that at the cellular level, TF staining may correlate with the expression of EGFRvIII in GBM cells. We also demonstrate that in the EGFRvIII-driven xenograft model of GBM, targeting tumor TF activity and signaling prolongs survival of tumor bearing mice. Notably, in this setting host TF plays a minor and site-specific role. Moreover, genetic disruption of TF expression in the neuroectodermal lineage delays, but does not prevent N-*ras*-driven spontaneous brain tumourigenesis. Thus, we suggest that the inhibition of TF activation and signaling could be explored as a therapeutic target in the subset of GBMs harboring oncogenic EGFR.

2. Materials and methods

2.1. Cells, culture conditions and databases

A431 (human squamous carcinoma) EGFR-driven cell line was maintained under standard culture conditions, DMEM media (10% FBS) along with antibiotics (Penicillin–Streptomycin 1%, GIBCO). U373vIII glioma cells were maintained with the addition of geneticin/G418 (5 μg/μL, GIBCO) and hygromycin (5 μg/μL, Invitrogen), as described earlier [10,16]. REMBRANDT: National Cancer Institute (2005), (http://rembrandt.nci.nih.gov) accessed July 10 2012.

2.2. Mice, treatments and tumor analysis

Low-TF/SCID mice expressing hypomorphic human TF minigene (1% activity) on the background of mouse TF-null mutation (mTF−/−, hTF+/+) were described earlier [17,18]. Cre-Nestin/TF−/− mice express no TF in nestin-positive neuroectodermal cells (Pawlinski et al., manuscript in preparation). (i) *Subcutaneous inoculation*: Immunodeficient SCID mice (Charles River), SCID mice harboring the YFP transgene (YFP/SCID) [17] or low-TF/SCID were injected subcutaneously (s.c.) into the flank with 0.2 mL of a single cell suspension in PBS or Matrigel (BD Biosciences), with either 5×10^6 or $2–3 \times 10^6$ viable cancer cells (>90% trypan blue exclusion), or as indicated. The emerging tumors were measured with a vernier's caliper and tumor volume (TV) was calculated according to the formula: $TV = (a^2 \times b) \times 0.52$, where "*a*" and "*b*" are, the smaller and the larger perpendicular diameter, respectively. Mice were treated intraperitoneally (i.p.) with CNTO859 (500 μg or 350 μg/mouse initially followed by subsequent injections with 200 μg/mouse) or 10H10 (both from Centocor/Janssen Research and Dev.) (initially 500 μg/mouse; subsequent 200 μg/mouse or as indicated) or saline daily (Day 0–4 unless otherwise stated). In some experiments treatment was re-applied until the endpoint once tumors reached >2 mm in diameter. (ii) *Intracranial inoculation*: YFP transgenic mice and low-TF/SCID mice were injected intracranially with (5×10^4 cells/μL with total volume of 2 μL) using a Stoelting Stereotaxic Injector at the coordinates (2.5:−1.5:−3.0) of bregma and sagittal suture. All procedures involving animals were performed in accordance with the guidelines of the Canadian Council of Animal Care (CCAC) and the Animal Utilization Protocols (AUP) approved by the Institutional Animal Care Committee (ACC) at MUHC RI and McGill University. When possible, luminescence data was obtained using IVIS 200 scanner after administering D-Luciferin Firefly potassium salt (Caliper Life Science; 15 μg/mL).

2.3. Histology, immunohistochemistry and immunofluorescence

Tissue microarrays containing formalin fixed paraffin embedded specimens of normal human brain and brain tumors (204 cores) were purchased from US BioMax Inc (9 – normal; 9 – Grade I; 21 – Grade II; 16 – Grade III, 13 – Grade IV). Additional GBM blocks were sectioned using American Optical microtome into 4 μm thick tissue sections and processed for immunostaining with sheep anti-human TF (Cat SATF-IG, Affinity Biologicals, Lancaster, Ontario, Canada) and mouse anti-human EGFRvIII (Cat 08-1330, Zymed, San Francisco, Cal., USA) antibodies followed by alexa flour donkey anti-sheep 488 (A11015) and Alexa flour goat anti-mouse 594 (A11020) secondary antibodies, respectively. Slides were mounted using Vectashield Hard Set Mounting media (H-1400, Vector Laboratories, Burlingame, Cal, USA).

2.4. Expression of mRNA

Cells and tissues were extracted in 1 mL of TRIzol Reagent (Invitrogen), processed and PCR amplified as described previously [16]. PCR primers used included (forward/reverse): hTF: GCTGACTTCAATCCATG/GAAGGTGCCCAGAATACCAA, hGAPDH: GAGTCAACGGATTTGGTCGT/TTGATTTTGGAGGGATCTCG; mTF: TGCTTCTCGACCACAGACAC/TAAAAACTTTGGGGCGTTTG; mPAR1: CTCCTCAAGGAGCAGACCAC/AGACCGTGGAAACGATCAAC; mFVII: TCCAGGGACCTCTAGGGACT/CCTCCGTTCTGACATGGATT; mGAPDH: AA CTTTGGCATTGTGGAAGG/ACACATTGGGGGTAGGAACA.

2.5. Data analysis

All experiments were reproduced at least twice with similar results and presented as number of replicates (n) and mean value of replicates +/−S.D. Statistical analyses were performed using JMP 10.0 (SAS Institute Inc). Differences were considered statistically significant when $P < 0.05$. A Wilcoxon & Log-rank testing were performed for all mouse experiments. Otherwise, for all other experiments statistical analysis was performed using one-tailed, unpaired *t*-test.

3. Results

3.1. TF expression correlates with poor prognosis in glioma but not in GBM

TF mRNA expression is thought to be elevated in aggressive brain tumors [7,19], but its impact on disease outcomes remains unclear. We assessed this link *in silico* using the clinically annotated public database (REMBRANDT) which contains gene expression profiles of 343 human glial brain tumors. Indeed, higher levels of TF mRNA (⩽2-fold increase) significantly correlated with poor patient survival in the whole glioma population (Fig. 1A). However, when we restricted this analysis to GBM (grade IV astrocytoma) there was only a trend toward better survival of low expressors, with no statistical significance (Fig. 1B). This could be explained by the generally poor prognosis in all GBM cases [1],