Effect of Ionizing Radiation Induced Damage of Endothelial Progenitor Cells in Vascular Regeneration

Mi-Ok Lee, Seung-Hyun Song, Seokyun Jung, Seulgi Hur, Takayuki Asahara, Hyongbum Kim, Sang-Mo Kwon, Hyuk-Jin Cha

Objective—A number of studies have revealed that stress signaling and subsequent stress responses in stem/progenitor cells are responsible for attenuated regeneration or degenerative disease. Because ionizing radiation (IR), which sensitizes diverse types of stem cells, reportedly induces cardio-circulatory diseases, we hypothesized that IR-induced vascular abnormalities are associated with defects in endothelial progenitor cells (EPCs) that are responsible for vascular homeostasis.

Methods and Results—We used an irradiated mouse model to mimic the IR effect on vasculogenesis. Mouse EPCs isolated from irradiated mice and human EPCs exposed to IR were used for functional analysis and gene expression study. Under IR exposure, EPCs were depleted, and their function for vasculogenesis in vitro and in vivo was significantly reduced. In such IR-mediated stress responses, upregulating p21Cip1 and downregulating vascular endothelial growth factor (VEGF) were mediated by p53 transcriptional activity.

Conclusion—The results of the present study suggest that suppression of p53 would be clinically applicable to (1) minimize the functional defects in EPCs in order to prevent the onset of vascular diseases caused by radiation therapy or radiation exposure and also to (2) provide novel insight into the mechanisms of IR-induced vascular damage and a possible strategy to minimize vascular damage by IR. (Arterioscler Thromb Vasc Biol. 2012;32:343-352.)

Key Words: aging ■ signal transduction ■ DNA damage ■ ionizing radiation ■ p53

Stem cell abnormality has been considered a main cause of a variety of cancers and degenerative diseases. Likewise, decreased stem/progenitor cell function has been suggested to result in reduced regeneration potential in aged tissue. This hypothesis is supported by a number of studies demonstrating progenitor cell depletion and decreased differentiation potential during aging. Cellular senescence is caused not only by replication stress, which is caused by continuous cell division, but also by a variety of physiological stresses such as nutrient deprivation, oxidative stress, and DNA damage, whose accumulation results in “premature senescence.” Premature senescence in stem cell populations grown under diverse stress conditions may be closely linked to depletion and/or dysfunction of progenitor cells resulting in subsequent physiological responses (eg, aging or degenerative diseases).

Endothelial progenitor cells (EPCs) have been widely studied because of their regenerating potential and ability to maintain homeostasis of the endothelial system, which has potential application in cell-based therapies. However, similar to other stem/progenitor cells, EPCs undergo premature senescence and dysfunction under a variety of stress conditions such as hypertension, diabetes, and hyperglycemia. Thus, abnormalities of the circulatory system, which are frequently found in stress-induced vascular diseases, may be strongly associated with senescence or dysfunction of EPCs. This idea was supported by studies demonstrating a strong correlation between cell numbers of EPCs and a variety of cardiovascular diseases.

Exposure to ionizing radiation (IR; γ or X-rays) triggers diverse physiological responses such as cancer, gastrointestinal failure, bone marrow failure, and accelerated aging depending on a wide range of radiation doses. On the cellular level, IR results in serious DNA damage triggering DNA damage responses (DDR) such as cell cycle arrest and senescence, which are largely dependent on p53 transcriptional activity and subsequent induction of p21Cip1, a cell cycle inhibitor protein. Interestingly, accumulation of DNA damage under normal conditions has been observed in stem cells isolated from aged organisms. Additionally, a mouse

Received on: November 2, 2010; final version accepted on: October 24, 2011.
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Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org

DOI: 10.1161/ATVBAHA.111.237651

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model with deficiencies in the DNA repair system causing a massive DDR suffered from accelerated aging and significant stem cells loss. Therefore, chronic DNA damage accumulation in stem/progenitor cells, and subsequent DDR during aging, will influence both the onset of senescence and the dysfunction of stem cells that occurs with aging. Recently, acute or chronic radiation exposure, such as that of A-bomb survivors or nuclear industry workers, has been revealed to be strongly associated with circulatory diseases. However, with few exceptions, little is known about how IR exposure induces cardiac or circulatory diseases. EPCs play crucial roles in maintaining circulatory system homeostasis and are sensitive to a variety of stresses; therefore, it is readily hypothesized that IR-induced abnormalities of the circulatory system would be derived from stress responses of EPCs (especially DDR) under IR exposure.

The present study is the first to demonstrate that radiation exposure in either mice- or human-cultured EPCs induces senescent growth arrest and functional defects, which lead to attenuated vascular regeneration. The growth arrest and senescent phenotype, as well as functional defects in EPCs, are dependent on p53 transcriptional activity on IR, which leads to p21Cip1 upregulation and vascular endothelial growth factor (VEGF) downregulation. Therefore, the present study implied that functional defects in EPCs from IR in a p53-dependent manner would be responsible for the failure of circulatory homeostasis that may be linked to IR-induced circulatory diseases. Furthermore, the study suggested that transient inhibition of p53 transcriptional activity would be applicable to minimize the damage to EPCs from IR exposure. The present study provides a novel mechanism to address IR-induced cardiovascular damage, and it indicates that transient p53 inhibition could protect EPCs from IR damage.

Materials and Methods

Animals

Experiments were performed in male 8- to 10-week-old C57BL/6J mice (Orient, Sung-Nam, Korea) maintained under a 12-hour light/dark cycle and in accordance with the regulations of Pusan National University. The experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Pusan National University. All procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication no. 85-23, revised 1996).

In Vivo Neovascularization Assay Using a Hind-Limb Ischemic Model

To develop the hind-limb ischemic model, C57BL/6 mice were anesthetized by IP injection of 300 mg/kg Avertin (cat. #T48402-5G, Sigma-Aldrich, St. Louis, MO). An incision was made in the skin overlying the middle portion of the left hind-limb. After ligation of the proximal end of the femoral artery, the distal portion of the saphenous artery was ligated as well as all side branches. Cell transplantation of mouse or human EPCs was performed with 2 or 5 × 10^5 cells per mouse by intravascular or intramuscular injection. In cases of transplantation of human late EPCs into C57BL/6 mice, Cyclosporin A (cat. #C1832, Sigma-Aldrich, St. Louis, MO) was subcutaneously injected daily to suppress immune response.

Monitoring of Hind-Limb Blood Flow and Capillary Density

After anesthesia, hind-limb perfusion was measured using a laser Doppler perfusion imaging system (LDPI; Moor Instruments, Wilmington, DE). The stored perfusion values behind the color-coded pixels representing the microvascular blood flow distribution were used for analysis. Color photographs were recorded, and the analysis was performed by calculating the average perfusion of the ischemic and nonischemic foot. To account for variables such as ambient light and temperature, the results are expressed as the ratio of perfusion in the ischemic versus normal limb. To analyze capillary density of recovered tissue, frozen sections (10 mm) of adductor muscles from ischemic mice were prepared for immunohistochemistry with rabbit antimonouse CD31 antibody (BD).

Monitoring of Survived Cells in Ischemic Sites

To monitor the survived EPCs in the ischemic area, 5 × 10^5 of human EPCs (hEPCs: control hEPCs and 5 Gy-irradiated hEPCs) were labeled by Dil-conjugated Ac-LDL (Dil-Ac-LDL) (Biomedical Technologies Inc., MA, USA). At 7 days after generation of hind-limb ischemia followed by transplantation of control hEPCs and IR-hEPCs, frozen sections (10 mm) of adductor muscles from the ischemic area were prepared for immunohistochemistry for CD31 (BD). The surviving cells were analyzed using photomicrographs of chemical staining for acetyl LDL (Ac-LDL)-Dil uptake (red) or immunostaining for endothelial cell (EC) markers, CD31 (PECAM-1, green) to visualize the regenerated vessels in the ischemic tissue.

Cell Culture

Human late EPC and human umbilical vein endothelial cells were cultured in EBM2 medium (cat. #CC-3156, Lonza, Basel, Switzerland) containing EGM-2 MV SingleQuots (cat. #CC-4147, Lonza, Basel, Switzerland) on fibronectin-coated dishes, and cells were passaged every 3 days.

Ionizing Radiation

Mice or cells were exposed to various doses of IR in an IBL 437C 137Cesium γ-iradiator at a rate of 2.4 Gy/min.

