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SCIENCE DIRECT

Experimental Cell Research 303 (2005) 22–31

Experimental Cell Research

www.elsevier.com/locate/yexcr

Angiostatin directly inhibits human prostate tumor cell invasion by blocking plasminogen binding to its cellular receptor, CD26

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Received 2 July 2004, revised version received 17 September 2004
Available online 26 October 2004

Abstract

Previous studies demonstrate that one of the six plasminogen type 2 glycoforms, plasminogen 2ε, enhances invasiveness of the 1-LN human prostate tumor cell line in an in vitro model. Binding of plasminogen 2ε to CD26 on the cell surface induces a Ca^{2+} signaling cascade which stimulates the expression of matrix metalloproteinase-9, required by these cells to invade Matrigel®. We now report that angiostatin, a fragment derived from plasminogen which prevents endothelial cell proliferation, is also a potent, direct inhibitor of 1-LN tumor cell invasiveness. We studied the effect of individual plasminogen 2 glycoform-derived angiostatins and found that only angiostatin 2ε binds to CD26 on the surface of 1-LN cells at a site also recognized by plasminogen 2ε. As a result, the plasminogen 2ε-induced Ca^{2+} signaling cascade is inhibited, the expression of matrix metalloproteinase-9 is suppressed, and invasion of Matrigel® by 1-LN cells is blocked. Angiostatin 2ε is also the only angiostatin glycoform which is able to inhibit in vitro endothelial cell proliferation and tubule formation. These studies suggest that, in addition to its ability to inhibit tumor vascularization, angiostatin 2ε may also directly block tumor metastasis.

Keywords: Angiostatin and prostate cancer; Plasminogen receptors; CD26 and angiostatin binding; Angiostatin glycoforms; Tumor invasion and angiostatin; Angiogenesis

Introduction

Invasion of tumor cells into the basement membrane requires proteolytic modification of proteins of the extracellular matrix (ECM). In this process, plasminogen (Pg), urinary-type Pg activator (u-PA), and a variety of matrix metalloproteinases (MMPs), including matrix metalloproteinase-9 (MMP-9), are essential for tumor growth and dissemination [1–5]. We recently reported that Pg type 2 (Pg 2) plays an important role in the regulation of expression of MMP-9 in the highly invasive 1-LN human prostate tumor cell line via binding to CD26 (dipeptidyl peptidase IV) on the cell-surface [6]. Pg 2 is composed of six glycoforms that differ in their degree of sialylation [7]; however, only one of these glycoforms, Pg 2ε, is able to significantly stimulate expression of MMP-9 via a Ca^{2+}-dependent signaling cascade in 1-LN cells [6]. As a consequence, the ability of 1-LN cells to invade the synthetic basement membrane Matrigel® is greatly enhanced [6]. Angiostatin, a kringles 1–4 fragment of Pg, is a potent inhibitor of angiogenesis, tumor growth, and metastasis [8,9]. MMP-9 can generate angiostatin from Pg, and their expression in the peritumoral environment may serve to regulate angiogenesis, thereby controlling tumor growth [10].

The in vivo generation of angiostatin has been proposed to follow a sequential order of events beginning with conversion of Pg to plasmin (Pm), followed by reduction of Pm by disulfide reductases, then serine proteinase-dependent release of kringles 1–4 1/2, and finally matrix metalloproteinase-dependent trimming of kringles 1–4 1/2 to either kringles 1–4 or 1–3 [11]. Pg binds via its 2,3-linked sialic acid residues

Abbreviations: Pg, plasminogen; Pm, plasmin; MMP, matrix metalloproteinase; CD26, dipeptidyl peptidase IV; u-PA, urinary-type plasminogen activator; HUVEC, umbilical vein endothelial cell.

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doi:10.1016/j.yexcr.2004.09.008

attached to Thr^{345} *O*-linked carbohydrate chains to CD26 [12]. Pg also binds via its kringle 4 to adenosine deaminase in complex with CD26 [13]. Both these interactions with CD26 result in an augmented Pm generation which, in addition to facilitating cell invasion, may also stimulate generation of angiostatin directly on the cell surface.

