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Plasmin-clipped β_2-glycoprotein-I inhibits endothelial cell growth by down-regulating cyclin A, B and D1 and up-regulating p21 and p27

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ABSTRACT

β_2-Glycoprotein-I ($\beta_2$gpI), an abundant plasma glycoprotein, functions as a regulator of thrombosis. Previously, we demonstrated that plasmin-clipped $\beta_2$gpI (c$\beta_2$gpI) exerts an anti-angiogenic effect on human umbilical vein endothelial cells (HUVEC). The present study was focused on the molecular background responsible for this phenomenon. c$\beta_2$gpI strongly reduced HUVEC growth and proliferation as evidenced by the MTT and BrdU assay and delayed cell cycle progression arresting HUVEC in the S-and G2/M-phase. Western blot analysis indicated that c$\beta_2$gpI inhibited cyclin A, B and D1, and enhanced p21 and p27 expression. Activity of p38 was down-regulated independently from the c$\beta_2$gpI incubation time. Phosphorylation of ERK1/2 was not changed early (30 and 60 min) but became enhanced later (90 min, 4 h). JNK activity was reduced rapidly after c$\beta_2$gpI treatment but compared to controls, increased thereafter. Annexin II blockade prevented growth inhibition and cell cycle delay evoked by c$\beta_2$gpI. We assume that c$\beta_2$gpI's effects on HUVEC growth is mediated via cyclin A, B and D1 suppression, up-regulation of p21 and p27 and coupled to modifications of the mitogen-activated protein (MAP) kinase signalling pathway. c$\beta_2$gpI may represent a potential endogenous angiogenesis-targeted compound, opening the possibility of a novel tool to treat cancer.

1. Introduction

β_2-Glycoprotein-I ($\beta_2$gpI; syn. apolipoprotein H) is a single-chain plasma glycoprotein composed of 326 amino acid residues with four homologous repeated units of 60 amino acids (domains I through IV) and a distinct C-terminal kringle domain (domain V). Domain V carries a lysine-rich sequence motif that binds negatively charged molecules and a hydrophobic loop that embeds the protein into anionic lipid-containing target membranes [1].

Due to these properties, the protein inhibits ADP-induced platelet aggregation and competes for the assembly of coagulation cascade proteins on procoagulant cell surfaces. $\beta_2$gpI is involved in lipid metabolism, atherosclerosis and clearance of apoptotic cells [2–4]. Other studies have shown that $\beta_2$gpI binds with high affinity to endothelial cells, acting as an endothelial cell viability factor [5]. Furthermore, $\beta_2$gpI has been identified as the primary target antigen recognized by autoantibodies in patients with the antiphospholipid syndrome [6].

Very recently, we discovered that treatment of $\beta_2$gpI with plasmin resulted in a clipped version of $\beta_2$gpI (c$\beta_2$gpI) with novel biological properties. c$\beta_2$gpI, but not $\beta_2$gpI

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strongly inhibited endothelial cell growth and tube formation, mediated by endothelial annexin II surface receptors. This points to distinct anti-angiogenic properties of $c\beta_2$gpI [7]. Thus, $c\beta_2$gpI may represent a potential endogenous anti-tumor compound, opening the possibility of a highly efficient tool to treat cancer.

The anti-angiogenic characteristics of $c\beta_2$gpI have meanwhile been corroborated by others [8,9]. However, the molecular background responsible for blocking angiogenesis is still a matter of debate. We, therefore, analyzed the influence of $c\beta_2$gpI versus $\beta_2$gpI on the endothelial cell cycle, on the expression of cell cycle regulating proteins and on activation of the mitogen-activated protein (MAP) kinase signalling pathway.

