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# Vitronectin improves cell survival after radiation injury in human umbilical vein endothelial cells

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ABSTRACT

**Vitronectin (VN) is a multi-functional protein involved in extracellular matrix (ECM)-cell binding through integrin receptors on the cell surface, which is an important environmental process for maintaining biological homeostasis. We investigated how VN affects the survival of endothelial cells after radiation damage. VN attenuated radiation-induced expression of p21, an inhibitor of cell cycle progression, and selectively inhibited Erk- and p38 MAPK-dependent p21 induction after radiation exposure through regulation of the activity of GSK-3β. VN also reduced the cleavage of caspase-3, thereby inhibiting radiation-induced apoptotic cell death. These results suggest that VN has important roles in cell survival after radiation damage.**



## 1. Introduction

After accidental exposure to a high dose of ionizing radiation (IR), therapeutic strategies for patients with acute radiation syndrome (ARS) remain a major problem. At relatively high doses, gastrointestinal and vascular syndromes emerge in a dose-dependent manner, which lead to multi-organ dysfunction [1]. Moreover, damage to the target organ is related to systemic inflammatory response mainly caused by the release of cytokines from damaged vascular endothelial cells [1]. Vascular endothelial cells restrict the passage of inflammatory cells and circulating molecules into underling tissues. Following endothelial cell death after radiation exposure, para-cellular permeability is increased, which leads to unrestricted passage of circulating molecules and inflammatory cells and eventually triggers uncontrollable inflammation and further tissue damage [2]. Therefore, it is important to improve survival and to maintain the function of IR-damaged vascular endothelial cells in order to control and mitigate ARS.

A recent report showed that IR enhanced the expression of integrin αvβ3 in human umbilical vein endothelial cells (HUVECs), and HUVECs were sensitized to IR by blocking this receptor [3]. Vitronectin (VN) recognizes the integrin αvβ3 receptor on the cell surface, resulting in receptor clustering for the subsequent initiation of intracellular signaling pathways [4,5]. Therefore, it is reasonable to postulate that vascular endothelial cells demand binding of VN by its integrin receptor in order to sustain its own survival signaling after radiation damage.

VN is a cell-adhesive glycoprotein found in the extracellular matrix (ECM) of various tissues and in circulating blood, and VN is implicated in various pathophysiological processes including inflammation, cell migration, angiogenesis, and regulation of plasminogen activation [4]. More than 95% VN in plasma exists as a latent form (monomeric/dimeric form) and others in the ECM as multimeric forms [4]. The function and activity of VN strongly depend on its binding partners, such as plasminogen activator inhibitor 1 (PAI-1), heparin, or cell surface receptors including integrin receptors [4]. However, it remains unclear how abundant latent VN (concentration: 200–400 μg/mL) in plasma impacts on the fate of vascular endothelial cells after IR damage. Therefore, in this study, we investigated the effects and mechanisms of latent VN in plasma against IR-damaged HUVECs.

## 2. Materials and methods

### 2.1. Reagents

Human purified plasma VN protein was purchased from MILLIPORE (CC808, Lot NG1888899). Antibodies against GAPDH (#5174), GSK-3β (#9315), phosphoglycogen-synthase kinase-3β (GSK-3β) at Ser9 (#9336), p44/42 (extracellular signal-regulated kinase; ERK) kinase (#9102), phospho-ERK at Thr202/Tyr204 (#9101), p38 mitogen activated protein kinase (MAPK) (#9212), phospho-p38 MAPK at Thr180/Tyr182 (#9211), phospho-stress activated protein kinase/c-Jun-N-terminal kinase (SAPK/JNK) at Thr183/Tyr185 (#9251), AKT (#9272), AKT1 (#2938), AKT2 (#3063), phospho-AKT at Ser473

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(#4051), and poly (ADP-ribose) polymerase (PARP) (#9532) were purchased from Cell Signaling Technology. An antibody against SAPK/JNK (sc-571) was purchased from Santa Cruz Technology, an antibody against p21 (556431) was purchased from BD Bioscience, and an antibody against integrin αv/CD51 was purchased from EPITOMICS. Inhibitors of PI3K (LY294002), MEK1 (PD98059), p38 (SB203580), and JNK (JNK inhibitor II, Anthra[1,9-*cd*]pyrazol-6(2*H*)-one) were purchased from CALBIOCHEM. An inhibitor of GSK3-β (LiCl) was purchased from Wako.

### 2.2. Cell lines

HUVECs were purchased from the Health Science Research Resource Bank and maintained in MCDB107 (COSUMO BIO Co., Ltd.) supplemented with 15% heat-inactivated FBS, penicillin/streptomycin (Invitrogen), 10 ng/mL bFGF (Sigma), and 50 μg/mL heparin. Cells were incubated in a humidified atmosphere at 37 °C with 5% $CO_2$. Col-I coated dishes (11-018-002, IWAKI) were used for cell culture.

### 2.3. γ-Ray irradiation

Cells were irradiated using a Cesium-137 ($Cs^{137}$) gammator (model M Gammator, Irradiation Machinery), at a dose rate of 8.0 Gy/min on a rotating platform.

### 2.4. Cell cycle analysis

Cells were irradiated and cultured with or without VN (10 μg/mL) for 28 h. The harvested cells were treated with PBS containing 0.1% Triton X-100 (Wako) for 5 min on ice, and stained with propidium iodide (PI; 50 μg/mL, Sigma). An analysis of the cell cycle distribution was performed using a fluorescent cell analyzer (FACSCalibur, Becton Dickinson).

