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The cell-membrane prothrombinase, fibrinogen-like protein 2, promotes angiogenesis and tumor development

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ABSTRACT

The aim of the study was to further investigate the role of fibrinogen-like protein 2 (FGL-2), a transmembrane prothrombinase that directly cleaves prothrombin to thrombin, in angiogenesis and tumor development and the mechanism(s) underlying these processes. To study angiogenesis HUVEC clones with decreased fgl-2 mRNA were generated by specific siRNA. To study tumorigenesis SCID mice were implanted with intact (wild type) and fgl-2-silenced PC-3 clones. IFN-γ treated HUVEC expressing increased fgl-2 mRNA exhibited significant capillary sprouting that was not inhibited by hirudin, whereas fgl-2 silencing completely inhibited blood-vessel formation. Tumors (poorly differentiated carcinoma) developed in all 12 mice injected with wild type PC-3 compared with 8/12 mice injected with the fgl-2-silenced PC-3 clone. The tumors developed by fgl-2-silenced PC-3 clones were smaller and less aggressive and contained significantly fewer blood vessels ($p < 0.05$). All tumors' sections were negative for thrombin staining, indicating that FGL-2-induced tumorigenesis was not mediated by thrombin. In fgl-2-silenced tumors there was a decrease in fgl-2 mRNA ($p = 0.02$) and ERK1/2 phosphorylation ($p < 0.05$) by 80% and a 20%, respectively. The mechanism underlying these processes, studied in PC-3 clones, revealed that fgl-2 silencing was associated with a 65% decrease in FGF-2 mRNA ($p < 0.01$) and a 30% down regulation of ERK1/2 phosphorylation ($p < 0.05$). Together, these results suggest that FGL-2 mediates angiogenesis and tumorigenesis not by thrombin-mediated mechanism but rather through FGF-2/ERK signaling pathway. FGL-2 may serve as a valuable therapeutic target in the future.

Introduction

The bidirectional relationship between cancer and thrombosis has been known for almost two centuries [1–3]. Thrombosis often precedes the diagnosis of cancer, and its presence is associated with a detrimental disease course [4,5]. These findings support the paradigm that coagulation and tumor growth form a vicious circle in which hypercoagulability facilitates the aggressive biology of cancer and *vice versa*. The mechanism underlying these events is still unclear. Malignant cells are known to directly activate blood coagulation in three ways: by producing procoagulant, fibrinolytic, and proaggregating factors; by releasing pro-inflammatory and proangiogenic cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin (IL)-1ß; and by interacting directly with host endothelial cells, leukocytes, and platelets via adhesion molecules [5]. However, the precise procoagulant proteins that stimulate tumorigenesis have not been identified.

One potential candidate is the cell-membrane-associated protein, fibrinogen-like protein 2 (FGL-2)/fibroleukin, which has been shown to induce sprouting in vascular endothelial cells [6] and to be overexpressed in tumor cells [7]. FGL-2, also known as FGL-2-prothrombinase, is a member of the fibrinogen family of proteins [8]. It exerts serine protease activity and is capable of directly cleaving prothrombin to thrombin in the absence of factor VII or factor X. Like plasmatic prothrombinase, factor Xa, FGL-2 prothrombinase requires phospholipids, calcium, and factor Va for optimal catalytic activity [9]. However, unlike factor Xa, FGL-2 is a transmembrane protein which is not inhibited by antithrombin in the presence of heparin or by other protease inhibitors that inhibit factor Xa [9].

Abbreviations: ERK, extracellular-signal-regulated kinases; FGF-2, basic fibroblast growth factor; FGL-2, fibrinogen-like protein 2; HUVEC, human umbilical vein endothelial cells; IFN-γ, human interferon-gamma; MAPK, mitogen-activated protein kinases; SCID, Severe Combined Immunodeficiency

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The human gene encoding FGL-2, originally cloned from cytotoxic T lymphocytes, spans approximately 7 kb on chromosome 7 and contains 2 exons. The 70-KD protein is predicted to be 439 amino acids (aa) long, with the N-terminus including a 2-aa-long cytoplasmic domain and a 21-aa-long transmembrane domain. The remaining 416 aa constitute the extracellular domain. FGL-2 shares 36% sequence homology with the fibrinogen β and γ chains and 40% homology with the fibrinogen-related domain (FRED) of tenascin [10]. The murine and human proteins share 78% overall identity, with greater conservation at the C terminus [11,12].

FGL-2 is expressed on the surface of activated macrophages and endothelial cells and also secreted by peripheral blood CD4 + and CD8 + T cells. The secreted protein is devoid of coagulation activity. It has potent modulatory effects on the adaptive immune system and was reported to inhibit the maturation of dendritic cells [13,14]. The prothrombinase and immune activities of FGL-2 are located on distinct domains on the FGL-2 molecule [9,14].

The prothrombinase activity of FGL-2, first described in a murine fulminant hepatitis model [15], is exhibited when FGL-2 is expressed on activated macrophages and endothelial cells in the form of a membrane-associated protein. It has shown to be associated with both experimental and human allograft rejection that was abrogated following neutralization of FGL-2 by antibodies or in FGL-2 knockout mice [16]. Macrophage and endothelial cell induction of FGL-2 occurs *via* interferon gamma (IFN –γ) [17].

FGL-2 also plays a role in tumor development. Overexpression of FGL-2 has been detected in tumor and interstitial inflammatory cells but not in the normal surrounding tissue [7]. A recent study found that knockdown of FGL-2 delayed tumor growth and angiogenesis in mice injected with the human hepatocellular carcinoma (HCC) cell line [18]. The authors hypothesized that the protumorigenic activity of FGL-2 is a result of FGL-2-induced generation of thrombin leading to thrombin-induced tumorigenesis [7,18]. However, this does not explain the upregulation of FGL-2 in tumor cells.

