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Intra- and extracellular plasminogen activator inhibitor-1 regulate effect of vitronectin against radiation-induced endothelial cell death

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ABSTRACT

Plasminogen activator inhibitor-1 (PAI-1) is induced by radiation resulting in endothelial cell impairment, potentially leading to multiple organ failure. Vitronectin (VN) is a 75-kDa glycoprotein (VN_{75}) cleaved into two forms (VN_{75} or $VN_{65/10}$) by furin, which is regulated by intracellular PAI-1. VN protects against radiation-induced endothelial cell death, but the mechanisms involved in VN processing and its interactions with intra- and extracellular PAI-1 remain unclear. We examined these processes in cells *in vitro* using recombinant proteins or overexpression of VN and PAI-1 genes, including furin-susceptible (T^{381}) and furin-resistant VN (A^{381}). VN processing was analyzed using a mutant PAI-1 with relatively weaker binding to VN. VN function was evaluated by survival of radiation-damaged endothelial cells. Wild-type, but not mutant PAI-1 inhibited furin-dependent VN processing. Gene transfer revealed that furin-susceptible VN was processed more than the furin-resistant form, but processing of both was inhibited by PAI-1 overexpression. Intracellular PAI-1 formed a complex with VN_{75} (T^{381}) in cells and media, and the VN_{75} form was secreted preferentially. Only VN_{75} protected against radiation-induced endothelial cell death, in which its effect was abolished by wild-type but not mutant PAI-1. These findings indicate that intracellular PAI-1 inhibits VN processing and protects against radiation-induced endothelial cell death.

1. Introduction

Plasminogen activator inhibitor-1 (PAI-1) is a primary regulator of tissue-type and urokinase-type plasminogen activators, with important effects on cell adhesion, migration, and apoptosis [1,2]. PAI-1, as an acute-phase protein and with a virtually silent gene, is not expressed in normal tissues, but is rapidly produced and released primarily from the liver, adipose tissue, and vasculature in response to a variety of signals released during trauma, inflammation, and sepsis [3]. PAI-1 can occur in an active or latent form in plasma [4,5], and active PAI-1 is metastable and converts spontaneously into latent PAI-1 [5,6]. Vitronectin (VN) is thought to prolong the half-life of PAI-1 and alter its functions, as well as stabilizing and maintaining active PAI-1 [3,7,8]. However, the nature of the interactions between PAI-1 and VN remain unclear, partly because of the unusual properties of PA-1, including being a trace protein, its short half-life, and its rapid induction by diverse molecules, as well as methodologic problems [1,9,10].

Excessive PAI-1 expression has been suggested to exacerbate numerous diseases through initial impairment or apoptosis of endothelial cells [11]. Serum PAI-1 may be a pathogenetic factor for endothelial damage, and may also act as a diagnostic or prognostic marker for diseases involving endothelial damage, such as sepsis, hepatic sinusoidal endothelial cell obstruction syndrome (SOS), thrombotic microangiopathy, and radiation-induced gastrointestinal syndrome (GIS), which frequently occurs following radiation-based conditioning in patients undergoing hematopoietic stem cell transplantation [12–14]. Radiation also increases PAI-1 expression levels and induces apoptosis in endothelial cells, resulting in SOS and GIS. These diseases of vascular endothelial impairment may become severe, resulting in multiple organ failure associated with high mortality. Furthermore, there are currently no standard treatments for these conditions. Protecting against endothelial cell damage is therefore crucial to preventing these critical pathologies. We previously demonstrated a novel function of VN in protecting against radiation-induced human umbilical vein endothelial cell (HUVEC) death through upregulation of p21 and blockade of extracellular signal-regulated kinase and p38 mitogen-activated protein kinase signaling pathways [15]. This suggested that VN released from hepatic cells may protect against radiation-induced endothelial cell death or injury, such as SOS and GIS.