### 2.5. SDS–PAGE and western blotting analysis

Harvested cells were lysed in SDS sample buffer (300 mM Tris–HCl, pH 6.8, 10% SDS, 1% 2-mercaptoethanol, 1% glycerol) with sonication, and then heated for 7 min at 95 °C. Proteins were separated by SDS–PAGE and transferred onto PVDF membranes (Advantec Toyo). The membrane was reacted with each primary antibody in Tris-buffered saline (10 mM Tris–HCl, pH 7.4, 100 mM NaCl, 0.1% Tween-20) supplemented with 2% blocking agent (GE Healthcare) for 1 h at room temperature after blocking of the membrane. After the primary antibody reaction, membranes were labeled with each corresponding HRP-conjugated antibody (GE Healthcare) and then detected using ECL Prime western blotting substrate (GE Healthcare).

### 2.6. MAPK, AKT and GSK-3β inhibition assay

HUVECs with incubated with inhibitors; LY294002 (20 μM), PD98059 (50 μM), SB203580 (20 μM), JNK II (200 nM) and/or LiCl (20 mM), for 2 h at 37 °C before irradiation. Then, the cells were irradiated (8 Gy), and cultured for a further 28 h. Harvested cells were subjected to SDS–PAGE and immunoblotting analysis in accordance with the procedure described above.

### 2.7. Statistical analysis

The significance of differences between the control and experimental groups was determined using Student's *t*-test depending on the data distribution. Statistical analysis was performed using Excel 2003 software (Microsoft) with add-in software Statcel 2.

## 3. Results

### 3.1. Exogenously added latent vitronectin prevents γ-ray-induced cell death in HUVECs

Radiation-enhanced expression of integrin αv was detected in HUVECs by western blotting analysis (Fig. 1(A)), suggesting that cellular signaling from integrin affects the survival of radiation-damaged cells. Therefore, we examined whether VN, one of the ligands of integrin αvβ3, affects the survival of radiation-damaged HUVECs. As shown in Fig. 1(B), exogenously added latent VN increased the survival ratio of radiation-damaged HUVECs in a dose-dependent manner, which was also observed by recombinant VN treatments (Supplementary Fig. S2(A)). The addition of VN to HUVECs 10 min after irradiation inhibited the production of cleaved active-caspase3 and also inhibited the cleavage of a downstream substrate of caspase3, PARP (Fig. 1(C)). These results indicated that latent VN can prevent radiation-induced apoptotic cell death in HUVECs.

### 3.2. Vitronectin suppresses cell cycle arrest by inhibiting γ-ray-induced up-regulation of cell cycle inhibitor p21 in HUVECs

In general, radiation induces DNA damage and subsequent cell cycle arrest before activation of apoptotic cell death. Therefore, we next examined the effect of VN on cell cycle distribution of irradiated HUVECs by flow cytometric analysis. As shown in Fig. 2(A), radiation exposure induced cell cycle arrest at G1 and G2/M phase. However, VN treatment abolished G1 phase arrest, which was supported by a 1.3-fold increase in the G2/M population compared with radiation treatment alone (Fig. 2(A)). VN treatment also decreased the sub-G1 phase population by one-third, an indicator of apoptotic DNA fragmentation (Fig. 2(A)). These results again support the conclusion that VN treatment prevents radiation-induced apoptotic cell death in HUVECs. Because p21 is an inhibitor of the G1-S phase transition and its expression is induced by DNA damage, we determined whether VN affects DNA damage-induced p21 expression. As shown in Fig. 2(B), VN treatment decreased radiation-induced expression of p21 protein. Similarly, recombinant VN also suppressed p21 expression (Supplementary Fig. S2(B) and (C)). Although the levels of p53, a major transactivator of p21, also increased in response to DNA damage, p53 protein levels were unchanged by VN treatment (data not shown). These results suggest that other mechanisms may accommodate the regulation of p21 expression by VN treatment (Fig. 2(B)).

### 3.3. Vitronectin suppresses γ-ray-induced up-regulation of p21 through prevention of activation of ERK and p38 MAP kinase pathways in HUVECs

A number of reports indicate that signal transduction, such as the MAPK pathways, is involved in the regulation of p21 expression, although the involvement and effect of p21 expression depend on each cell type [6]. Therefore, we examined the involvement of the PI3K/AKT, ERK, p38 MAPK, and SAPK/JNK pathways in the radiation-induced p21 expression in HUVECs. For this purpose, we used chemical inhibitors, LY294002 for PI3K/AKT, PD98059 for ERK, SB203580 for p38 MAPK, and JNK inhibitor II for SAPK/JNK pathways. Except for the JNK inhibitor II, the other inhibitors, including LY294002, PD98059, and SB203580, prevented the radiation-induced expression of p21 in HUVECs (Fig. 3(A)). When pairs of inhibitors, including LY294002, PD98059, and SB203580, were added, the inhibitory effect was enhanced. Furthermore, treatment with all three inhibitors inhibited the expression of p21 much more strongly. Therefore, these results suggest the involvement of the PI3K/AKT, ERK, and p38 MAPK pathways in the radiation-induced p21 expression in HUVECs. We subsequently examined whether VN treatment affected the radiation-induced activation of the protein kinases in these signal transduction pathways.