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Lumican inhibits angiogenesis by interfering with α2β1 receptor activity and downregulating MMP-14 expression

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ABSTRACT

Introduction: Previous studies showed that lumican, a small leucine-rich proteoglycan that binds to α2 integrin I domain, is an efficient inhibitor of cell adhesion and migration. In this report, we tested its effect on angiogenesis *in vitro* and *in vivo*.
Materials and methods: Effect of lumican on angiogenesis was evaluated by *in vitro* capillary tube formation test performed between Fibrin II Gels or in Matrigel™ and *in vivo* by Matrigel™ plug assay in BALB/c mice. Changes in matrix metalloproteinases expression caused by lumican were analyzed in endothelial cells by real-time PCR, Western immunoblotting and gelatin zymography.
Results: In unchallenged endothelial cells, Matrigel™ induced robust capillary morphogenesis. In contrast, tube formation was dramatically reduced by lumican, and by siRNA to β1 integrin subunit mRNA but not by control siRNA. Similarly, lumican effectively inhibited neovascularization *in vivo* in assays using Matrigel™ plugs formed in BALB/c mice. Interestingly, lumican significantly reduced expression of matrix metalloproteinases, particularly MMP-14 that is known to activate other MMPs in close vicinity of endothelial cell membranes.
Conclusions: Our results provide strong evidence that lumican affects angiogenesis both by interfering with α2β1 receptor activity and downregulating proteolytic activity associated with surface membranes of endothelial cells.

Introduction

Lumican is the major leucine-rich keratan sulfate proteoglycan of the corneal stroma and other extracellular matrices, such as skin, muscle, and cartilage. As evidenced by knocking-out of its gene in mouse, it plays a critical role in collagen fibrillogenesis. The lumican null mice ($Lum^{-/-}$) were found to have serious functional defects including corneal opacity as well as skin and tendon fragility associated with disorganized and loosely packed collagen fibers [1,2]. Furthermore, there seems to be a direct link between lumican expression and cancer progression. Elevated lumican mRNA in human breast carcinomas was associated with higher tumor grade [3] but no prognostic factors correlated to lumican protein expression were observed [4]. In colorectal cancer lumican was highly expressed, both in cancer cells and adjacent stroma cells, and it was suggested to contribute to colorectal cancer progression [5]. In pancreatic cancer tissues, lumican was localized to stromal fibroblasts and cancer cells [6] or exclusively synthesized by pancreatic stellate cells and secreted to extracellular matrix [7]. Though, human melanoma cell lines express lumican [8], in malignant melanoma lumican appears to be located to the peritumoral stroma but not in cancer cells [9]. Increased expression of cytoplasmic lumican was found in carcinoid tumors [10] and seems to be positively correlated with the differentiation and negatively correlated with the progression of osteosarcoma [11]. Unfortunately both positive [3] and negative [12] correlation to tumor growth has been reported rendering its exact role in tumorogenesis elusive.

Under pathological conditions, the lumican protein is highly expressed [13] and due to direct interactions with cell membrane proteins it may influence key biological processes, such as cell proliferation, adhesion and migration [11,14], cell growth and apoptosis [14]. Previously, we have shown that the inhibition of cell migration by lumican depends on a direct binding between the core protein of lumican and the α2β1 integrin [15]. In this study, we provide evidence

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that this mechanism may be more complex and in addition to blocking integrins, lumican affects expression of metalloproteinases such as MMP-14 and MMP-9. Furthermore, we show that lumican blocks pro-angiogenic activity of endothelial cells to the same extent as siRNA to β1 mRNA and produces the synergistic effect when used together with anti-β1 siRNA.

Material and methods

Reagents

All standard tissue culture reagents including Dulbecco's modified Eagle's medium, fetal bovine serum, and Lipofectamine 2000 reagent were from Invitrogen (Eggenstein, Germany). Enhanced Chemiluminescence (ECL) Western blotting substrate and BCA Protein Assay Kit were obtained from PIERCE (Rockford, IL). Horseradish peroxidase-conjugated goat anti-mouse antibody was purchased from Jackson ImmunoResearch (West Grove, PA). All synthetic short interference RNA duplexes were from Dharmacon (Lafayette, CO). All other reagents, except where noted, were from Sigma.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from freshly collected umbilical cords by collagenase treatment according to the previously established protocol [10]. Cells were cultured in M199 (Sigma) medium supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml), heparin (90 μg/ml), L-glutamine (1 mM), sodium bicarbonate (2 mg/ml), 20% fetal bovine serum (FBS), and epidermal growth factor (EGF, 15 ng/ml). Primary cultures were harvested at confluence with trypsin/EDTA and transferred into gelatin-coated dishes. Primary cultures between passage three and four were used in the experiments. The human endothelial cell line EA.hy926 was derived by fusion of human umbilical vein endothelial cells with continuous human lung carcinoma cell line A549. The cells were cultured in growth medium DMEM (Sigma) containing HAT (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine) and 10% fetal bovine serum. Cells were harvested at confluence with trypsin/EDTA and transferred into cell culture treated dishes. Culture flasks were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Zymography