The aim of the present study was to substantiate the role of FGL-2 in angiogenesis and tumor development and to uncover the mechanism underlying these processes. Using an *in vitro* pro-angiogenic assay and an *in vivo* mice model of tumor development, we established that FGL-2 exerts direct, non-thrombin-mediated, angiogenic and tumorigenic activity.

Material and Methods

Fgl-2 Expression

The expression of fgl-2 was analyzed in human umbilical vein endothelial cells (HUVEC) in the presence or absence of IFN-γ (20 ng/ml) and in a wild type (WT) human prostate carcinoma cell line, PC-3, established from bone metastasis (ATCC, Beit-Haemek, Israel). Total RNA was isolated using RNAqueous™ (Ambion #AM1912, Invitrogen, Austin, TX, USA) and analyzed by real-time polymerase chain reaction (RT-PCR) using the Rotor-gene RG-3000 (Corbett, Australia). The difference in cycle time (ΔCT) was measured by comparing fgl-2 gene with abl-1 gene (housekeeping gene). The relative quantification was calculated with the formula $RQ = 2^{-\Delta\Delta CT}$.

Generation of fgl-2-silenced Clones of HUVEC and PC-3 Cells

Small interfering RNA (siRNA), which interferes with the expression of specific genes, was employed to evaluate the impact of inhibiting fgl-2 expression on angiogenesis and tumorigenesis. The effect of siRNA was tested on HUVEC and PC-3 cells. The specific siRNA was purchased from Thermo Scientific (Pittsburg, PA, USA). Using pGIPZ lentiviral vector (Thermo Scientific), stable specific fgl-2-silenced clones and non-fgl-2-inhibited HUVEC and PC-3 clones (nonspecific siRNA clone) were generated. Stable transfectants were selected with 0.2 μg/ml puromycin.

In vitro Angiogenesis Assay

HUVEC (5 x 10^3/well) were cultured in F12 (HAM) medium complemented with 1% penicillin (10,000 UI/ml)/streptomycin (10,000 UI/ml) and 10% fetal calf serum (FCS). FCS was tested for prothrombin by one-stage prothrombin-time coagulation assay (ACL-1000 Coagulometer). The cells were maintained at 37 °C in a humidified incubator (85%) in 5% CO_2 atmosphere. The angiogenesis assay was performed with the Chemicon *in vitro* ECMatrix assay kit (ECM625, Millipore, Billerica, MA, USA) using HUVEC treated with 20 ng/ml IFN-γ, fgl-2-silenced clone of HUVEC, and nonspecific siRNA clone of HUVEC, in the presence or absence of 10 u/ml of hirudin (H0393, Sigma, St. Louis, Mo, USA). The extent of tube formation was monitored by light microscopy, as described by the kit manufacturer. Pattern recognition was defined by photographing the cells at the end point of the assay. A numerical value representing the degree of angiogenesis progression was assigned to each pattern according to the manufacturer's instructions.

Mouse Model

Ten-week-old male severe combined immunodeficiency (SCID) mice were purchased from Harlan Laboratories (Rehovot, Israel). The mice were maintained in laminar flow cabinets under specific pathogen-free conditions and a daily cycle of 12 h light/12 h dark. All food, water, and litter were sterilised prior to use, and temperature (20-21 °C) and humidity (50-60%) were controlled. Cages were changed fully once a week. Animals were manipulated under sterile conditions. The mice were managed in accordance with the NIH Guidelines on Laboratory Animal Welfare. Study and all protocols were approved and monitored by the Animal Care Committee of Rabin Medical Center.

Twenty-four mice underwent subcutaneous cell implantation as follows: 10 unmanipulated (WT) PC-3 cells; 12 fgl-2 siRNA-silenced PC-3 clone; 2 PC-3 transfected with nonspecific siRNA. In each case, 2.5 x 10^6 tumor cells suspended in 100 μl of phosphate-buffered saline were injected without anesthesia using a 27-gauge needle and a 1-ml disposable syringe. Tumor progression was monitored by palpation 3 times a week. Subcutaneous tumor dimensions (length (L) and width (W)) were measured with a caliper, and volume was calculated according to the formula $(L \times W^2)/2$ [19]. Mice were sacrificed by cervical dislocation in week 6 after implantation. Autopsy was performed to assess the distribution of metastases. Tumors, spleen, liver, and lung were harvested. The tissues were fixed in 4% formaldehyde for histological analysis.

Immunohistological Analysis and FGL-2 Expression in Mouse Tumor Tissues

Histological sections of the tumors and other mouse tissues were stained with haematoxylin-eosin. Immunohistochemistry study was performed on formalin-fixed paraffin-embedded sections (4 microns thick) using anti-FGL-2 monoclonal antibody (Abnova, St. Louis, MO, USA) diluted 1:400; mouse monoclonal anti- thrombin antibody F-1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:200; and anti-basic fibroblast growth factor (FGF-2) monoclonal antibody diluted 1:100 (#SC-79, Santa Cruz Biotechnology). Normal hepatic tissue served as a positive control for the anti-thrombin antibody. Endogenous reactions were blocked with DAKO blocking kit. The slides were stained with the automatic Bench Mark XT kit (Ventana Medical Systems, Oro Valley, AZ, USA) consisting of labelled streptavidin-biotin reagents and counterstained with haematoxylin. For quantitation of blood vessels, the histological sections were stained with polyclonal anti-human von Willebrand factor (VWF) antibody diluted 1:300 (#A0082, DAKO,