Chapter 6 - Dual targeting of cancer cell-derived TF isoforms: a new approach to block breast cancer progression

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Abstract

The primary initiator of the coagulation cascade, full length Tissue Factor (flTF), is also pro-angiogenic. By forming a complex with FVIIa, flTF facilitates signaling events through Protease Activated Receptor-2 on tumor cells. Alternative splicing of the TF pre-mRNA leads to the formation of alternatively spliced Tissue Factor (asTF) that lacks a transmembrane domain and features a unique C-terminus. asTF induces breast cancer (BrCa) cell proliferation by ligating β1 integrins, which activates several signaling cascades that promote tumor growth. It is not clear to what relative extent BrCa progression is dependent on flTF and/or asTF function. Therefore, we carried out a side-byside comparison study to investigate the relative impact of flTF- and asTF-driven signaling on BrCa progression. Using isoform-specific antibody-blockade, we show that both flTF and asTF significantly contribute to tumor growth. Combined flTF/asTF blockade decreased tumor size most effectively, indicating that the two TF isoforms likely contribute to BrCa growth using distinct pathways. Compared to flTF blockade, asTF blockade inhibited metastasis to a similar degree, emphasizing the importance of both isoforms in BrCa spread. Interestingly, when two isoforms were simultaneously blocked, metastatic load was only modestly decreased further, suggesting that the flTF and asTF pathway are likely engaging common as well as distinct elements to fuel BrCa metastasis. In sum, our data indicates that flTF and asTF both promote BrCa growth and metastasis through a variety of shared and isoform-specific pathways, raising the possibility that dual-isoform TF blockade may be a qualitatively superior TF-targeting treatment modality in BrCa.

Introduction

The initiator of the coagulation cascade, full length Tissue Factor (flTF), is overexpressed in tumor cells leading to the formation of a thrombogenic cell population [1-4]. Increased flTF expression is associated with pathological parameters: cancer patients with high TF expression levels have decreased survival rates [5, 6], increased metastasis [7, 8], higher tumor grade, stage [9], and increased tumor vessel density [7, 10, 11]. In addition, activated or apoptotic cells may release microparticles exposing flTF that interact with downstream coagulation factors residing in the circulation. This interaction has been hypothesized to increase the occurrence of thrombotic complications in cancer patients [12, 13]. The interaction of flTF with other coagulation factors also leads to activation of Protease Activated Receptors (PARs) [14]. There are four PAR family members, and they show specificity towards their activating proteases [14-19]. Thrombin can activate PAR1, PAR3 and PAR4 [20, 21]. PAR1 can also be activated by activated protein C [22], FXa [21] and matrix metalloproteinases [23]. In contrast, PAR2 is cleaved by the flTF/FVIIa binary

complex, FXa [21], trypsin [24] and matriptase [16]. Among the four PARs, PAR2 is thought to be the main receptor that influences BrCa progression [25, 26].

Alternative splicing of the TF primary transcript leads to the exclusion of exon 5 and consequently an mRNA frameshift. This alternative splicing event gives rise to a distinct TF isoform termed alternatively spliced tissue factor (asTF) that features a unique C-terminus. Unlike flTF, asTF is a soluble secreted protein whose procoagulant activity is extremely low [27-29], and it does not activate PARs [30]. asTF is present in organized mural thrombi, lung, placenta, pancreas [27], and cancer tissues such as pancreatic ductal adenocarcinoma [31], BrCa [32], non-small cell lung carcinoma [33] and cervical cancer [34]. asTF levels in tumor tissues significantly correlate with low survival rate [33], higher stage [35] and higher grade [32].