Isolation of Bone Marrow Mononuclear Cells, Lineage Negative Cells, and Lin^neg/c-kit+/Sca1+ Cells

Bone marrow mononuclear cells (BM-MNCs) and lineage negative (Lin^-) cells were isolated as described previously. Two days after total body irradiation (3 Gy), mononuclear cells were isolated from bone marrow using the Ficol gradient method. Lineage-determined (Lin^-) cells were removed from BMMNCs using a hematopoietic stem cell enrichment set (BD bioscience, cat. # 558452), and then Lin^- cells were counted. For FACS analysis, Lin^- cells were stained with PE-SCA1 antibody (BD Pharmingen™, cat. #, 553336) and APC-C-kit antibody (BD Pharmingen™, cat. #553356) and then analyzed with BD FACS Calibur™.

siRNA Delivery to EPCs

The siRNAs for the human TP53 and negative control were purchased from Dharmacon, Lafayette, CO) according to the manufacturer’s protocol. After 24 to 48 hours, siRNA transfected-cells were used for further experiments.

EPC Colony Forming Assay

CD34+ cells were cultured in methylcellulose-containing medium SF H4236 (StemCell Technologies, Vancouver, Canada) with 100 ng/mL stem cell-derived factor (Kirin, Tokyo, Japan), 50 ng/mL.
vascular endothelial growth factor (R&D Systems, Minneapolis, MN), 20 ng/mL interleukin-3 (Kirin), 50 ng/mL basic fibroblast growth factor (Wako, Osaka, Japan), 50 ng/mL epidermal growth factor receptor (Wako), 50 ng/mL insulin-like growth factor-1 (Wako), 2 U/mL heparin (Ajinomoto, Tokyo, Japan), and 30% FBS on 35 mm dishes for 21 days. Cell densities for each sample were as follows: CB-CD34+ cell, 500 cells/dish; PB-CD34+ cells, 1.5 × 10^6 cells/dish. The EPC-colony forming units (CFUs) were identified as large EPC-CFUs or small EPC-CFUs by visual inspection with an inverted microscope under 40x magnification. Large EPC-CFUs were composed of spindle-shaped cells, and small EPC-CFUs were composed of round adhesive cells.

**Protein Assay**

1 × 10^6 cells were plated in a 6-well dish, and the following day the cells were irradiated with X-rays (5 Gy) for various lengths of time. At the indicated times, the cells were counted using a hemocytometer.

**β-Galactosidase Activity Assay**

Irradiated EPCs were cultured in EBM2 media for 4 days. Following the culturing time, senescence cells were detected using a Senescence β-Galactosidase Staining Kit according to the manufacturer’s protocol (cat. #9860, Cell Signaling Technology, Danvers, MA).

**Matrigel Tubule Formation Assay**

Cultured human late EPCs were irradiated with 5 Gy of X-rays and then serum-depleted in EBM-2 media with 0.02% FBS for 24 hours. Next, the cells were transferred into growth factor-reduced Matrigel (cat. # 354230, BD Biosciences, San Jose, CA) in 96-well plates (1 × 10^4 cells/wells). After 24 hours, tubule formation by the EPCs was observed and analyzed.

**Migration Assay**

Late EPC cells were cultured on fibronectin-coated 12-well plates and then irradiated with 5 Gy of X-rays. Cells were serum-depleted in EBM-2 media with 0.02% FBS for 24 hours. After the serum-depletion, a line was scratched into the cell cultures. Areas refilled by EPC migration were monitored and compared among each experimental group.

**Quantitative Real-Time PCR and RT-PCR**

RNA extraction and cDNA synthesis were performed as described elsewhere. The sequences for the gene-specific primers were as follows; human GAPDH: F 5'-AAGGTCTCATACTTCTGGC-3', R 5'-GTGATGCGGATGCTTGTT-3', hVEGF: F 5'-AAAGGAGGAGCCGACCAAT-3', R 5'-CTCCTCATGTTGATTTAGA-3', human p53: F 5'-TCTTCGAGACCTTAC-3', R 5'-CCATCGAGAACTTAC-3', human p21: F 5'-GGGAGGAAGGTGTTGATGG-3', R 5'-GGGAGGAAGGTGTTGATGG-3', mouse GAPDH: F 5'-GGTCTGCATGATTGTC-3', R 5'-CAACATGTTCTTTTCACT-3', mouse vEGF: F 5'-AACGCTGTCATGTGAACT-3', mouse p53: F 5'-ATCGATGCTTAC-3', R 5'-AGAGAGATGTTG-3', mouse p21: F 5'-GGGAGGAAGGTGTTGATGG-3', R 5'-GGGAGGAAGGTGTTGATGG-3'.

**VEGF ELISA**

Human late EPCs with or without irradiation (X-ray, 5 Gy) were cultured with EBM2 media containing 0.5% FBS for 3 days. Then media was collected and concentrated 30-fold with Amicon Ultra-4 (Millipore, cat. # UFC800324). Protein levels of VEGF were determined by Human VEGF QuantGlo ELISA Kit (R&D Systems, cat # QVE00B).

**Immunoblotting**

Immunoblotting analysis was performed as described elsewhere. Primary antibodies used in the present study were as follows: anti-p53 (cat. # sc-126, Santa Cruz Biotechnology, Santa Cruz, CA), anti-H2AX (cat. #5971, Cell Signaling Technology, Danvers, MA).

**Statistical Analysis**

The graphical data are presented as the mean ± SD. Statistical significance among groups and between groups was determined using 1-way or 2-way ANOVA following application of Bonferroni multiple comparison posttest and Student t-test, respectively. Significance was assumed for P < 0.05 (*). Statistical analysis was performed using SAS statistical package, v.9.13 (SAS, Cary, NC).

**Results**

**Depletion of EPCs by IR**

To examine the damage of IR in mice, bone marrow cells were first investigated because bone marrow is considered to be one of the most sensitive tissues to IR-mediated damage. Considering the relatively high resistance of bone marrow stromal cells (eg, bone marrow mesenchymal stem cells), we focused on monitoring BM-MNCs that contained a variety of circulating cells including hematopoietic stem cells and EPCs. Male mice, 6 weeks old, were exposed to a sublethal dose (3 Gy) of total body irradiation (TBI), and their mononuclear cells (MNCs) were isolated on the indicated day. As shown in Figure 1A, the total population of MNCs in the mouse bone marrow was significantly reduced 1 day after TBI (Supplemental Figure I, available online at http://atvb.ahajournals.org) and gradually recovered over 10 days after TBI as previously described. However, even 31 days after radiation exposure, the population of MNCs in IR-exposed mice still failed to recover completely (Figure 1A). Cultured mouse EPCs were obtained from MNCs of TBI mice as previously described, and resultant EPCs, which were positively stained with both lectin and Ac-LDL-Dil (Figure 1B, inserted panel) were counted. As shown in Figure 1B, the number of EPCs isolated from MNCs of TBI mice was significantly reduced 1 day after IR and gradually recovered in a pattern similar to MNCs. Reduced numbers of EPCs from the MNCs of TBI mice were again confirmed by immunofluorescence analysis (Figure 1C). For further clarification, MNCs of either control or TBI mice were further separated through Lin+ depletion as described. The resultant cells (Lin− cells) were further quantified by FACS using Sca1 and c-kit antibodies, for which dually positive populations are determined as EPCs. Consistently, the number of Lin−/c-kit+ cells isolated from TBI-mice appeared to be markedly lower than that from control mice (Figure 1D, right panel). Of interest, the percentage of Lin−/c-kit+/Sca1+ cells was dramatically decreased in the total BM-MNCs population, whereas the total Lin− population was only marginally decreased by IR exposure suggesting a possible difference in IR sensitivity between the 2 different types of cells (Figure 1E).

Because IR-mediated stress induces premature senescence in mouse bone marrow stem cells, we reasoned that the depletion of EPCs after IR would be associated with premature senescence. To prove this hypothesis, β-galactosidase staining, which is widely used to identify senescent cells, was performed in EPCs from TBI mice. As predicted, strong β-galactosidase staining, which is indicative of senescent cells, was predominant in EPCs isolated from TBI mice (Figure 1F), as were cultured mouse EPCs that were positively

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stained with both lectin and Ac-LDL-Dil and revealed strong /H9252-galactosidase staining after IR exposure (Supplemental Figure II). This result implies that decreases in EPCs in TBI mice may occur in parallel with the onset of cellular senescence of EPCs after radiation exposure.

