혈관내피세포에서 Vascular Endothelial Growth Factor가 방사선에 의해 유도된 apoptosis에 미치는 영향

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국 문 초 록

혈관내피세포 성장 인자(Vascular endothelial growth factor, VEGF)는 혈관내피세포 특이하게 성장요인으로 작용하는 물질로 알려져 있다. 전리방사선에 대한 혈관내피세포의 효과는 정상조직에 대한 반응에 있어 아주 중요한 요소일 것으로 생각된다. 본 연구는 방사선 조사에 의해 배양시킨 혈관내피세포에서 apoptosis가 유도되는지, 유도가 된다면 VEGF에 의해 apoptosis가 억제되는지 그리고 apoptosis의 억제가 어떤 경로를 경유하는지를 실험하였다. 혈관내피세포에 방사선를 조사한 결과, 대조군에 비하여 선량이 증가함에 따라 apoptosis가 증가하였다. 같은 조건하에서 VEGF는 농도 의존적으로 apoptosis를 억제하였다. Antiapoptotic factor로서 VEGF가 어떤 신호 과정을 경유하는지를 밝히고자, 혈관내피세포에 방사선을 조사하여 apoptosis를 유도하면서 Flt-1과 Flk-1/KDR receptor를 처리하였다. 그결과 VEGF에 유도된 apoptosis 억제효과가 차단되었다. Phosphatidylinositol 3'-kinase(PI3-kinase) 특정 억제 물질인 Wortmanin과 LY294002를 방사선 조사한 혈관내피에 VEGF와 함께 처리했을 때 VEGF에 의해 유도된 apoptosis를 억제하였다. 이같은 결과는 VEGF가 방사선 조사로 일어나는 세포 치사를 억제하는 중요한 역할을 담당하며, In Vivo의 실험이 더 이루어져야 할 것으로 생각된다.

Vascular Endothelial Growth Factor Inhibits irradiation-induced Apoptosis in Human Umbilical Vein Endothelial Cells

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Abstract

Vascular endothelial growth factor (VEGF) has been identified as a peptide growth factor specific for vascular endothelial cells. In this study, we examined the effect of VEGF on radiation induced apoptosis and receptor/second messenger signal transduction pathway for VEGF effect in human umbilical vein endothelial cells (HUVECs). VEGF was found to protect HUVECs against the lethal effects of ionizing radiation by inhibiting the apoptosis induced in these cells by radiation exposure. VEGF (1-30 ng/ml) dose dependently inhibited apoptosi s by irradiation. Pre-treatment with Flt-1 and Flk-1/KDR receptor blocked the VEGF-in duced antiapoptotic effect. Phosphatidylinositol 3'-kinase (PI3-kinase) specific inhibitor, Wortman in and LY294002, blocked the VEGF-induced antiapoptotic effect. These data suggest that VEGF may play an important role in survival of HUVECs due to the prevention of apoptotic cell death caused by some stresses such as ionizing radiation.

I. Introduction

Vascular endothelium, a monolayer of cells lining the intima of the blood vessels, is involved in a variety of functions, including coagulation, vascular permeability, vascular tonus, and remodeling [1]. Endothelial cells, which are direct contact with plasma and cellular components of blood, are the targets of many cytokines and growth factors [2]. Vascular endothelial cells are important targets of radiation therapy and chemotherapy. It is suspected that the toxic response of the endothelium in normal tissues is a key limitation of these cancer treatment[3].

In more recent studies, in vitro radiation-induced damage and modification of endothelial functionality have been investigated on endothelial cells from other tissues. Part of these studies are showed induction of apoptosis [4, 5, 6], increased adhesion molecule expression [7, 8], augmented cytokine production [9, 10, 11], and increased production of von Willerbrand factor(VWF) [12, 13], increased KDR expression [14, 15].

Apoptosis, a strategic biologic process of eliminating unwanted cells, is involved in the regulation of cell number under physiological and certain pathological condition[16]. Apoptosis is associated with distinctive morphological and biological events, such as cellular shrinkage, nuclear condensation, and fragmentation of

chromatin caused by cleavage endonuclease at the internucleosomal linker region, yielding mono- and polynucleosomal DNA fragments. Among apoptosis-inducing agents, particularly ionizing radiation is sensitive to the effects of endothelial cells in vitro [17] and in vivo[18]. However, irradiation induces apoptosis in endothelial cells, thereby inducing vasomotor dysfunction[19] or reducing re-endothelialization[20]. Apoptosis induced by irradiation in vascular endothelial cells can be prevented by several growth factors and cytokines, such as, basic fibroblast growth factor (bFGF) [5, 18, 21], vascular endothelia growth factor (VEGF) [15, 19], endothelin-1 [22]. These molecules not only stimulate cell proliferation rate but also suppress apoptosis, thereby maintaining the surviving cell number.