Cell lysates (10 μl) were added to non-reducing sample buffer (1/3, v/v) and loaded on to 10% SDS–polyacryamide gels containing copolymerized gelatin (1 mg/ml), and electrophoresis was performed at 75 V for 0.5 h then at 100 V for 2 h at 4 °C. The gels were then rinsed twice with 2.5% Triton X-100 to remove SDS and renature the MMPs. After a brief wash in distilled water gels were incubated overnight at 37 °C in 50 mM Tris buffer, pH 7.6 containing 5 mM $CaCl_2$, 200 mM NaCl and Brij 35 (0.02%) with gentle agitation. The gels were stained with Coomassie® Brillant Blue R-250 (Sigma) and MMP activities were detected as transparent bands on the blue background.

Western immunoblotting

The protein content of lysates was determined by the BCA method [16]. Cell lysate aliquots containing 20 μg protein were boiled with 4x concentrated sample buffer and separated by SDS PAGE [17] then electrophoretically transferred to nitrocellulose membrane (0.45 μm pore size). β1 integrin subunit, MMP-2, MMP-9, and MMP-14 were detected with monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and then the level of β-actin was detected with rabbit polyclonal antibody (Abcam, Cambridge, MA). Immunodetection was accomplished using the enhanced chemiluminescence kit (ECL Kit, PIERCE), then films were scanned and protein bands quantitated using the Gel-Image system (Pharmacia LKB, Midland, Canada). To quantify the densitometric scans, the background was subtracted and the area for each protein peak was determined.

Capillary tube formation between Fibrin II Gels or in Matrigel™

Fibrinogen (1.5 mg/ml) was dialyzed into 0.05 M Tris, 0.15 M NaCl and passed through collagen immobilized on Sepharose 4B to remove fibronectin. To make the underlying fibrin gel, 250 μl of fibrinogen solution was placed into each well of a 24-well culture plate, and human thrombin (100 units/ml) was added to a final concentration of 2 units of thrombin/mg of fibrinogen. After the gels were polymerized (at least 5 min at 37 °C), 1-ml aliquots of EA.hy926 cells (5×10^5cells/ml), suspended in serum-free endothelial cell basal medium supplemented as indicated above, were seeded onto each well. After 24 h, unattached cells were aspirated, and the same procedure was used to generate a second fibrin II gel overlying the apical surface of the cells. This fibrin II gel was allowed to polymerize for 5 min at 37 °C, and then 1-ml aliquots of fresh, supplemented, serum-free endothelial cell basal medium were added to each well. Tube formation was assessed at several different focal planes through the gel. The extent of capillary tube formation was judged in relation to the amount of endothelial cell monolayer and to the number, width, and length of the tubes formed.

Wells of a 48-well plate were coated with Matrigel™ according to the manufacturer's instructions (Becton Dickinson, Bedford, MA) and were incubated at 37 °C for 30 min. HUVECs or EA.hy926 cells were grown in 6-well dishes in the presence of lumican (25 μg per cm^2), siRNA to β1 mRNA (40 pmol/ml) mixed with lipofectamine (5 μg/ml) or MMPs inhibitors for 24 hours. The following inhibitors were used: TIMP-1, -2, or −3 (1 μg/ml), MMP-2 inhibitor II (3 μM), MMP-9 inhibitor (1 μM), GM6001 (Ilomastat, 25 μM), ε-amino capronic acid (1 mg/ml). Then, they were detached with 1 mM EDTA, sedimented by centrifugation for 5 min and resuspended in cell culture medium. HUVECs or EA.hy926 cells were added to Matrigel™-coated wells and incubated for 24 hours. Photomicrographs at 100 x magnification were taken using a digital camera Photo CL50 AGFA attached to a Nikon TMS-F microscope and average cell length was measured for at least 100 cells.

Matrigel™ plug assay

Matrigel™ plug assays were performed as described previously with modifications [18]. BALB/c mice were anesthetized and injected at the abdominal midline with 0.5 ml of Matrigel™ (n = 12) supplemented with 500 ng of bFGF (R&DSystems, Minneapolis, MN). Control mice were injected with Matrigel™ without added growth factor or with acetic acid in place of lumican. A single dose of lumican (5 μg dissolved in 18 mM acetic acid) was administered with Matrigel™ during formation of plugs (0 time). Three doses of lumican (5 μg each) were administered every 3 days starting 72 h after Matrigel™ injection. Matrigel™ plugs were removed 3 days after last injection and photographed. Vascularization of plugs was determined by measuring hemoglobin concentration by Drabkin's reagent (Sigma, St Louis, MO) and calculated per 100 mm^3.

Data analysis

We have analyzed the statistical significance of our data using paired Student's *t* test. Data are expressed as means ± S.D.

Results

Since lumican *via* direct interaction with the I domain of the α2 integrin subunit can inhibit cell migration [15] and α2β1 integrin receptor plays a key role in angiogenesis, the goal of these experiments