Defective Vascular Regeneration by Radiation Exposure

Considering that radiation exposure decreases the number of both MNCs and EPCs in vivo, radiation exposure would be expected to have an impact on vascular regeneration potential where EPCs have a major role.31 To test this idea, mice were subjected to TBI, and their blood flow rates were monitored in the hind-limb ischemic model as previously described.32 Consistent with the reduced EPCs under radiation exposure, TBI mice suffered from severe reduction of vascular regeneration compared to the control (Figure 2A). Such reduced vascular regeneration potential was fully recovered by the transplantation of EPCs that were isolated from nonirradiated mice. These results suggest that depletion of EPCs by radiation exposure (Figure 1B) may be closely associated with decreases in vascular regeneration potential in TBI mice.

Figure 1. Depletion of endothelial progenitor cells (EPCs) by ionizing radiation (IR). C57BL/6 mice were exposed to 3 Gy of total body irradiation (TBI: -ray). A, Bone marrow mononuclear cells (BM-MNCs) were isolated from TBI mice at indicated times and counted. B, BM-EPCs derived from isolated mononuclear cells from TBI mice were counted, and the data are presented as a graph. A representative EPC image of dual positive to Lectin-FITC and Ac-LDL-Dil is presented in the inserted panel. C, The same number of BM-MNCs isolated from either TBI-exposed or control mice was replated 24 hours after IR, and EPCs were obtained by in vitro culture with EBM2 media. EPCs, dual positive with Lectin and Ac-LDL-Dil (left panel), were quantified, and their percentage is presented as a graph (right panel). D, The relative population of EPCs was quantified by FACS analysis using PE-Sca1 and APC-c-kit antibodies post depletion of Lin positive population (Lin−/c-kit−/Sca1−: LKS) (left panel). Its percentage is presented as a graph (left panel). E, Relative ratio of Lin− (left panel) or LKS (right panel) is presented as a graph. **P<0.01. F, Senescent cells of EPCs isolated from TBI (at 10 days after TBI) or control mice were determined by β-galactosidase activity assay (top panel). DAPI was used for nuclear counterstaining (bottom panel).

Figure 2. Defective vascular regeneration by radiation exposure. Total body irradiation (TBI; 24 hours post-IR with 3 Gy of -ray) and control mice were subjected to hind-limb ischemic surgery (6 mice per condition). Cultured mouse endothelial progenitor cells (EPCs) were transplanted into a group of TBI mice (6 mice each) intramuscularly. On the indicated day, blood flow rate was measured by a laser Doppler imager. A, Recovery of the blood flow rate is presented as a graph. ***P<0.001. B, Percentage of tissue necrosis in the hind-limb area is presented as a graph. C, Representative images of blood flow rates captured by the Laser Doppler imager are shown.
dominantly in TBI mice unlike mice with transplanted EPCs (Figure 2B). Representative laser Doppler images determining the blood flow of the ischemic model demonstrate the notable impact of radiation exposure on vascular regeneration potential (Figure 2C). These results clearly indicate that radiation exposure leads to impaired vascular regeneration that is overcome by the transplantation of EPCs.

Stress Response of EPCs Under Radiation Exposure

DDR by IR-triggering senescence or growth arrest is largely indicated by specific nuclear foci generation referred to as γ-H2AX foci. Therefore, we attempted to determine γ-H2AX foci in EPCs isolated from TBI mice to link DDR with the onset of senescence. As shown in Figure 3A, strong γ-H2AX foci were observed in IR-exposed EPCs, indicating that senescence onset of EPCs occurs in parallel with DDR as determined by γ-H2AX foci (Figure 3A). Of interest, the level of γ-H2AX-positive cells indicating the DNA-damaged cells were similar in both non-EPC cells (Lin+/Sca1+ and Lin/Sca1) and EPC cells (Lin+/Sca1+, white arrow) (Figure 3B and C). Thus, these results indicate that DNA damage occurs equivalently in all cells of bone marrow under IR exposure.

In response to a variety of stresses (eg, IR), cellular senescence is largely mediated by p53 transcriptional activity, upregulating a variety of cell-cycle inhibitor proteins such as p21Cip1 in cell-line models and primary stem/progenitor cells. Consistent with these results, we found that IR-mediated stress in isolated EPCs significantly upregulates p21Cip1 (Figure 3D). Early EPCs isolated from MNCs function in vascularization by secreting a variety of angiogenic factors such as VEGF. Therefore, we measured the VEGF expression level to reason the inhibited vascular regeneration potential in TBI mice as shown in Figure 2. The VEGF mRNA expression (Figure 3E) and protein levels, as determined by ELISA (Supplemental Figure III), were significantly suppressed in the cultured EPCs after radiation exposure. Thus, we concluded that the stress response in EPCs by radiation exposure occurs with senescence and VEGF suppression, which may account for defective vascular regeneration in TBI mice (Figure 2).

Defective Human Cord Blood EPCs Under IR

Because murine-cultured EPCs after TBI or direct IR exposure ex vivo were demonstrated to undergo senescence and/or acquire functional defects, we extended our studies to human EPCs in a similar manner. Because EPC populations were dramatically reduced in TBI mice (Figure 1B and C), development (or differentiation) of EPCs from stem/progenitor cells may be affected by IR exposure. To test this idea, a CFU assay was carried out to determine the differentiation potential of human EPCs from CD34+ positive stem cells isolated from human cord blood as previously described (Supplemental Figure IV, for characterization). As predicted, CFUs of late EPCs were markedly reduced compared to the control (Figure 4A) suggesting that IR may also impair the developmental cascade into endothelial lineage progenitors. In addition, the proliferation potential of cultured human EPCs was markedly reduced after irradiation (Figure 4B), which occurred in parallel with increased senescent populations as determined by β-galactosidase staining (Figure 4C). Considering that stem/progenitor cells senescence may be relevant to impaired function of stem cells in vivo or in vitro, the proliferation potential of cultured human EPCs was markedly reduced after irradiation (Figure 4B), which occurred in parallel with increased senescent populations as determined by β-galactosidase staining (Figure 4C). Considering that stem/progenitor cells senescence may be relevant to impaired function of stem cells in vivo or in vitro,
The senescence of EPCs may result in functional defects. To test this idea, human EPCs with or without IR pre-exposure were subjected to functional analysis to determine tubule-forming capability as well as re-endothelialization.40 As predicted, radiation-exposed EPCs revealed significant functional defects in both tubule formation (Figure 4D) and re-endothelialization (Figure 4E) compared to the control. Obvious defects in EPC function after IR were repeatedly validated by the low efficiency of neovascularization in vivo. Using the hind-limb ischemic model, vascular regeneration potential between the control and IR-exposed EPCs was compared. Consistent with the result from TBI mice (Figure 2), vascular regeneration of ischemic mice by EPC transplantation was significantly reduced when IR-exposed EPCs were transplanted compared to the control (Figure 4F and Supplemental Figure V). For further quantification of retarded vasculogenicity of IR-exposed EPCs compared to the control, chimeric vessel formation by transplanted hEPCs was determined by IFC with CD31 antibody as described previously.32 Consistent with the result in Figure 4F, chimeric blood vessel formation of IR-exposed hEPCs was markedly reduced, indicating that DNA damage by IR in EPCs (Figure 3 and 4H) significantly affects their vascular regeneration potential (Figure 4G). DNA damage after radiation exposure to hEPCs was revealed by the level of 53-H2AX and accumulated p53 protein levels, which leads to p53-dependent gene transcription such as p21Cip1 (Figure 4H). Both senescent growth arrest and defective function of EPCs leading to impaired vascular regeneration activity would be dependent on p53 transcriptional activity considering distinct p53 accumulation and subsequent p21Cip1 induction as well as VEGF suppression after IR (Figure 4I). These results strongly indicate that radiation exposure induces DNA damage in EPCs triggering senescence and functional defects in EPCs. The DNA damage stress responses, which are responsible for the senescence and function defect in EPCs, may be correlated with p53...
dependent alteration in gene expressions such as p21Cip1 and VEGF.