While tumor growth is highly dependent on angiogenesis, tumor metastasis also depends on the direct interaction of these cells with matrix components of the vessel wall. It has been suggested that angiostatin may control tumor invasive activity by blocking matrix-enhanced Pg activation [14]; however, a direct effect of this molecule on tumor cells has not been reported. In the present study, we assessed the efficacy of angiostatin (Pg kringles 1–3) as an inhibitor of 1-LN cell invasiveness stimulated by Pg 2. We studied single Pg 2-derived angiostatins and found that only angiostatin 2ε was able to significantly inhibit invasiveness of 1-LN cells stimulated by Pg 2ε. Direct binding experiments demonstrated that angiostatin 2ε binds to CD26 on the surface of these cells at a site also recognized by Pg 2ε. As a result, angiostatin 2ε inhibits the Pg 2ε-induced Ca^{2+} signaling cascade required for stimulation of the expression of MMP-9 by 1-LN cells [6]. We also evaluated the effect of Pg 2-derived angiostatins on umbilical vein endothelial cell (HUVEC) growth and tubule formation and show that only angiostatin 2ε is able to inhibit both processes. In addition to inhibiting angiogenesis, these results suggest an alternative, direct mechanism by which angiostatin, via down-regulation of MMP-9 expression, might regulate human tumor cell invasion and migration.

Materials and methods

Purification of proteins

Pg was purified from human plasma by affinity chromatography on L-lysine-sepharose [15] and separated into its two major classes of glycoforms, Pg 1 and Pg 2, by affinity chromatography on concanavalin A-sepharose [16]. Fractionation of Pg 2 into its six individual glycoforms using chromatofocusing on a mono P column linked to an FPLC system was performed as described previously [7]. Isoelectric focusing was employed to demonstrate purification to apparent homogeneity as in the previous report [7]. Single Pg 2 glycoform-derived angiostatins (kringles 1–3) were prepared by digestion of each glycoform with elastase and then purified to homogeneity employing a combination of gel filtration on G-75 Sephadex and affinity chromatography on L-lysine-sepharose as described previously [17]. Purity was confirmed by isoelectric focusing. Since Pg 2ϕ does not bind to 1-LN cells and is only a minor glycoform [6,7], it was not purified for study. Desialylation of total angiostatin, containing the six glycoforms, was performed with VC-neuraminidase (Sigma, St. Louis, MO) as described previously [18]. Each angiostatin glycoform was filtered through ENDOTRAP® mini-columns (Boca Scientific, Boca Raton, FL) for endotoxin removal. Radioiodination was carried out by the method of Markwell [19]. Radioactivity was measured in a Pharmacia LKB Biotechnology 1272 γ-radiation counter (Pharmacia, Rockville, MD). Incorporation of ^{125}I was at a level of 8×10^6 cpm/nmol of protein. ^{125}I-labeled angiostatin was repurified by affinity chromatography on L-lysine-sepharose and then employed for the binding experiments.

Protein sequence analysis

The proteins (100 pmol) were sequenced by automated Edman degradation in a gas/liquid phase sequencer (Model 477A; Applied Biosystems, Inc., Foster City, CA) with on-line phenylthiohydantoin analysis using HPLC (Model 120A, Applied Biosystems). The instruments were operated as recommended in the user bulletins and manuals distributed by the manufacturer.

Cell cultures

The human prostate tumor cell line 1-LN was grown in RPMI 1640 supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin G and 100 g/ml streptomycin, as previously described [6]. The 1-LN cell line was a kind gift of Dr. Philip Walther, Department of Urology, Duke University Medical Center.

Ligand-binding analysis

Cells were grown in tissue culture plates until the monolayers were confluent. Prior to use in binding assays, the cells were washed in Hanks balanced salt solution (HBSS). All binding assays were performed at 4°C in RPMI 1640 containing 2% (w/v) bovine serum albumin (BSA). Increasing concentrations of ^{125}I-labeled angiostatin 2ε were incubated with cells for 60 min in 48-well or 96-well culture plates. Free ligand was separated from bound ligand by aspirating the incubation mixture and washing the cell monolayers rapidly three times with RPMI 1640 containing 2% BSA. The cells were then lysed with 0.1 M NaOH, and bound radioactivity was determined in a Pharmacia LKB Biotechnology 1272-γ counter. Molecules of ligand bound were calculated after subtraction of nonspecific binding measured in the presence of 100 μM unlabeled angiostatin 2ε. Estimates of dissociation constant (K_d) values and maximal binding (B_{max}) were determined by fitting data directly to the Langmuir isotherm using the statistical program SYStat® for Windows.

Cross-linking of Pg 2ε and angiostatin 2ε to 1-LN cell membrane proteins

Cell monolayers cultured on 150 cm^2 Falcon flasks were rinsed with serum-free RPMI 1640 culture medium and gently detached with a plastic scraper. Cell suspensions