2. Materials and methods

2.1. Endothelial cells

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical veins and harvested by enzymatic treatment with chymotrypsin. HUVEC were grown in Medium 199 (M199; Biozol, Munich, Germany), supplemented with 10% fetal calf serum (FCS, Gibco, Karlsruhe, Germany), 10% pooled human serum, 20 μg/ml endothelial cell growth factor (Boehringer, Mannheim, Germany), 0.1% heparin, 100 ng/ml gentamaycin, and 20 mM HEPES-buffer (pH 7.4). To control the purity of HUVEC cultures, cells were stained with fluorescein isothiocyanate (FITC)-labelled monoclonal antibody against Factor VIII-associated antigen (Von Willebrand factor; clone F8/86; Dako, Hamburg, Germany) and analyzed microscopically or by FACscan (Becton Dickinson, Heidelberg, Germany; FL-1H (log) channel histogram analysis; 1×10^4 cells/scan). Cell cultures with a purity >95% were serially passaged. Subcultures from passages 2 to 4 were selected for experimental use. Mouse hemangioendothelioma EOMA cells (LGC Promochem, Wesel, Germany) were maintained in DMEM supplemented with 10% FCS, 100 ng/ml gentamycin, 20 mM HEPES-buffer and 1% penicillin/streptomycin.

2.2. Plasmin digest of human β_2-glycoprotein I

Human $\beta_2$gpI was derived from CellSystems Biotechnology (St. Katharinen, Germany). As described in an earlier publication [7] $\beta_2$gpI (50 μg) was incubated with 5.3 μg plasmin, with a molar ratio of plasmin to substrate of 1–15. The reaction took place in serum deprived HUVEC medium at 37 °C for 2 h and was terminated by snap freezing the digests in dry ice. In all experiments, effects of plasmin digested $\beta_2$gpI ($c\beta_2$gpI) were compared with effects of non-digested material, i.e. (a) untreated $\beta_2$gpI, (b) plasmin, (c) control medium.

2.3. Purification of $c\beta_2$gpI

Untreated and plasmin treated $\beta_2$gpI samples were purified using a Hi-Trap Heparin-Sepharose affinity column according to the protocol described elsewhere [9]. The protein samples were loaded on the column in 50 mM Tris, pH 8.0, and 20 mM NaCl. The elution was performed using a linear NaCl gradient from 20 mM to 500 mM in 50 mM Tris, pH 8.0, and 20 mM NaCl, with a flow rate of 0.5 ml/min.

2.4. Annexin II blockade

For blocking studies, an anti-annexin II monoclonal antibody was used (clone 5; Becton Dickinson). Prior to use, 200 μl antibody solution was dialysed for 5 h using a Slide-A-Lyzer cassette (Perbio Science, Bonn, Germany). This was necessary to remove NaN_3 and to prevent toxic effects which might have been evoked by this chemical adjuvant. The antibody was finally diluted 1:5 or 1:10 in HUVEC medium.

2.5. Endothelial cell proliferation assay

HUVEC and EOMA cells were adapted to medium containing 2% fetal bovine serum (Endothelial Cell Basal Medium, Cambrex, Apen, Germany), and then seeded onto 6-well plates (0.5×10^6 cells/ml) in the presence of test materials containing (a) untreated $\beta_2$gpI, (b) $\beta_2$gpI treated with plasmin ($c\beta_2$gpI), (c) plasmin, (d) culture medium without supplements (control). 24 h after incubation, cell growth was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay (Roche Diagnostics, Penzberg, Germany). HUVEC (0.5×10^4 cells/well) were seeded onto 96-well culture plates and incubated as described above. After 24 h, MTT (0.5 mg/ml) was added for an additional 4 h. Thereafter, cells were lysed in a buffer containing 10% SDS in 0.01 M HCl. The plates were allowed to stand overnight at 37 °C, 5% CO_2. Absorbance at 570 nm was determined for each well using a microplate ELISA reader. After subtracting background absorbance, results were expressed as cell number. Cell proliferation was measured using a BrdU cell proliferation enzyme-linked immunosorbent assay (ELISA) kit (Calbiochem/Merck Biosciences, Darmstadt, Germany). HUVEC or EOMA cells, seeded onto 96-well microtitre plates, were incubated with 20 μl BrdU-labeling solution per well for 8 h, fixed and detected using anti-BrdU mAb according to the manufacture's instructions. Absorbance was measured at 450 nm.

2.6. In vitro angiogenesis assay

The formation of tubular like structures was assessed using EOMA cells. EOMA were treated with (a) $\beta_2$gpI, (b) $c\beta_2$gpI, (c) plasmin, (d) culture medium without supplements (control) and then transferred to 24-well culture plates which had been precoated before with basement membrane matrix (Matrigel, BD Biosciences, Heidelberg, Germany; 250 μl/well). After 24 h at 37 °C, tube formation was monitored by an inverted phase contrast microscope.