Functional Defects in IR-Exposed EPCs is Mediated by p53

In order to examine whether p53 is responsible for the DNA damage stress response in EPCs, senescence and functional defects were determined either with or without p53. To achieve suppression of p53, p53 siRNA (siTP53) was introduced to human EPCs and considerable p53 suppression was achieved (Figure 5A). In the absence of p53, reduced proliferation potential of cultured EPCs and senescence onset that were both caused by IR was reversed (Figure 5B and 5C). Additionally, the tubule-forming capability of EPCs (determined by both the number of tubule junctions and the average length of the tubules) that was strongly repressed by IR (Figure 4D) was significantly reversed by suppressing p53 (Figure 5D). In a similar fashion, the migration capability of EPCs that was inhibited by IR (Figure 4E) was also remarkably recovered by p53 suppression under IR (Figure 5E).

Because upregulation of p21Cip1 and downregulation of VEGF expression, which both occur in a p53-dependent manner, were found in IR-exposed EPCs along with decreased proliferation potential and senescence onset as well as functional defects in vascularization, the restoration of function of cultured EPCs with p53 suppression may result from altered p21Cip1 and VEGF expression. As shown in

![Figure 5](image-url)
Figure 5F, levels of p21Cip1 mRNA (left panel) and protein (right panel), which are upregulated by IR, were markedly reduced in p53-suppressed EPCs (siTP53) compared to the control (siNC) after IR exposure. In the case of VEGF expression, p53 suppression markedly increased basal levels of VEGF expression even in the control. Considering the negative effect of p21Cip1 expression on the functional regulation of EPCs, a significant loss of p21Cip1 expression by the knock-down of p53 (Figure 5G, top panel) may be linked to higher expression of VEGF in either control or irradiated EPCs (Figure 5H).

These results clearly imply that increased p53 transcriptional activity and altered gene expression by radiation exposure induce stress responses in EPCs such as cell growth arrest, senescence, and vascularization. Such stress responses occurring in IR-exposed EPCs in a p53-dependent manner would be responsible for defective vascular regeneration.

**Discussion**

As stem/progenitor cells are responsible for regenerating damaged or senescent somatic mature cells, maintenance of an adequate pool of stem/progenitor cells would be critically important for retaining tissue homeostasis. Thus, abnormalities in stem/progenitor cells are considered to be a cause of several serious diseases such as cancers, degenerative disease, and even aging. Therefore, stress induced by external or internal factors such as IR, oxidative damage, lack of nutrients, or exposure to carcinogens would dramatically influence the pool of stem/progenitor cells. Consequently, both oxidative stress and IR have a significant effect on hematopoietic stem cell lifespan.

Because IR has a serious negative impact on hematopoietic stem cells leading to bone marrow failure or leukemia, functional defects of stem cells should be closely associated with diverse physiological responses. Therefore, we attempted to explore the pathophysiological effects on EPCs by IR because cardiac or circulatory disease has been shown to be caused by both acute and chronic IR exposure, and EPCs, which play crucial roles in maintaining the circulatory system, are susceptible to a variety of stresses.

The present study demonstrated that growth arrest or senescence onset and functional defects in EPCs by IR exposure occurred along with retarded vascular regeneration. We also showed evidence that the stress response of EPCs after IR exposure is mediated by p53 activation because suppression of p53 reversed the effects of IR on EPCs, growth arrest, and functional defects (Figure 5). These results are consistent with previous studies demonstrating the important roles of p53 in the functional regulation of EPCs under various stress conditions. For example, circulating EPCs are decreased and undergo senescence-like growth arrest, which are both dependent on p53 activation, and follow p21Cip1 expression in a diabetes-induced stress model. Likewise, p21Cip1, a downstream target of p53, which induces cell-cycle arrest and senescence onset under various stress conditions, has been shown to contribute to the turnover rate of mature EPCs. Lack of p21Cip1 not only increases the proliferation and survival of EPCs but also promotes vascular regeneration. Because ECs reportedly play an important role in radiation-induced vascular injury by increasing proliferation, migration, and fibrinogenic phenotype, the possibility of crosstalk between ECs and smooth muscle cells may be an important factor in retarded vascular regeneration phenotype and/or endothelial apoptosis by IR exposure. Considering the increased expression of transforming growth factor-β, subsequently activated Smad signaling by IR, and its strong correlation to apoptosis, it would be interesting to study the possible role of transforming growth factor-β signaling in ECs by IR during radiation-induced vascular injury and retarded vasculorigenesis. In addition, we also provided evidence that EPCs appeared to be more sensitive to the DNA damage stress than ECs (Supplemental Figure IX).

Thus, we surmised that defects in EPCs by IR-induced DNA damage stress, would be more closely associated with reduced vascular regeneration by IR exposure. Of note, vascular damage by IR exposure can be ameliorated by cell types other than EPCs. For example, irradiated tissue may be rescued by adipose tissue-derived stromal cells, suggesting that cell therapy is a possible therapeutic modality for vascular damage by irradiation.

These studies strongly imply that p53 and/or p21Cip1 are important for regulating EPC growth and function following radiation exposure. Furthermore, we provided evidence that VEGF, a crucial growth factor for EPC function and survival, is suppressed by p53, which has also been demonstrated in diverse cancer cell models. Thus, suppression of VEGF by activated p53 due to radiation exposure is likely to be responsible for functional defects in EPCs, as shown by tubule formation and migration. This notion is well supported by results showing that increased VEGF by p53 suppression, even under exposure, occurred with the restoration of EPC function. Further mechanistic studies of VEGF suppression by p53 transcriptional activation are currently under active investigation. Considering that cardiac and circulatory diseases are serious side effects of radiation exposure, understanding the molecular mechanism of IR-mediated circulatory diseases would be important for minimizing risk. Accordingly, we suggest that transient inhibition of p53 transcriptional activity by a pharmacological inhibitor may minimize the damage to EPCs by IR and possibly reduce circulatory disease caused by radiation exposure. This interesting idea is being actively investigated. Taken together, we demonstrated that radiation exposure caused cell senescence and functional defects in EPCs, which may be responsible for inhibited vascular regeneration. Such radiation-related effects were reduced by p53 suppression, lowering p21Cip1 induction and retaining VEGF expression, suggesting that p53 transcriptional activation and the resultant gene response would contribute to the stress response of EPCs.

**Sources of Funding**

This work was supported by the National Research Foundation of Korea (2011006300) and Basic Science Research Program (M-2011-A0403-00080) (H.J.C.), and by a National Research Foundation grant funded by the Korean government (2010-0020260) (S.M.K.).
Disclosures

None.

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Arterioscler Thromb Vasc Biol. 2012;32:343-352; originally published online November 10, 2011;
doi: 10.1161/ATVBAHA.111.237651

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Supplement Material.

Supplemental Figure I. Depletion of BM-MNC by IR

The numbers of BM-MNCs after TBI are presented as a graph (***, p < 0.01). Mice (n = 4, each group) were irradiated with 3Gy of γ-ray, and BM-MNCs were isolated 2 days after IR exposure.

Supplemental Figure II. Senescent phenotype of the EPCs by IR

Senescence of EPCs by IR was determined by β-galactosidase staining. EPCs were revealed by Ac-LDL-Dil uptake and Lectin-FITC positive staining.

Supplemental Figure III. Decrease of VEGF protein secretion from hEPCs by IR

Level of VEGF proteins, collected from conditioned EPC medium was analyzed with an ELISA kit, and the relative ratio is graphically presented (***, p < 0.01).

Supplemental Figure IV. Identification of typical surface markers expressing in Endothelial Progenitor Cells (EPCs)

Representative surface markers of EPCs were analyzed by flow cytometry. (A) Stem/progenitor cell markers, CD34 and c-kit, as well as the functional EPC markers KDR (VEGFR-2) and CXCR4 were markedly expressed in EPCs. (B) Expression level of hematopoietic markers such as ICAM3 (CD50), CD11b, and CD45 were determined by flow cytometry.

Supplemental Figure V. Impaired functional activity of hEPCs after IR
Functional activity of transplanted hEPCs was examined in the hind-limb ischemic nude mouse model (n = 4–6, each group). (A) Representative laser Doppler images of blood flow rates are displayed (red: intact blood flow, blue: low blood flow). (B) Recovery of blood flow rates examined on the 28th day after surgery is presented as a graph. (**, p < 0.01., ***, p < 0.001).

Supplemental Figure VI. Modulation of anti-angiogenic protein TSP-1 by IR

mRNA expression levels of the hTSP-1 were determined by real-time PCR in the hEPC (A) at 8 hs after IR, (B) at an indicated time after IR, (C) 9 h after treatment with p53 activator (Nutrin) and (D) 24 h after IR in control siRNA (siNC) or p53 siRNA (siTP53) treated hEPCs.