Associations of TF isoform with histological parameters encouraged studies employing pharmacological or antibody-based TF blockade to stem tumor growth. Blockade of the flTF/FVIIa complex by rNAPc2, but not FXa blockade by rNAPc5, leads to the formation of smaller tumors in a Lewis lung carcinoma model and diminished tumor angiogenesis [36]. Similarly, targeting flTF/FVIIa complex by TFPI decreased tumor mass [36], tumor cell-triggered coagulation and metastasis [37]. Ixolaris, a tick salivary anticoagulant protein with TFPI-like properties, is effective in blocking metastasis [38] as well as inhibiting flTF/PAR signaling [39]. Further, it reduces tumor expansion as well as vessel density [40]. The use of two unique monoclonal antibodies that inhibit either flTF-dependent coagulation (mAb-5G9) or PAR2 mediated signaling (mAb-10H10) identified activation of PAR2 as a key process that is critical to angiogenesis and primary tumor growth, while coagulation activation was critical to metastasis in a tail vein injection assay [25]. More recently, we showed that orthotopic injection of BrCa cells in the presence of a specific inhibitory anti-asTF antibody (mAb-Rb1) delays tumor growth significantly, and our mechanistic studies demonstrated that, in BrCa setting, asTF acts predominantly as a pro-mitogenic molecule augmenting tumor cell proliferation [32]. Both flTF and asTF can promote formation of new vessels, yet the signaling pathways and the cellular events engaged by the two TF isoforms to promote angiogenesis are not identical [29,35]; at present, it remains to be determined whether dual flTF/asTF blockade is superior to single-isoform blockade in suppressing tumor growth. Therefore, in this study we aimed to delineate the relative contributions of the proteolysis-driven flTF/PAR pathway, and the non-proteolytic proliferative asTF/integrin pathway, to BrCa progression. We report that, while both mAb-10H10 and mAb-Rb1 by themselves significantly delay tumor onset and growth rates, combined targeting of both TF isoforms delays tumor growth more efficiently. Thus, flTF/asTF-dependent angiogenesis, as well as asTF-dependent proliferation, contribute significantly to BrCa progression.

Materials and Methods

Reagents and cell culture

The flTF- (mAb-10H10; mouse) and asTF- (mAb-Rb1; rabbit) specific antibodies were described previously [25, 32]. To avoid a possible natural killer cell immune attack against rabbit mAb-Rb1, F(ab')1 fragment is prepared by using Fab preparation kit (Thermo Scientific, Waltham, MA). The MDA-MB-231-mfp cell line was cultured in DMEM (GE Healthcare, Buckinghamshire, UK) with 10% bovine serum, 2 mM L-glutamine, penicillin, and streptomycin.

Orthotopic breast cancer injection

Animal experiments were approved by the animal welfare committee of the Leiden University Medical Center (LUMC). Five animals per group were used. Orthotopic injections were performed as described previously [32]. In short, the antibody concentration was determined based on previous work (mAb-10H10 [unpublished data] and mAb-Rb1 [32]). Mice were anesthesized using isoflourane and $5x10^5$ MDA-MB-231-mfp cells were mixed with 500 µg mouse mAb-10H10, 100 µg F(ab')1 mAb-Rb1, 500 µg mouse IgG1 (TIB115) or 500 µg mAb-10H10 + 100 µg F(ab')1 mAb-Rb1 and injected into inguinal fat pads of NOD-SCID mice (Charles River, Wilmington, MA); temgesic (0.05mg/kg, Schering-Plough, Kenilworth, NJ) was injected as analgesic. Tumor volume was measured with calipers using the formula length x width x width)/2. Mice were sacrificed on day 98 and tumors extracted for analysis; lungs were snap frozen in liquid nitrogen for qPCR analysis.

qPCR

Lungs were homogenized in Trizol (Invitrogen, Carlsbad, CA) and RNA isolation was performed using phenol/chloroform extraction. Total RNA was converted into cDNA using Super Script II reverse transcriptase (Invitrogen). Real time PCR was conducted using SYBR Green (Applied Biosystems, Carlsbad, CA). The following primers were used to quantify metastatic burden: human GAPDH forward 5' TTGCAGGAGCGAGATCCCT 3 ', human GAPDH reverse 5' CACCCATGACGAACATGGG 3', murine β-actin forward 5' AGGTGATGACTATTGGCAACGA 3' and murine β-actin reverse 5' CCAAGAAGGAAGGCTGGAAAA 3'. ΔCt values of the individual samples were related to the mean ΔCt of the IgG group. Student's t-test was used to assess significance.

Results

Targeting TF isoforms suppresses breast tumor growth

To investigate the relative impact of flTF and asTF inhibition on BrCa progression, we used MDA-MB-231-mfp cells, an aggressive subclone of the MDA-MB-231 triple negative breast cancer cell line that expresses both TF isoforms and PAR-2 – the key players in TF-mediated signaling events that drive BrCa progression [26, 32]. We co-injected $5x10^5$ cells in fat pads of NOD-SCID mice in the presence of 500 µg mAb-10H10, 100 µg F(ab')1 mAb-Rb1, their combination, or 500 µg control IgG . Rb1 F(ab')1 fragments were used to prevent natural killer cell-mediated effects [41]. Individual blockade of TF isoforms yielded a significantly smaller average final tumor volume **(Fig. 1A, 1B)** and weight **(Fig.1C)**. Interestingly, the F(ab')1 mAb-Rb1/mAb-10H10 combination significantly reduced tumor growth compared to mAb-10H10 alone. Although not statistically different, combined F(ab')1 mAb-Rb1/mAb-10H10 treatment showed a trend towards more efficient tumor growth inhibition, compared to F(ab')1 mAb-Rb1 alone. These data point to comparable importance of angiogenic and proliferative signals elicited by flTF and asTF in breast cancer progression **(Fig.1A-C)**.