VEGF has been identified as a peptide growth factor specific for vascular endothelial cells. VEGF not only has mitogenic activity for vascular endothelial cells but also has permeability-enhancing activity for blood vessels. Notably, VEGF exerts its antiapoptotic effect through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway after binding to its specific receptor, flt-1 and flk-1/KDR, in endothelial cells [23, 24]. But, an important role of VEGF as radiation induced-apoptosis modulation and signal transduction pathway in endothelial cells has not been described carefully to date.

We examined the effect of VEGF on radiation-induced apoptosis and the VEGF receptor/second signal transduction pathway on human umbilical vein endothelial cells (HUVECs). These data indicate that VEGF is a apoptosis survival factor in endothelial cells. In addition, our findings indicate that the flk-1/KDR receptor and the PI3-kinase/Akt signal transduction pathway are crucial elements in the processes leading to endothelial cell survival induced by VEGF.

Material and methods

Cell culture and reagents

Human umbilical vein endothelial cells (HUVECs) were prepared from human umbilical cords by collagenase digestion as previously discribed [24]. The endothelial origin of the cultures was confirmed by the presence of factor VIII by immunofluoresence detection. HUVECs were maintained in M-199 medium supplemented with 20% (vol/vol) fetal bovine serum at 37°C in a 5% CO2 atmosphere. The primary cultured cells used in this study were of between passage 2 and 4. M-199 and fetal bovine serum were purchased from Life Technology, Inc. Collagenase Type II was purchased from Worthington. Culture dishes and flasks were purchased from Falcon. Recombinant human VEGF was purchased from R&D systems. Anti-phosphotyrosine an

tibody was purchased from Upstate Biotechnology. Wortmanin and LY294002 were purchased from RBI. Hoechst 33425 and Sytox Green were purchased from Molecular Probes, Inc. All other reagents were purchased from Sigma.

Induction of Apoptosis in Endothelial Cells

HUVECs were plated onto gelatinized 24-well plates (5 x 104 cells per well) in M-199 medium supplemented with 20% (v/v) fetal bovine serum and incubated for 24 hr. To produce irradiation-induced apoptosis, before irradiation the wells were extensively washed with PBS, and the medium was changed to M-199 medium with 5% FBS. Then, the cells were uniformly irradiated at dose rate of approximately 2Gy/min in X-irradiation using a linear accelerator (Simens, 6MV X-ray).

Quantitative Determination of Apoptosis

Quantitative determination of apoptosis in cultured endothelial cells was performed as described previously [24]. All floating cells were collected with two PBS washes, and all adherent cells were also collected after trypsinization. The numbers and size distributions of the floating an d adherent cells were determined with a Coulter Model Z1 Dual Counter System. To detect the apoptotic cells in the adherent cells, the cells in parallel wells were washed with 0.9% sodium chloride, fixed for 15 min with 0.5% glutaraldehyde, and stained with Hoechst 33425 and Sytox green nucleic acid stainings. Apoptotic cells were counted by two independent, blinded investigators, in four different random locations using fluorescence microscope (Zeiss). The number of apoptotic cells in the adherent cells was confirmed by CytoDeath kit according to the manufacturer's protocol (Boehringer Manheim) in the part of this experiment. Both the nuclear stainings and the CytoDeath kit gave similar results. Therefore, in this study, the total apoptotic events were presented as a percent from the apoptotic (determined by nuclear staining) and floating cells in a given cell population.