Supplemental Figure VII. Dose dependent effect of transplanted hEPCs

Each indicated number of hEPC was transplanted into a hind-limb ischemic nude mouse model (n = 5, each group). Recovery of blood flow rate on the 28th day was determined by a laser Doppler; its relative ratio is presented as a graph.

Supplemental Figure VIII. Different survival rates of transplanted hEPCs

hEPCs with pre-uptake of Ac-LDL-Dil (shown in red) were transplanted into the hind-limb ischemic mouse model, and the survival of transplanted hEPCs was determined by fluorescence microscopy. (A) Representative fluorescence microscopy image. (B) Graph for the quantification of red fluorescence positive cells (*, p < 0.05).

Supplemental Figure IX. Higher IR sensitivity of EPCs
hEPCs or mouse EPCs (mEPCs) were irradiated by 5Gy of IR, and the damage response was determined by growth rate or tubule forming capability. (A) Growth rate of hEPCs and HUVEC cells was determined by cell counting. The graph for the quantification is presented (*, \(p < 0.05\)). (B) Representative images of tubule-forming capability of hEPCs and HUVEC with and without 5Gy of IR (left panels). The graph for the quantification is presented (*, \(p < 0.05\), **, \(p < 0.01\), ns: not significant). (C) Representative fluorescence microscopy image of mEPCs and mouse Endothelial cells (mECs) with and without 5Gy of IR. (Green: Ki-67, Blue: DAPI, Scale bar: 100 \(\mu m\)) (left panels). Graph for the quantification of Ki-67 positive cells (*, \(p < 0.05\)) (right panel).
Supplemental Figure. I

![Bar chart showing the number of MNC in BM (x10^7) for Cont and IR conditions.](image)
Supplemental Figure. II

A. Cont
  - Dil-ac-LDL
  - FITC-Lectin
  - Merge
  - SA-β-gal

B. IR
  - Dil-ac-LDL
  - FITC-Lectin
  - Merge
  - SA-β-gal
Supplemental Figure. III

[Diagram showing relative VEGF levels across Media, Control (Cont), and IR conditions.]

*** indicates significant differences.
Supplemental Figure. IV

A. CD133  KDR  CD31
   CD34
   CXCR4  c-Kit
   CD34

B. ICAM3 (CD50)
   CD11b
   CD45

0.58%
0.31%
0.39%
Supplemental Figure. V

A.

PBS  hEPCs  hEPCs + IR

B.

Recovered blood flow (%)
Supplemental Figure. VI

A.

B.

C.

D.
Supplemental Figure. VII

- Graph showing the relationship between recovered blood flow (%) and transplanted cell number.
Supplemental Figure. IX

A. Relative growth rate (%)

B. hEPC and HUVEC images and tubule length graphs.

C. mECs and mEPCs images and fold decrease of Ki67 positive cell rates graphs.
전리 방사선에 의해서 유발되는 혈관내피 전구세포의 손상은 혈관의 재생을 지연시킨다.

황경국 교수
충북대학교병원 순환기내과

Summary

배경
병적, 생리적 스트레스 및 이로부터 속발되는 반응들은 줄기세포/전구세포에 영향을 주어 재생과 퇴행성 질환에 관여하는 것으로 알려져 있다. 전리 방사선(ionizing radiation, IR) 역시 다양한 종류의 줄기세포에 영향을 주는데, 여기에서는 IR에 의해 유발되는 혈관병변 형성에 혈관내피 전구세포(endothelial progenitor cell, EPC)의 수적, 기능적 이상이 관여하는지, 관여한다면 그 기전은 무엇인지를 확인하고자 하였다.

방법 및 결과
3.0Gy의 방사선(감마선)을 온 몸에 조사한 생쥐에서 분리된 EPC와 인체 제대혈에서 분리된 후 5.0Gy의 방사선에 노출된 EPC를 대상으로 혈관손상의 기능, 즉 하지혈관손상모델을 이용한 생체 내 기능과 증식, 관 형성을 포함하는 생체 외 기능 및 유전자 발현양상을 분석하였다. EPC의 수는 감소하였으며, 생체 내외 혈관손상 기능도 현저히 감소하였다. 특히 이 과정은 중앙억제자인 p53의 전사능력에 의존적이었으며, 세포주기 억제단백질인 p21Cip1의 과발현과 혈관신생인자인 VEGF (vascular endothelial growth factor)의 저발현에 의한 것으로 확인되었다.

결론
이상의 결과는 IR에 의해 유발되는 혈관손상에 대한 새로운 기전을 시사하는 것이며, IR 유발성 혈관손상을 최소화하는데 이용될 수 있었다. 임상적으로는 p53을 억제함으로써 방사선 치료 혹은 방사선 노출에 동반되는 EPC의 기능손상을 최소화함으로써 혈관손상을 줄이는 전략적 치료에 이용될 수 있었다.
줄기세포의 이상은 암, 퇴행성 질환의 주요 병인 중 하나로 여겨지고 있다. 특히 노화 조직에서 관찰되는 분열능력의 감소는 줄기세포의 수적인 감소와 더불어 기능적 이상에 의한 것으로 여겨지고 있다. 세포의 노화는 지속적인 세포분열에 의한 스트레스 즉, 분열 스트레스(replication stress) 뿐 아니라 영양결핍, 산화 스트레스, DNA 손상과 같은 다양한 생리적 스트레스에 의해서도 유발되며, 이들의 누적은 '조기 노화(premature senescence)'를 가져오게 된다. 혈관의 신생 및 항상성 유지에 중요한 역할을 하는 EPC의 경우도 고혈압, 당뇨와 같은 스트레스에서 조기 노화가 진행됨이 보고된 바 있다.

IR(감마선 혹은 X-선) 노출도 그 양에 따라 암, 골수부전, 노화 등을 다발성하여 나타내는데, 특히 세포수준에서는 심각한 DNA 손상을 유발하여 세포주기 정지(cell cycle arrest)나 노화와 같은 DNA 손상반응들(DNA damage responses, DDR)을 촉발한다. 세포주기에서는 주로 p53에 의존적으로 세포주기 억제단백질인 p21Cip1의 발현을 증가시키는 것으로 알려져 있다. 뿐만 아니라 노화가 진행된 성체에서 추출한 줄기세포에서도 이러한 DNA 손상의 축적이 관찰되며, 손상된 DNA 치료 시스템에 문제가 있는 생쥐모델의 경우 과양의 DDR이 나타나며, 노화가 촉진되고 줄기세포의 기능이 소실됨이 보고되었다. 원자폭탄 피해자 중 생존자나 핵 관련산업 노동자들과 같이 급성 혹은 만성 IR 노출과 혈관질환 발생은 연관성이 깊다고 알려져 있지만, 이러한 IR 유발성 혈관질환의 발생기전과 이 과정에서 혈관신생, 혈관항상성에 핵심적으로 작용하는 EPC의 역할에 대해서는 거의 알려져 있지 않다.

본 연구는 IR에 노출된 생쥐 및 인체의 EPC의 경 우 그 수가 감소하고, 세포성장 중지와 함께 기능 이상이 유발되며 혈관재생이 지연된다는 것을 보여준 첫 연구라는 데 큰 의미가 있다. 또한 이 과정에서 p53에 의존적인 세포주기 억제단백질 p21Cip1의 발현 증가와 VEGF의 발현 감소가 확인되어, IR 유발성 혈관손상의 새로운 기전을 제시한 부분에 대해서도 의미를 찾을 수 있었다.

이 연구의 결과를 좀 더 자세히 살펴보면(Figure 1), 3.0Gy의 감마선을 온 몸에 조사한 생쥐에서 분리된 EPC는 1일 후 급격히 감소하다가 서서히 증가하여 보통 10일 전후로 회복되는 정상(대조군: non IR) EPC와는 달리 31일이 지나도 회복되지 않았고, IR후 10일 째의 EPC는 β-galactosidase 강양성을 보여 조기노화가 진행되었음을 확인하였다. 하지허혈 손상모델에서도 하지의 혈류량을 측정한 결과, IR군은 10일이 지나도 정상군에 비해 혈류가 감소하였으나, 정상 EPC를 동시에 국소 주입한 IR군은 혈류가 회복되었다. 이들의 p21의 mRNA 발현은 방사선 조사 직후 증가하기 시작하여 8시간째에 가장 높았으며, VEGF의 mRNA 발현은 이와는 정반대로 나타났다. 이러한 결과는 인체의 EPC에서도 같은 양상이었으며, p53에 의존적인 양상을 보였다. 마지막으로 siRNA를 이용하여 p53을 선택적으로 억제한 EPC의 경우, IR에 의한 p21의 mRNA 발현 증가와 VEGF의 mRNA 발현 감소가 완전히 차단되었는데, 이는 p53 의존적 변화임을 재확인한 결과였다.