Blockade of TF isoforms decrease metastasis significantly

We next analyzed the impact of flTF and asTF antibodies on the systemic spread in tumor bearing mice. To assess the metastatic burden, we performed real-time PCR using a human specific primer set to detect human cancer cell populations, and a mouse specific primer set as a loading control. Both mAb-10H10 and F(ab')1 mAb-Rb1 treatment decreased the metastatic burden in the lungs dramatically (> 100 fold). Compared to individual antibody blockade, dual antibody blockade did not decrease the metastatic burden much further **(Fig.1D)**, although we did observe a trend for lower metastasis (F(ab')1 mAb-Rb1 vs. combination p=0,354 and mAb-10H10 vs. combination p=0,208). Taken together, this data show that both flTF and asTF are important contributors to the metastatic process in BrCa **(Fig.2)**.

Discussion

In this paper, we evaluated the relative contribution of flTF and asTF to BrCa progression, by blocking their function with an antibody specific to each TF isoform. Although there have been reports demonstrating the effects of TF blockade and the resultant outcome [25, 32], this study is the first to make a side by side comparison of the effects of individual as well as dual inhibition of the TF isoforms on BrCa progression. mAb-10H10, which selectively recognizes flTF, suppresses flTF-dependent PAR2 signaling, tumor growth, and angiogenesis [25, 42]. mAb-Rb1, which selectively recognizes and blocks asTF, leads to a

decrease in tumor size *in vivo* and BrCa cell proliferation *in vitro* [32]. Thus, both flTF and asTF contribute to primary tumor growth in BrCa. Importantly, dual blockade elicited a stronger effect on tumor growth compared to mAb-10H10 alone. The advantage of dual targeting over mAb-10H10 and/or mAb-Rb1 might be due to the presence of a unique asTF-dependent pathway that does not overlap with flTF/PAR2 dependent pathways [43, 44]. On the other hand, the lack of difference in tumor size upon F(ab')1 mAb-Rb1 and mAb-10H10 treatment also suggests the presence of common downstream components that regulate BrCa progression.

Previous studies have shown that highly coagulant flTF plays a crucial role in metastasis. In an experimental metastasis model, injection of MDA-MB-231 cells with mAb-5G9 hampered metastasis to lungs. Most likely, flTF coagulant activity shields these cells from immune cell attack by forming a layer of fibrin and activated platelets around cancer cells [25, 45]. Of note, mAb-10H10 has no effect on metastasis *in vivo* [25]; in this model, cancer cells directly injected into venous circulation are soon detectable in the lung tissue. This method does not fully represent metastasis as it does not recapitulate the invasion of primary tumor cells into adjacent normal tissue and/or their entry into the circulation [46]. Undoubtedly, flTF/PAR2 signaling is important for invasion [47] and angiogenesis [25] and, in our model, inhibition of those processes may very well explain the decreased metastatic burden in the lungs in response to mAb-10H10 treatment. Overexpression of asTF in pancreatic ductal adenocarcinoma increased the metastatic capacity of these cells, showing for the first time a role for asTF in metastasis [31]. We here demonstrate that the use of F(ab')1 mAb-Rb1 hampers metastasis of BrCa cells to the lungs (Figure 1D). Interestingly, the combination of mAb-10H10 and F(ab')1 mAb-Rb1 did decrease the systemic spread of BrCa cells somewhat more effectively than either mAb alone. Possibly, TF/PAR2 and asTF/integrin complexes activate similar pathways to promote metastasis. Of note, both flTF and asTF expressing BrCa tumors show higher vessel density [25, 32] which is likely to facilitate the entry of cancer cells into the circulation. In addition, both signaling via flTF and asTF activates genes involved in invasion [32, 47] which, in turn, might trigger metastasis.

In conclusion, our findings show that the proteolysis-dependent flTF pathway and the non-proteolytic, integrin-mediated proliferative asTF pathway both contribute significantly to breast cancer progression. Because dual targeting of flTF and asTF is clearly superior in suppressing primary BrCa growth *in vivo* compared to selective targeting of either TF isoform, it opens a new approach in developing TF-based treatment modalities in cancer. Our future studies will focus on delineation of the shared and isoform-specific pathways employed by flTF and asTF in promoting BrCa growth and spread.

