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Inhibition of tissue factor by ixolaris reduces primary tumor growth and experimental metastasis in a murine model of melanoma

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ABSTRACT

Melanoma is a highly metastatic cancer and there is strong evidence that the clotting initiator protein, tissue factor (TF), contributes to its aggressive pattern. TF inhibitors may attenuate primary tumor growth and metastasis. In this study, we evaluated the effect of ixolaris, a TF inhibitor, on a murine model of melanoma B16F10 cells. Enzymatic assays performed with B16F10 and human U87-MG tumor cells as the TF source showed that ixolaris inhibits the generation of FX in either murine, human or hybrid FVIIa/TF complexes. The effect of ixolaris on the metastatic potential was further estimated by intravenous injection of B16F10 cells in C57BL/6 mice. Ixolaris (250 μg/kg) dramatically decreased the number of pulmonary tumor nodules (4 ± 1 compared to 47 ± 10 in the control group). Furthermore, a significant decrease in tumor weights was observed in primary tumor growth assays in animals treated with ixolaris (250 μg/kg) from days 3 to 18 after a subcutaneous inoculation of melanoma cells. Remarkably, immunohistochemical analyses showed that inhibition of melanoma growth by ixolaris is accompanied by a significant downregulation of both vascular endothelial growth factor (VEGF) expression and microvascular density in the tumor mass. Our data demonstrate that ixolaris targets B16F10 cell-derived TF, resulting in the reduction of both the primary tumor growth and the metastatic potential of melanoma, as well as the inhibition of tumor angiogenesis. Therefore TF may be a potential target for the treatment of this aggressive malignancy.

Introduction

In 1865, Armand Trousseau first described an important relationship between cancer and thrombosis. Blood disorders involving the hyperactivation of the coagulation system and formation of intravascular fibrin clots (thrombosis) may be the first sign of a malignant tumor [1,2]. This correlation is exemplified by the abnormally elevated expression of the clotting initiator protein, tissue factor (TF), on the surface of tumor cells. The presence of elevated TF levels in plasma, mainly incorporated into tumor-derived microvesicles, would allow the formation of the TF/factor VIIa (FVIIa) complex and the subsequent activation of blood coagulation reactions. This condition has been correlated with both disseminated intravascular coagulation and thrombosis occurrence [3,4].

TF is expressed in many types of cancers [5–7] and studies employing cultured cells as well as patient specimens have demonstrated a strong correlation between TF expression and aggressive tumor behavior [8–10]. In particular, TF procoagulant activity has been correlated with the metastatic potential of tumor cells [11,12]. In addition, TF expression correlates with increased tumor angiogenesis, as reported in studies on patient samples from non-small cell lung, colorectal, hepatocellular, and pancreatic cancer [13–16]. It is therefore proposed that TF expression leads to an unbalanced production of anti- and/or proangiogenic factors such as vascular endothelial growth factor (VEGF), which favors increased tumor vasculature [6,17]. Along with its role in initiating blood coagulation reactions, the TF/FVIIa complex may modulate intracellular signaling through cleavage of the G protein-coupled protease-activated receptor-2 (PAR2). *In vitro* and *in vivo* assays have demonstrated that PAR2 activation is correlated with the production of tumor-promoting molecules, primary tumor growth and the proangiogenic and invasive properties of tumors [18]. Remarkably, some of these effects seem to be independent of the procoagulant activity of the TF/FVIIa complex [19].

Melanoma, the most common fatal form of skin cancer, is an aggressive, therapy-resistant malignancy of melanocytes that represents a significant public health burden because its incidence is

Abbreviations: TF, tissue factor; VEGF, vascular endothelial growth factor; PAR1/2, protease activated receptor-1/2; ERK, extracellular-signal regulated kinase.

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increasing in Caucasian populations across the world [20]. The prognosis of patients diagnosed with metastatic melanoma is very poor, with a limited number of agents available for treatment [21]. Several lines of evidence indicate a key role for blood clotting activation in melanoma progression, suggesting that TF is a target for adjuvant treatment of this aggressive cancer [22–24].

Ixolaris, a tick salivary 140 amino acid protein containing 10 cysteines and 2 Kunitz-like domains, binds to FXa or FX as scaffolds for the inhibition of the TF/FVIIa complex, in which the FVIIa catalytic site is inactivated, as previously demonstrated by inhibition of macromolecular (i.e., FX and FIX) substrates [25]. Ixolaris does not bind to the active site cleft of FXa. Instead, complex formation is mediated by the FXa heparin-binding exosite [26]. In addition, ixolaris interacts with zymogen FX through a precursor state of the heparin-binding exosite [27]. Because ixolaris displays potent and long-lasting antithrombotic activity [28], we hypothesized that this molecule might interfere with melanoma progression. Here, we demonstrate that ixolaris inhibits human and murine TF procoagulant activity to a similar extent. Consequently, ixolaris decreases both primary tumor growth and experimental metastasis in a murine melanoma model. Inhibition of tumor growth was accompanied by the downregulation of VEGF expression and a decrease in vessel density in tumor mass. Therefore, ixolaris displays potential antitumor effects, emerging as a valuable tool for studying the role of TF in melanoma biology.