Application of VEGF, Soluble flt-1 and flk-1/KDR Receptor, and PI3-Kinase Inhibitors to HUVECs

The endothelial cells were exposed to growth factor or cytokine beginning 30 min before irradiation. Fresh growth factor or cytokine was added to the cells and continued presence of them in the medium after irradiation. A ten-fold excess of flt-1 or flk-1/KDR(300ng/ml) was added 30 min before VEGF (30ng/ml) treatment. The same concentration of flt-1 or flk-1/KDR was freshly added to the medium at the same time as the addition of VEGF. In the assay of PI3-kinase inhibitors, wortmanin (WT, 30 nM) or LY294002 (LY, 100 nM) was added to HUVECs 1hr before VEGF (30ng/ml) treatment. The same concentration of wortmanin or LY294002 was freshly added to the cells at the time that VEGF was added. All agents applied to the cells continued to be present during and after irradition. Control cultures received the same amount of PBS or dimethylsulfoxide.

Statistic.

Data are expressed as mean±tandard deviation. Statistical significance between 2 groups was tested using the unpaired Student's t-test. Statistical significant between more than 2 group were tested using one-way ANOVA followed by the Student-Newmam-Keuls test. Statistical significance was set at $p<0.05$.

Results

Morphological studies

HUVECs were grown to confluence in 35-mm dishes or 24-well culture plate coated with gelatin. The medium was then replaced either with medium lacking VEGF or containing VEGF (30 ng/ml) and the cells were then irradiated. 24 h later they were photographed. Cultured human endothelial cells have been demonstrated to undergo apoptosis by irradiation and apoptotic cells were detached from culture plates (Fig. 1. A). The nuclear morphology studies were conducted using HUVECs with or without VEGF by irradiation and stained with Hoechst 33425 and Sytox green (Fig. 1. B).

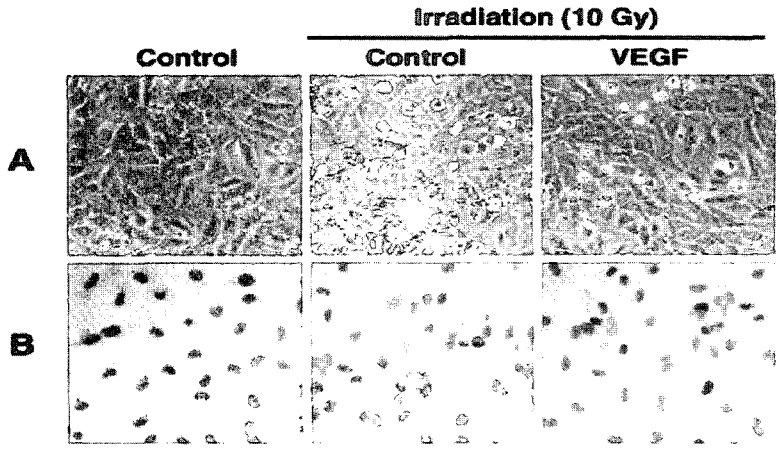

Fig. 1. (A). Phase microscopy illustrating the effect of VEGF deprivation and irradiation on HUVECs morphology. HUVECs were irradiated (10Gy) with and without VEGF (30ng/ml) 24 h later they were photographed. All magnifications are X200. (B). Detection of apoptotic cells by morphological analysis of Sytox Green nucleic acid-stained nuclei at 24 h after irradiation. Arrows indicate the apoptotic cells. All magnifications are X400.

VEGF is an apoptosis survival factor for endothelial cells

The percentage of apoptotic cells was examined at 24 hr after irradiation. The percent of apoptotic cells under control condition(0Gy) was 8.2%. Irradiation(1, 5, 10, and 15Gy) induced apoptosis in a dose-dependent manner(Fig. 2 and 3). The percent of apoptotic cells was approximately 34.9% after 10 Gy of irradiation. Under these conditions, pretreatment with VEGF(1, 3, 10, and 30 ng/ml) inhibited the apoptotic rate in a dose-dependent manner(Fig. 3). Thirty ng/ml inhibited approximately 40-50% of the apoptotic events that occurred in the 10 Gy-irradiated cells(Fig. 1, 3)

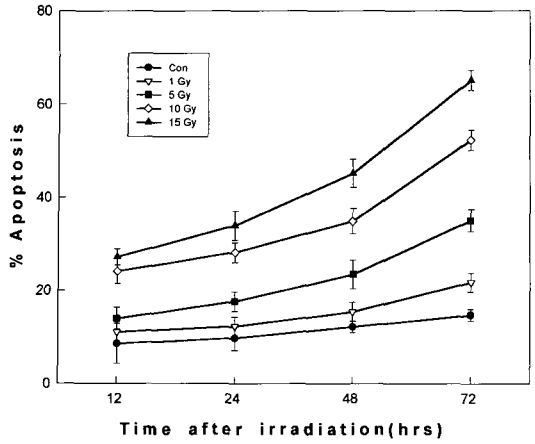

Fig. 2. Irradiation induce apoptosis in HUVECs. HUVECs were grown for 24 h in the presence of 5% serum. The cells were irradiated (0, 1, 5, 10, and 15 Gy) using a linear accelerator and then incubated for another 24 h.