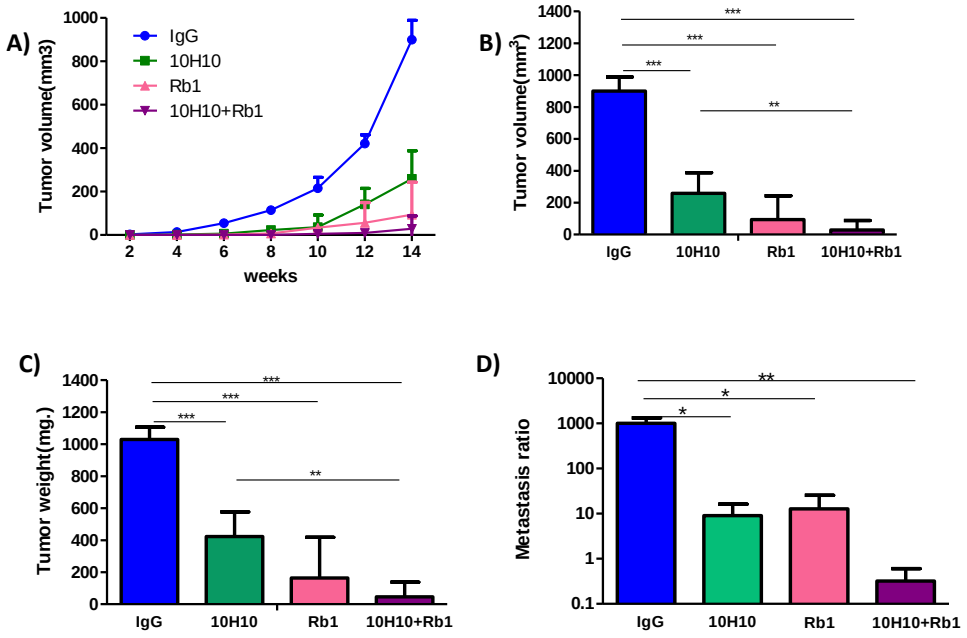

Fig.1 Dual blockade of TF isoforms is superior in stemming BrCa tumor size and metastasis when compared to single-isoform blockade. MDA-MB-231-mfp cells were co-injected with 500 µg IgG control, 500 µg mAb-10H10, 100 µg F(ab')1 mAb-Rb1, or a combination of mAb-10H10 and F(ab')1 mAb-Rb1 into mammary fat pads of NOD-SCID mice. A) Tumor growth was followed for 14 weeks; tumor volume (B) and weight (C) at 14 weeks are indicated. D) MDA-MB-231-mfp breast cancer cells were orthotopically injected into NOD-SCID mice with the indicated antibodies. 14 weeks after engraftment, mice were sacrificed, lung tissue was collected, and metastatic burden evaluated using real time-RT-PCR performed with human and mouse specific primer sets. The results are shown on a log scale. Mean and SEMs are depicted. (*p<0.05, **p<0.01, ***p<0,001)

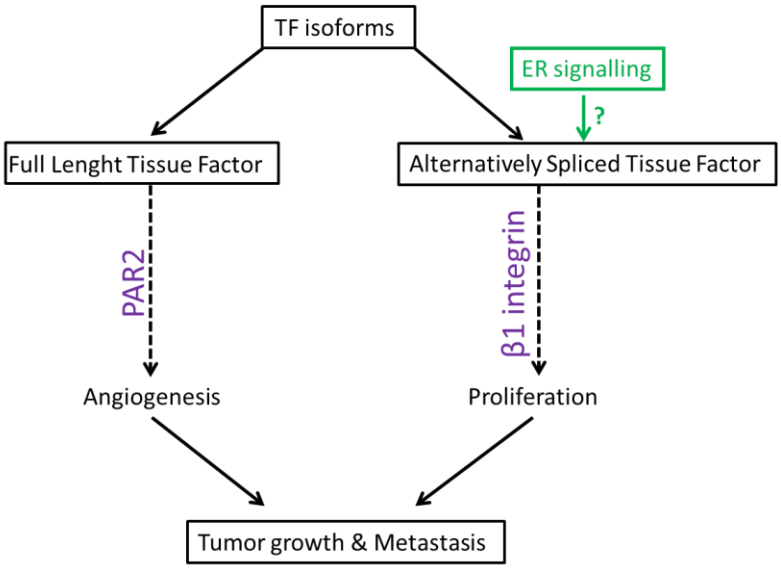

Fig.2 Schematic representation of the roles of the two TF isoforms in BrCa progression. Dashed lines indicate the pathways predominantly engaged by the respective TF isoform, solid lines indicate common pathways/functions.

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