VN contains binding sites for PAI-1, integrin, and urokinase-plasminogen activator receptor in its N-terminal domain, and sites for heparin, complements, perforin, and a second PAI-1 binding site in its C-terminal domain [16,17]. VN exerts pleiotropic functions by interacting with

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these cofactors, and is implicated in a number of pathophysiologic processes, including migration, angiogenesis, thrombosis, tissue repair, and survival [1,18–21]. VN is mostly produced in the liver, but not in endothelial cells, and the liver also produces plasma PAI-1 [1,22]. A VN polymorphism (methionine to threonine substitution at position 381 in the C-terminal domain) results in cleavage by furin at Arg^{379}-Ala^{380} to generate a disulfide-linked two-chain $VN_{65/10}$ [23–25]. Furin, which is abundantly expressed in the liver and present in systemic cells [22], is a master regulator converting latent precursors into active proteins, and shuttling between the Golgi network and the cell surface [26]. Furin activity is regulated by serine protease inhibitors, including proteinase inhibitor 8 and the recently defined PAI-1 [22,23,27,28]. Intracellular PAI-1 levels may thus determine the form of VN secreted from the liver into the plasma. Conversion of VN_{75} to $VN_{65/10}$ may modify its affinity for cooperating factors and thereby change its biologic properties in extracellular spaces. Indeed, a previous study demonstrated that $VN_{65/10}$ increased the risk of hemangioblastoma in patients with von-Hippel-Lindau gene defects [29]. Although numerous studies have examined the mutual interactions between PAI-1 and VN in the extracellular matrix and plasma [1,18], few have investigated intracellular PAI-1 functions [30,31], and the intracellular interactions between PAI-1 and VN have not been reported.

In this study, we analyzed the role of intracellular PAI-1 in furin-dependent VN processing in relation to radiation in hepatic cells using recombinant proteins. We also investigated the roles of furin and intracellular PAI-1 in intracellular VN processing in VN- and/or PAI-1-overexpression assays using furin-resistant and furin-susceptible forms of VN genes in HEK293 cells, which express furin but not endogenous PAI-1 or VN. We finally compared the inhibitory effects of VN_{75} and $VN_{65/10}$ against radiation-induced endothelial cell death in HUVECs. The results of these experiments will provide basic insights to help prevent vascular endothelial-damage diseases such as SOS and GIS, and contribute to the development of new therapeutic agents for these conditions.

2. Materials and methods

2.1. Reagents

Human recombinant VN (rVN) and PAI-1 (rPAI-1) were purchased from Wako (Osaka, Japan), and human recombinant furin was obtained from R&D Systems (Boston, MA, USA). Antibodies against furin, VN, and PAI-1 were obtained from Santa Cruz Biotechnology (Dallas, TX, USA), BD Biosciences (Tokyo, Japan), and Abcam (Burlingame, CA, USA), respectively. Fluorescence-labeled antibody against integrin αv/β3 was obtained from Cell Signaling Technology (Tokyo, Japan). Small interference RNAs (siRNAs) against furin and integrin αv were purchased from Santa Cruz Biotechnology.

2.2. Cell culture

Hep G2 cells (DS Pharma Biomedical, Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium without 10% heat-inactivated fetal bovine serum (FBS), and HEK293 cells (RIKEN Cell Bank, Tsukuba, Japan) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated (FBS and 1% non-essential amino acids (Invitrogen, Carlsbad, CA, USA). HepG2 with production of VN and PAI-1 is a perpetual cell line which was derived from the liver tissue. HEK293 without production of VN and PAI-1 is a cell line originally derived from human embryonic kidney cells. Collagen type I-coated dishes (35-mm diameter; IWAKI, Japan) were used for HUVECs (Health Science Resource Bank, Tokyo, Japan) [15], which were maintained in MCDB107 (Cosmo Bio, Tokyo, Japan) supplemented with 15% heat-inactivated FBS, penicillin/streptomycin (Invitrogen), 10 ng/ml basic fibroblast growth factor (Sigma-Aldrich, Tokyo, Japan), and 50 μg/ml heparin. Cells were incubated in a humidified atmosphere at 37 °C with 5% CO_2. All cell lines were used in experiments within passage five.