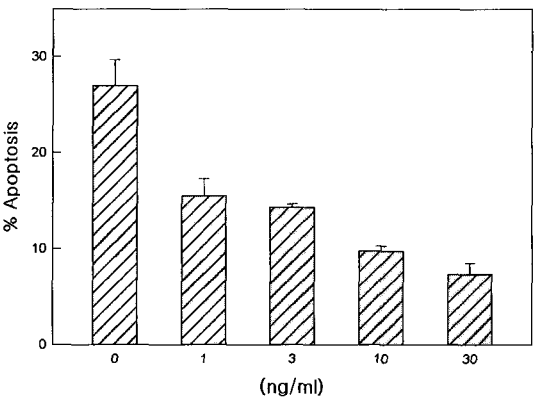

Fig. 3. VEGF inhibits irradiation-induced apoptosis in HUVECs. The cells were exposed to the indicated amount of VEGF before irradiation(10Gy) and were incubated for the next 24h in the presence of same concentration of VEGF.

VEGF is an apoptosis survival factor for endothelial cells through Flt-1 and Flk-1/KDR receptor binding

In the present study, we investigated the survival role of VEGF in HUVEC cells cultured in X-irradiation. VEGF signaling is itself mediated by two other endothelial cell-selective receptor thyrosin kinases, called Flt-1 and Flk-1/KDR. Then, we examined the receptor specificity for VEGF antiapototic effect. VEGF (30 ng/ml) was applied with or without a 10-fold excess of Flt-1 and Flk-1/KDR as the cells were put into irradiation medium. Both receptor competed VEGF from its cellular receptor, completely blocking the antiapoptotic effect of VEGF on HUVECs (Fig. 4). These results indicate that VEGF exerts its antiapoptotic effect through Flt-1 and Flk-1/KDR receptor binding.

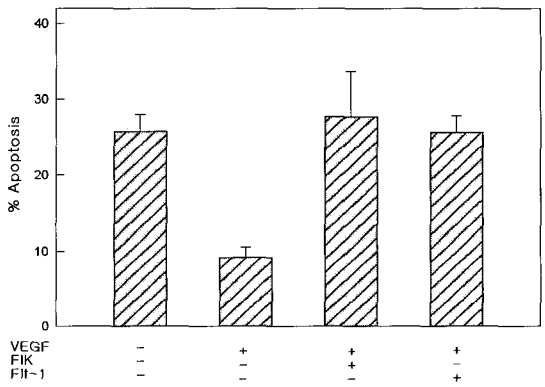

Fig. 4. The antiapoptotic effects of VEGF in HUVECs is blocked by Flk1/KDR and Flt-1 receptor. HUVECs were esposed to VEGF(30 ng/ml) with or without a 10- fold excess of Flk1/KDR and Flt-1 receptor immediately after irradiation.

VEGF induced antiapoptotic effect in HUVECs is mediated by PI3-kinase

The PI3-kinase-specific inhibitor, wortmanin, blocks the protective action of VEGF in serum-deprived HUVECs (23). These findings suggest that the PI3-kinase pathway is involved in the antiapoptotic effects of growth factors. To examine the involvement of PI3-kinase in VEGF antiapoptotic effect after irradiation, we incubated HUVECs with the two specific inhibitors of PI3-kinase, wortmanin, and the structurally unrelated synthetic compound LY294002. Wortmanin (30 nM) or LY294002 (100 nM) almost completely blocked the VEGF-induced antiapoptotic effect (Fig. 5). These results indicate that VEGF exerts its antiapoptotic effect under radiation-induced in apoptosis endothelial cells through a PI3-kinase-mediated pathway.