이상의 결과들은 임상적으로 p53을 억제하여 방사선 치료 혹은 방사선 노출에 동반되는 EPC의 수적 감소와 기능손상을 최소화함으로써 전리 방사선에 의해 유발되는 혈관질환을 줄이는 전략적 치료에 이용될 수 있을 것이다.
The irradiation-exposed EPCs showed a significantly reduced capability as well as re-endothelialization. As predicted, this idea, human EPCs with or without IR pre-exposure were subjected to functional analysis to determine tubule-forming defects in EPC function after IR were repeatedly validated by DNA damage by IR in EPCs (Figure 3 and 4H). DNA damage after radiation exposure to hEPCs was revealed by the level of p21Cip141 (Figure 4H). Both senescent growth senescence of EPCs may result in functional defects. To test senescence of EPCs may result in functional defects. To test

**Figure 4.** The relative population of EPCs was quantified by FACS analysis using PE-Sca1 and APC-c-kit antibodies post depletion of Lin12/c-kit.

**Figure 1.** Recovery of the blood flow rate is presented as a graph. ***

Bone marrow mononuclear cells (BM-MNCs) were isolated from TBI mice at indicated times and counted.

**Figure 2.** Hind-limb ischemia. The relative population of EPCs was quantified by FACS analysis using PE-Sca1 and APC-c-kit antibodies post depletion of Lin12/c-kit.

Effect of Ionizing Radiation Induced Damage of Endothelial Progenitor Cells in Vascular Regeneration

Mi-Ok Lee, Seung-Hyun Song, Seokyun Jung, Seulgi Hur, Takayuki Asahara, Hyongbum Kim, Sang-Mo Kwon, Hyuk-Jin Cha

Objective—A number of studies have revealed that stress signaling and subsequent stress responses in stem/progenitor cells are responsible for attenuated regeneration or degenerative disease. Because ionizing radiation (IR), which sensitizes diverse types of stem cells, reportedly induces cardio-circulatory diseases, we hypothesized that IR-induced vascular abnormalities are associated with defects in endothelial progenitor cells (EPCs) that are responsible for vascular homeostasis.

Methods and Results—We used an irradiated mouse model to mimic the IR effect on vasculogenesis. Mouse EPCs isolated from irradiated mice and human EPCs exposed to IR were used for functional analysis and gene expression study. Under IR exposure, EPCs were depleted, and their function for vasculogenesis in vitro and in vivo was significantly reduced. In such IR-mediated stress responses, upregulating p21Cip1 and downregulating vascular endothelial growth factor (VEGF) were mediated by p53 transcriptional activity.

Conclusion—The results of the present study suggest that suppression of p53 would be clinically applicable to (1) minimize the functional defects in EPCs in order to prevent the onset of vascular diseases caused by radiation therapy or radiation exposure and also to (2) provide novel insight into the mechanisms of IR-induced vascular damage and a possible strategy to minimize vascular damage by IR. (Arterioscler Thromb Vasc Biol. 2012;32:343-352.)

Key Words: aging ■ signal transduction ■ DNA damage ■ ionizing radiation ■ p53

Stem cell abnormality has been considered a main cause of a variety of cancers and degenerative diseases. Likewise, decreased stem/progenitor cell function has been suggested to result in reduced regeneration potential in aged tissue.1 This hypothesis is supported by a number of studies demonstrating progenitor cell depletion and decreased differentiation potential during aging.2,3 Cellular senescence is caused not only by replication stress, which is caused by continuous cell division, but also by a variety of physiological stresses such as nutrient deprivation, oxidative stress, and DNA damage, whose accumulation results in “premature senescence.”4 Premature senescence in stem cell populations grown under diverse stress conditions may be closely linked to depletion and/or dysfunction of progenitor cells resulting in subsequent physiological responses (eg, aging or degenerative diseases).5,6

Endothelial progenitor cells (EPCs) have been widely studied because of their regenerating potential and ability to maintain homeostasis of the endothelial system, which has potential application in cell-based therapies.7 However, similar to other stem/progenitor cells, EPCs undergo premature senescence and dysfunction under a variety of stress conditions such as hypertension, diabetes, and hyperglycemia.8-10 Thus, abnormalities of the circulatory system, which are frequently found in stress-induced vascular diseases, may be strongly associated with senescence or dysfunction of EPCs. This idea was supported by studies demonstrating a strong correlation between cell numbers of EPCs and a variety of cardiovascular diseases.11

Exposure to ionizing radiation (IR; γ or X-rays) triggers diverse physiological responses such as cancer, gastrointestinal failure, bone marrow failure, and accelerated aging depending on a wide range of radiation doses.12 On the cellular level, IR results in serious DNA damage triggering DNA damage responses (DDR) such as cell cycle arrest and senescence, which are largely dependent on p53 transcriptional activity and subsequent induction of p21Cip1, a cell cycle inhibitor protein.13 Interestingly, accumulation of DNA damage under normal conditions has been observed in stem cells isolated from aged organisms.14 Additionally, a mouse...
model with deficiencies in the DNA repair system causing a massive DDR suffered from accelerated aging and significant stem cells loss. Therefore, chronic DNA damage accumulation in stem/progenitor cells, and subsequent DDR during aging, will influence both the onset of senescence and the dysfunction of stem cells that occurs with aging.

Recently, acute or chronic radiation exposure, such as that of A-bomb survivors or nuclear industry workers, has been revealed to be strongly associated with circulatory diseases. However, with few exceptions, little is known about how IR exposure induces cardiac or circulatory diseases. EPCs play crucial roles in maintaining circulatory system homeostasis and are sensitive to a variety of stresses; therefore, it is readily hypothesized that IR-induced abnormalities of the circulatory system would be derived from stress responses of EPCs (especially DDR) under IR exposure.

The present study is the first to demonstrate that radiation exposure in either mice- or human-cultured EPCs induces senescent growth arrest and functional defects, which lead to attenuated vascular regeneration. The growth arrest and senescent phenotype, as well as functional defects in EPCs, are dependent on p53 transcriptional activity on IR, which leads to p21Cip1 upregulation and vascular endothelial growth factor (VEGF) downregulation. Therefore, the present study implied that functional defects in EPCs from IR in a p53-dependent manner would be responsible for the failure of circulatory homeostasis that may be linked to IR-induced circulatory diseases. Furthermore, the study suggested that transient inhibition of p53 transcriptional activity would be applicable to minimize the damage to EPCs from IR exposure. The present study provides a novel mechanism to address IR-induced cardiovascular damage, and it indicates that transient p53 inhibition could protect EPCs from IR damage.

Materials and Methods

Animals
Experiments were performed in male 8- to 10-week-old C57BL/6J mice (Orient, Sung-Nam, Korea) maintained under a 12-hour light/dark cycle and in accordance with the regulations of Pusan National University. The experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Pusan National University. All procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication no. 85-23, revised 1996).

In Vivo Neovascularization Assay Using a Hind-Limb Ischemic Model
To develop the hind-limb ischemic model, C57BL/6 mice were anesthetized by IP injection of 300 mg/kg Avertin (cat. #T48402-5G, Sigma-Aldrich, St. Louis, MO). An incision was made in the skin overlying the middle portion of the left hind-limb. After ligation of the proximal end of the femoral artery, the distal portion of the saphenous artery was ligated as well as all side branches. Cell transplantation of mouse or human EPCs was performed with 2 or 5×10⁵ cells per mouse by intravascular or intramuscular injection. In cases of transplantation of human late EPCs into C57BL/6 mice, Cyclosporin A (cat. #C1832, Sigma-Aldrich, St. Louis, MO) was subcutaneously injected daily to suppress immune response.

Monitoring of Hind-Limb Blood Flow and Capillary Density
After anesthesia, hind-limb perfusion was measured using a laser Doppler perfusion imaging system (LDPI; Moor Instruments, Wilmington, DE). The stored perfusion values behind the color-coded pixels representing the microvascular blood flow distribution were used for analysis. Color photographs were recorded, and the analysis was performed by calculating the average perfusion of the ischemic and nonischemic foot. To account for variables such as ambient light and temperature, the results are expressed as the ratio of perfusion in the ischemic versus normal limb. To analyze capillary density of recovered tissue, frozen sections (10 mm) of adductor muscles from ischemic mice were prepared for immunohistochemistry with rabbit antimmouse CD31 antibody (BD).