2.3. Preparation of VN and VN mutants

We prepared VN (T^{381}) with a threonine at position 381, which is highly susceptible to furin, and VN (A^{381}) with a methionine at this position, which is resistant to furin [23,24]. Human VN cDNA was amplified by polymerase chain reaction using previously described primer sets [24]. Polymerase chain reaction amplification was performed in a Thermal Cycler with an Ex Taq (Takara Bio, Shiga, Japan) under the following cycle conditions: an initial denaturation step at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 45 s, extension at 72 °C for 45 s, and a 10-min incubation at 72 °C after completion of the final cycle. The single A381T substitution was created using a Quick Change site-directed mutagenesis kit (Agilent Technology, Santa Clara, CA, USA). Polymerase chain reaction fragments were introduced into a pDONR221 vector for gene cloning, in accordance with the instructions for GATEWAY Cloning Technology (Life Technology, Tokyo, Japan), and confirmed by sequencing. Each gene was then transferred from its entry clone by recombination into the pT-Rex™-DEST31 vector (Life Technology).

2.4. Preparation of PAI-1 and PAI-1 mutant proteins

Human PAI-1 cDNA was purchased from OriGene (Rockville, IL, USA). We created the Q123K mutant [32] using GATEWAY system Cloning Technology (Invitrogen). Wild-type PAI-1 ($PAI\text{-}1_{wt}$) and mutant PAI-1 ($PAI\text{-}1_{Q123K}$) genes were transferred from the entry clone by recombination into a pDEST17 vector, which is an N-terminal fusion vector containing an ATG initiation codon upstream of a sequence encoding a His6 tag. $PAI\text{-}1_{Q123K}$ reveals weaker binding to VN compared to $PAI\text{-}1_{wt}$. Recombinant PAI-1 proteins such as $PAI\text{-}1_{wt}$ and $PAI\text{-}1_{Q123K}$ were generated in *Escherichia coli*. The supernatant from the bacterial culture lysates was applied to a Ni^{2+}-nitrilotriacetic acid affinity column and washed with the same buffer supplemented with 20 mM imidazole. Elution was carried out using the same buffer containing 200 mM imidazole. The protein concentration was determined according to the Bio-Rad Protein Assay, and purity was assessed by Coomassie brilliant blue-stained reducing sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE).

2.5. Transfection of VN and PAI-1 genes

We investigated the relationship between intracellular PAI-1 and VN processing in HEK293 cells transfected with VN and/or PAI-1 genes cloned into pT-Rex-DEST31 using Lipofectamine 2000 (Life Technology). HEK293 cells express furin, but not VN or PAI-1, thus avoiding any effects of endogenous VN/PAI-1 on transfected gene interactions. In contrast, HepG2 cells constantly express VN, furin, and a small amount of PAI-1. An empty-gene plasmid was transfected to maintain the DNA content. Control transfections were carried out using a green fluorescent protein (GFP) gene plasmid. Culture media and cells were harvested and stored after 12, 24, 36, and 60 h. Expressed proteins were evaluated by immunoblotting. Total VN and active PAI-1 concentrations in the culture media were determined using human Vitronectin Total Antigen and human PAI-1 ELISA kits (Innovate Research, Novi, MI, USA). HepG2 cells and HUVECs were transfected with siRNAs (furin and integrin αv) using Lipofectamine 2000 to investigate a relationship among furin, PAI-1, VN forms, and radiation.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting analysis

SDS-PAGE and western blotting procedures were followed as described previously [33]. Briefly, harvested cells or culture media were lysed directly in Laemmli sample buffer, subjected to SDS-PAGE, and transferred to polyvinylidene difluoride membranes. The membranes were blocked and probed with primary antibodies and peroxidase-conjugated secondary antibodies, followed by detection using an ECL Western