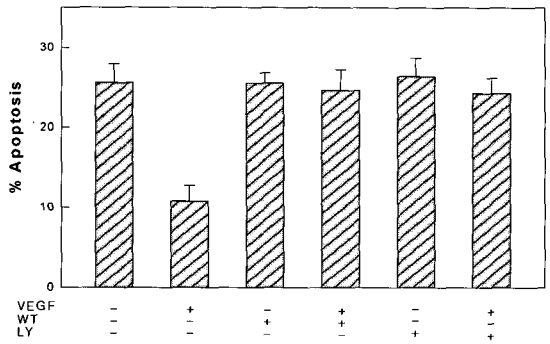

Fig 5. VEGF-induced antiapoptotic effect in HUVECs is blocked by PI3 kinase inhibitor. The cell were exposed to wortmanin(30nM), LY294002(100nM) and VEGF(30ng/ml) before irradiation (10Gy) and incubated for 24h.

Discussion

Many studies have examined the effects of radiation-induced apoptosis on endothelial cell. Few in vitro studies, however, have examined the radiation-induced antiapoptosis and its signal transduction pathway. Using the in vitro model system of radiation-induced apoptosis in HUVECs, we have elucidated the effect of VEGF and the receptor/signal transduction pathways resulting in the antiapoptotic effect of VEGF. We found that VEGF potently prevents apoptosis and promotes survival in these systems. We also demonstrate the critical role of the PI3-kinase-Akt pathway, leading to endothelial cell survival induced by VEGF. Our resulst show that flk-1/KDR is a primary mediator of such signal transduction pathway.

The radiation-induced apoptosis increased by a dose (from 1 to 15Gy) and a time (up to 72 h) dependent manner. Our results on HUVECs extend earlier reports on both macro- and microvascular endothelial cells from other tissues (4, 5).

Apoptosis induced by irradiation in vascular endothelial cells can be prevented by several growth factors and cytokines, such as, bFGF, VEGF, endothelin-1 , has been studied. VEGF, a highly active endothelial cell mitogen and angiogenic factor, has been implicated in several forms of physiological(developmental and reproductive) and pathological(tumor growth/metastasis and retinopathies) angiogenesis(25, 26). Previous studies have proposed a protective role of VEGF in radiation-induced cell damage, since VEGF was shown to hematopoietic stem cells(13). We also observed that

VEGF effectively inhibited in HUVECs in a dose-dependent manner at 10 Gy irradiation. Also, our results show that the antiapoptotic potency of VEGF in endothelial cells is higher than bFGF or endothelin-1(Fig. 3).

VEGF bind to two tyrosine-kinase receptor, flt-1 and Flk-1/KDR, which are expressed almost exclusively in endothelial cell(27). VEGF binding to the Flk-1/KDR send a classical proliferative signal. In contrast, VEGF binding to flt-1 elicits endothelial cell-cell interactions and capillary tube formation (28). Gerber et al(29) was shown previously that VEGF, selectively binding to the Flk-1/KDR receptor, displayed high levels of survival activity, whereas Flt-1-specific ligands failed to promote survival of serum-starved primary human endothelial cells. Our results indicate that VEGF antiapoptotic effect in endothelial cells is mediated through the Flt-1 and Flk-1/KDR receptor in irradiation-induced apoptosis. Also we suggest that an up-regulation of Flk-1/KDR may reduced radiation-induced apoptosis on endothelial cells by increased binding site for VEGF. Recent report indicates that Flk-1/KDR activates PI3-kinase and Akt. The PI3-kinase and Akt pathway are common features in the transduction of the antiapoptotic effects of growth factor(29). Therefore, we examined whether the PI3-kinase pathway was involved in VEGF antiapoptotic effect under irradiation-induced apoptosis in endothelial cells. Wortmanin or LY294002, two potent inhibitors of PI3-kinase, completely reversed VEGF antiapoptotic effect in both case. Therefor, VEGF-induced PI3-kinase activation could be an essential intracellular step in the VEGF-induced antiapoptotic effect. Thus, VEGF is therapeutic candidates for preventing endothelial cell apoptosis under certain pathologic condition such as radiation therapy.

In summary, pretreatment with VEGF provided an antiapoptotic effect in endothelial cells after irradiation. VEGF antiapoptotic effects are mediated through Flk-1/KDR binding and PI3-kinase activation. This data suggest that pretretment of VEGF may provide a benefical effect in maintaining normal endothelial cell integrity under certain pathologic condition such as radiation therapy.

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