Monitoring of Survived Cells in Ischemic Sites
To monitor the survived EPCs in the ischemic area, 5×10⁵ of human EPCs (hEPCs: control hEPCs and 5 Gy-irradiated hEPCs) were labeled by Dil-conjugated Ac-LDL (Dil-Ac-LDL) (Biomedical Technologies Inc., MA, USA). At 7 days after generation of hind-limb ischemia followed by transplantation of control hEPCs and IR-hEPCs, frozen sections (10 mm) of adductor muscles from the ischemic area were prepared for immunohistochemistry for CD31 (BD). The surviving cells were analyzed using photomicrographs of chemical staining for acetyl LDL (Ac-LDL)-Dil uptake (red) or immunostaining for endothelial cell (EC) markers, CD31 (PECAM-1, green) to visualize the regenerated vessels in the ischemic tissue.

Cell Culture
Human late EPC and human umbilical vein endothelial cells were cultured in EBM2 medium (cat. #CC-3156, Lonza, Basel, Switzerland) containing EGM-2 MV SingleQuots (cat. #CC-4147, Lonza, Basel, Switzerland) on fibronectin-coated dishes, and cells were passaged every 3 days.

Ionizing Radiation
Mice or cells were exposed to various doses of IR in an IBL 437C Cesium γ-iradiator at a rate of 2.4 Gy/min.

Isolation of Bone Marrow Mononuclear Cells, Lineage Negative Cells, and Lin⁺Neg/c-kit+/Sca1+ Cells
Bone marrow mononuclear cells (BM-MNCs) and lineage negative (Lin−) cells were isolated as described previously. Two days after total body irradiation (3Gy), mononuclear cells were isolated from bone marrow using the Ficoll gradient method. Lineage-determined (Lin−) cells were removed from BM-MNCs using a hematopoietic stem cell enrichment set (BD bioscience, cat. # 558452), and then Lin− cells were counted. For FACS analysis, Lin− cells were costained with PE-SCA1 antibody (BD Pharmingen™, cat. #, 555336) and APC-C-kit antibody (BD Pharmingen™, cat. #553356) and then analyzed with BD FACSCalibur™.

siRNA Delivery to EPCs
The siRNAs for the human TP53 and negative control were purchased from Bioneer (Daejeon, Korea). Human late EPC cells were transferred to 6-well plates (1×10⁵ cells/well) and incubated overnight. The following day, the cells were transfected with 20 mmol/L of the appropriate siRNA using DharmaFECT siRNA transfection reagent (cat. # T-2001-02, Thermo Scientific Dharmacon, Lafayette, CO) according to the manufacturer’s protocol. After 24 to 48 hours, siRNA transfected-cells were used for further experiments.

EPC Colony Forming Assay
CD34+ cells were cultured in methylcellulose-containing medium SP H4236 (StemCell Technologies, Vancouver, Canada) with 100 ng/mL stem cell-derived factor (Kirin, Tokyo, Japan), 50 ng/mL.
vascular endothelial growth factor (R&D Systems, Minneapolis, MN), 20 ng/mL interleukin-3 (Kirin), 50 ng/mL basic fibroblast growth factor (Wako, Osaka, Japan), 50 ng/mL epidermal growth factor receptor (Wako), 50 ng/mL insulin-like growth factor-1 (Wako), 2 U/mL heparin (Ajinomoto, Tokyo, Japan), and 30% FBS on 35 mm dishes for 21 days. Cell densities for each sample were as follows: CB-CD34+ cell, 500 cells/dish; PB-CD34+ cells, 1.5x10^4 cells/dish. The EPC-colony forming units (CFUs) were identified as large EPC-CFUs or small EPC-CFUs by visual inspection with an inverted microscope under 40x magnification. Large EPC-CFUs were composed of spindle-shaped cells, and small EPC-CFUs were composed of round adhesive cells.

**Proliferation Assay**

1x10^5 cells were plated in a 6-well dish, and the following day the cells were irradiated with X-rays (5 Gy) for various lengths of time. At the indicated times, the cells were counted using a hemocytometer.

**β-Galactosidase Activity Assay**

Irradiated EPC were cultured in EB2 media for 4 days. Following the culturing time, senescence cells were detected using a Senescence β-Galactosidase Staining Kit according to the manufacturer’s protocol (cat. #9860, Cell Signaling Technology, Danvers, MA).

**Matrigel Tube Formation Assay**

Cultured human late EPCs were irradiated with 5 Gy of X-rays and then serum-depleted in EB2-media with 0.02% FBS for 24 hours. Next, the cells were transferred into growth-factor-reduced Matrigel (cat. # 354230, BD Biosciences, San Jose, CA) in 96-well plates (1x10^3 cells/wells). After 24 hours, tube formation by the EPCs was observed and analyzed.

**Migration Assay**

Late EPC cells were cultured on fibronectin-coated 12-well plates and then irradiated with 5 Gy of X-rays. Cells were serum-depleted in EB2-media with 0.02% FBS for 24 hours. After the serum-depletion, a line was scratched into the cell cultures. Areas refilled by EPC migration were monitored and compared among each experimental group.

**Quantitative Real-Time PCR and RT-PCR**


**VEGF ELISA**

Human late EPCs with or without irradiation (X-ray, 5Gy) were cultured with EB2 media containing 0.05% FBS for 3 days. Then media was collected and concentrated 30-fold with Amicon Ultra-4 (Millipore, cat. # UFC800324). Protein levels of VEGF were determined by Human VEGF QuantiGlo ELISA Kit (R&D Systems, cat #QVE00B).

**Immunoblotting**

Immunoblotting analysis was performed as described elsewhere. Primary antibodies used in the present study were as follows: antip53 (cat. #sc-126, Santa Cruz Biotechnology, Santa Cruz, CA), antip21 (cat. #397, Santa Cruz Biotechnology, Santa Cruz, CA), and antipH2AX (cat. #9718s, Cell Signaling Technology, Danvers, MA).

**Statistical Analysis**

The graphical data are presented as the mean±SD. Statistical significance among groups and between groups was determined using 1-way or 2-way ANOVA following application of Bonferroni multiple comparison posttest and Student t-test, respectively. Significance was assumed for P<0.05 (*). Statistical analysis was performed using SAS statistical package, v.9.13 (SAS, Cary, NC).

**Results**

**Depletion of EPCs by IR**

To examine the damage of IR in mice, bone marrow cells were first investigated because bone marrow is considered to be one of the most sensitive tissues to IR-mediated damage. Considering the relatively high resistance of bone marrow stromal cells (eg, bone marrow mesenchymal stem cells), we focused on monitoring BM-MNCs that contained a variety of circulating cells including hematopoietic stem cells and EPCs. Male mice, 6 weeks old, were exposed to a sublethal dose (3 Gy) of total body irradiation (TBI), and their mononuclear cells (MNCs) were isolated on the indicated day. As shown in Figure 1A, the total population of MNCs in the mouse bone marrow was significantly reduced 1 day after TBI (Supplemental Figure I, available online at http://atvb.ahajournals.org) and gradually recovered over 10 days after TBI as previously described.

However, even 31 days after radiation exposure, the population of MNCs in IR-exposed mice still failed to recover completely (Figure 1A). Cultured mouse EPCs were obtained from MNCs of TBI mice as previously described, and then resultant EPCs, which were positively stained with both lectin and Ac-LDL-Dil, were counted. As shown in Figure 1B, the number of EPCs isolated from MNCs of TBI mice was significantly reduced 1 day after IR and gradually recovered in a pattern similar to MNCs. Reduced numbers of EPCs from the MNCs of TBI mice were again confirmed by immunofluorescence analysis (Figure 1C). For further clarification, MNCs of either control or TBI mice were further separated through Lin+ depletion as described. The resultant cells (Lin<sup>neg</sup> cells) were further quantified by FACS using Sca1 and c-kit antibodies, for which dually positive populations are determined as EPCs. Consistently, the number of L<sup>neg</sup>K<sup>+</sup>S<sup>+</sup> cells isolated from TBI-mice appeared to be markedly lower than that from control mice (Figure 1D, right panel). Of interest, the percentage of Lin<sup>neg</sup>/c-kit+/Sca1<sup>+</sup> cells was dramatically decreased in the total BM-MNCs population, whereas the total Lin− population was only marginally decreased by IR exposure suggesting a possible difference in IR sensitivity between the 2 different types of cells (Figure 1E).