Materials and Methods

Reagents

Recombinant ixolaris was produced in High Five insect cells (Invitrogen, San Diego, CA), as previously described by Francischetti et al. [25], and purified and quantified as described [28]. Recombinant nitrophorin-2 was produced in *Escherichia coli*, purified, and quantified as previously described [29]. Both human FX and murine FX were purchased from Haematological Technologies Inc. (Essex Junction, VT, USA). Human FVIIa was purchased from American Diagnostica (Greenwich, CT, USA). Murine FVIIa was expressed and purified as previously described [30]. The chromogenic substrate for FXa (S-2765, N-α-benzyloxycarbonyl-D-Arg-Gly-Arg-p-nitroanilide) was purchased from Diapharma (Westchester, OH). The PAR1 agonist peptide (PAR1-AP, TFLLR-NH2) and the PAR2 agonist peptide (PAR2-AP, SLIGKL-NH2) were synthesized by Biosynthesis Inc. (Lewisville, TX, USA).

Cell Culture

The murine melanoma cell line B16F10 was grown at 37 °C in a humidified, 5% CO_2 atmosphere, in Dulbecco´s modified Eagle medium (DMEM; GibcoBRL) at pH 7.2, containing 10% (v/v) foetal bovine serum (FBS) and supplemented with 2.4 g/L HEPES, 3.7 g/L sodium bicarbonate, 125 mg/L sodium dihydrogen phosphate, 110 mg/L sodium pyruvate, 100,000 U/L penicillin, 100 mg/L streptomycin and 2 mM L-glutamine. Cells were detached with Hank's solution containing 10 mM HEPES and 0.2 mM EDTA, spun at 350 ×*g* for 7 min, resuspended in DMEM (supplemented as described above) and transferred to another culture flask. The human glioblastoma cell line, U87-MG, was grown at 37 °C in a humidified, 5% CO_2 atmosphere, in DMEM-F12 (GibcoBRL) containing 10% (v/v) FBS and supplemented with 2 g/L HEPES, 60 mg/L penicillin, 100 mg/L streptomycin and 1.2 g/L sodium bicarbonate. Cells were washed twice with phosphate buffered saline (PBS), detached with Hank's solution containing 10 mM HEPES and 0.2 mM EDTA, spun at 350 ×*g* for 7 min, resuspended in DMEM-F12 (supplemented as described above) and transferred to another culture flask.

FX Activation as Measured by Hydrolysis of a Chromogenic Substrate

Activation of FX by FVIIa was performed as previously described [31] in 50 mM HEPES, 100 mM NaCl, 5 mM $CaCl_2$, and 1 mg/mL BSA, pH 7.5 (HEPES-BSA buffer). Murine FVIIa (1 nM) was incubated with U87-MG or B16F10 cells (5×10^4/mL) for 10 min at 37 °C. The reaction was initiated by the addition of murine FX (135 nM), and 25 μL aliquots were transferred after 30 min into microplate wells containing 25 μL of Tris-EDTA buffer (50 mM Tris–HCl, 150 mM NaCl, 20 mM EDTA, and 1 mg/mL polyethyleneglycol 6000, pH 7.5). Alternatively, human FVIIa (1 nM) was incubated with U87-MG cells (5×10^4/mL) for 10 min at 37 °C prior to the addition of human FX (135 nM). After the addition of 50 μL of 200 μM S-2765, prepared in Tris-EDTA buffer, the absorbance at 405 nm was recorded at 37 °C for 30 min using a Spectramax Microplate Reader (Molecular Devices, Menlo Park, CA, USA). The velocities (mOD/min) obtained in the first minutes of the reaction were used to calculate the amount of formed FXa, as compared to a standard curve using known enzyme concentrations. The appropriate controls performed in the absence of cells or in the absence of FVIIa showed no significant FXa formation.

The inhibitory effect of ixolaris was evaluated by incubating FX with varying amounts of the inhibitor (0–5 nM) for 10 min prior to adding it to the FVIIa and tumor cells. FXa formed in the absence of ixolaris was taken as 100%.

Quantification of Experimental Melanoma Metastasis

Eight week-old C57BL/6 mice maintained at our own facilities were injected via the lateral tail vein with a 100 μL-bolus of 2.5×10^5 B16F10 cells (resuspended in DMEM). The anti-metastatic effect of ixolaris or nitrophorin-2 was evaluated by the intravenous injection of 250 μg/kg ixolaris or nitrophorin-2 two hours before the inoculation of B16F10 cells. The control animals were injected with PBS instead of the coagulation inhibitor. After 15 days, the animals were sacrificed, the lungs were removed, and the number of pulmonary tumor nodules only on the convex face of the organ was counted. The animal experiments were performed under approved protocols of the institutional animal use and care committee (Institute of Medical Biochemistry, Federal University of Rio de Janeiro).

Primary Tumor Growth Assay

B16F10 cells (3.5×10^5, resuspended in DMEM) were subcutaneously inoculated into the flank of 8-week-old C57BL/6 mice. Treatment with 50 or 250 μg/kg ixolaris, or 250 μg/kg nitrophorin-2 (diluted in PBS, 100 μL of final volume intraperitonealy inoculated) was initiated 3 days after the tumor cell inoculation and continued daily for 15 days. The control animals were injected with PBS instead of the coagulation inhibitor. Tumor weights were determined at the time of sacrifice. The animal experiments were performed under approved protocols of the institutional animal use and care committee (Institute of Medical Biochemistry, Federal University of Rio de Janeiro).

Immunohistochemistry

Primary tumors were removed from mice at the time of sacrifice (day 18 after tumor cell inoculation) and fixed in 4% formaldehyde. Tissue staining was performed on paraffin-embedded sections (4 μm-thick), which were incubated overnight, following heat antigen retrieval, with either a monoclonal antibody, anti-mouse endoglin (CD105) (MAB-1320, R&D Systems, USA) at 1:10 dilution, or a monoclonal antibody against VEGF (SC-7269, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 dilution. In order to reduce nonspecific antibody binding, the sections were incubated with PBS containing 10% nonimmune goat serum, 5% BSA and 10% FBS for 30 min prior