Because IR-mediated stress induces premature senescence in mouse bone marrow stem cells, we reasoned that the depletion of EPCs after IR would be associated with premature senescence. To prove this hypothesis, β-galactosidase staining, which is widely used to identify senescent cells, was performed in EPCs from TBI mice. As predicted, strong β-galactosidase staining, which is indicative of senescent cells, was predominant in EPCs isolated from TBI mice (Figure 1F), as were cultured mouse EPCs that were positively stained by 1-Galactosidase Staining Kit according to the manufacturer’s protocol (cat. #9860, Cell Signaling Technology, Danvers, MA).
stained with both lectin and Ac-LDL-Dil and revealed strong β-galactosidase staining after IR exposure (Supplemental Figure II). This result implies that decreases in EPCs in TBI mice may occur in parallel with the onset of cellular senescence of EPCs after radiation exposure.

**Defective Vascular Regeneration by Radiation Exposure**

Considering that radiation exposure decreases the number of both MNCs and EPCs in vivo, radiation exposure would be expected to have an impact on vascular regeneration potential where EPCs have a major role.31 To test this idea, mice were subjected to TBI, and their blood flow rates were monitored in the hind-limb ischemic model as previously described.32 Consistent with the reduced EPCs under radiation exposure, TBI mice suffered from severe reduction of vascular regeneration compared to the control (Figure 2A). Such reduced vascular regeneration potential was fully recovered by the transplantation of EPCs that were isolated from nonirradiated mice. These results suggest that depletion of EPCs by radiation exposure (Figure 1B) may be closely associated with decreases in vascular regeneration potential in TBI mice. Similarly, tissue necrosis caused by ischemia occurred pre-
dominantly in TBI mice unlike mice with transplanted EPCs (Figure 2B). Representative laser Doppler images determining the blood flow of the ischemic model demonstrate the notable impact of radiation exposure on vascular regeneration potential (Figure 2C). These results clearly indicate that radiation exposure leads to impaired vascular regeneration that is overcome by the transplantation of EPCs.

**Stress Response of EPCs Under Radiation Exposure**

DDR by IR-triggering senescence or growth arrest is largely indicated by specific nuclear foci generation referred to as γ-H2AX foci. Therefore, we attempted to determine γ-H2AX foci in EPCs isolated from TBI mice to link DDR with the onset of senescence. As shown in Figure 3A, strong γ-H2AX foci were observed in IR-exposed EPCs, indicating that senescence onset of EPCs occurs in parallel with DDR as determined by γ-H2AX foci (Figure 3A). Of interest, the level of γ-H2AX-positive cells indicating the DNA-damaged cells were similar in both non-EPC cells (Lin⁺ and Lin⁻/Sca1⁻) and EPC cells (Lin⁻/Sca1⁺, white arrow) (Figure 3B and C). Thus, these results indicate that DNA damage occurs equivalently in all cells of bone marrow under IR exposure.

In response to a variety of stresses (eg, IR), cellular senescence is largely mediated by p53 transcripational activity, upregulating a variety of cell-cycle inhibitor proteins such as p21Cip1 in cell-line models and primary stem/progenitor cells. Consistent with these results, we found that IR-mediated stress in isolated EPCs significantly upregulates p21Cip1 (Figure 3D). Early EPCs isolated from MNCs function in vascularization by secreting a variety of angiogenic factors such as VEGF. Therefore, we measured the VEGF expression level to reason the inhibited vascular regeneration potential in TBI mice as shown in Figure 2. The VEGF mRNA expression (Figure 3E) and protein levels, as determined by ELISA (Supplemental Figure III), were significantly suppressed in the cultured EPCs after radiation exposure. Thus, we concluded that the stress response in EPCs by radiation exposure occurs with senescence and VEGF suppression, which may account for defective vascular regeneration in TBI mice (Figure 2).

**Defective Human Cord Blood EPCs Under IR**

Because murine-cultured EPCs after TBI or direct IR exposure ex vivo were demonstrated to undergo senescence and/or acquire functional defects, we extended our studies to human EPCs in a similar manner. Because EPC populations were dramatically reduced in TBI mice (Figure 1B and C), development (or differentiation) of EPCs from stem/progenitor cells may be affected by IR exposure. To test this idea, a CFU assay was carried out to determine the differentiation potential of human EPCs from CD34⁺ positive stem cells isolated from human cord blood as previously described (Supplemental Figure IV, for characterization). As predicted, CFUs of late EPCs were markedly reduced compared to the control (Figure 4A) suggesting that IR may also impair the developmental cascade into endothelial lineage progenitors. In addition, the proliferation potential of cultured human EPCs was markedly reduced after irradiation (Figure 4B), which occurred in parallel with increased senescent populations as determined by β-galactosidase staining (Figure 4C). Considering that stem/progenitor cells senescence may be relevant to impaired function of stem cells in vivo or in vitro,5,38,39
senescence of EPCs may result in functional defects. To test this idea, human EPCs with or without IR pre-exposure were subjected to functional analysis to determine tubule-forming capability as well as re-endothelialization. As predicted, radiation-exposed EPCs revealed significant functional defects in both tubule formation (Figure 4D) and re-endothelialization (Figure 4E) compared to the control. Percentages of re-endothelialized area after scratching were assessed and represented as a graph (right panel). F. Blood flow rate of hind-limb ischemic mice model was measured after cell transplantation with dPBS, control EPCs (NT_EPCs) or irradiated EPCs (IR_EPCs). Blood flow rate was increased in mice transplanted with NT_EPCs compared to IR_EPCs (left panel). Representative laser Doppler images of blood flow rate are displayed (right panel).

Figure 4. Defective human cord blood endothelial progenitor cells (EPCs) under ionizing radiation (IR) (A) CD34+ cells, isolated from human cord blood, were irradiated by 5 Gy of X-ray. Colony forming unit (CFU) of EPCs from CD34+ cord blood stem cells with and without IR exposure was determined (right panel); the percentage of colony number formed during culture is represented as a graph (left panel). B. Proliferation rate of cultured human EPCs with and without IR exposure was determined by cell counting on the indicated day (NT: control, IR: 5 Gy of X-rays). C. The senescent cells (indicated by arrows) were identified by β-galactosidase activity assay 4 days after IR. D. Tubule formation of EPCs with (5 Gy, 24 hours after IR) and without IR on the Matrigel (left panels) was determined by assessing the number of tubule junctions (top panel) and average tubule length (bottom panel); the percentage is represented as a graph. E. Migration level of EPCs with (5 Gy, 24 hours after IR) and without IR was determined by measuring the re-endothelialized area after scratching (left panel). Percentage of re-endothelialized area is represented as a graph (right panel). F. Blood flow rate of hind-limb ischemic mice model was measured after cell transplantation with dPBS, control EPCs (NT_EPCs) or irradiated EPCs (IR_EPCs) respectively. ***P<0.001 (left panel). Representative laser Doppler images of blood flow rate are displayed (right panel). G. Quantification of capillary density by staining with CD31, an endothelial marker, in ischemic tissues after cell transplantation with dPBS, control EPCs (NT_EPCs) or irradiated EPCs (IR_EPCs), respectively. **P<0.01, ***P<0.001. H, p53, p21, and γ-H2AX protein level by IR was determined by immunoblotting analysis. ERK2 levels were used as the equal loading control (bottom panel). I, mRNA level of p21Cip1 (left panel) and VEGF (right panel) were determined by qRT-PCR analysis, and the fold change is presented as a graph. 

Consistent with the result in Figure 4F, chimeric blood vessel formation of IR-exposed hEPCs was markedly reduced, indicating that DNA damage by IR in EPCs (Figure 3 and 4H) significantly affects their vascular regeneration potential (Figure 4G). DNA damage after radiation exposure to hEPCs was revealed by the level of γ-H2AX and accumulated p53 protein levels, which leads to p53-dependent gene transcriptions such as p21Cip1 (Figure 4H). Both senescent growth arrest and defective function of EPCs leading to impaired vascular regeneration activity would be dependent on p53 transcriptional activity considering distinct p53 accumulation and subsequent p21Cip1 induction as well as VEGF suppression after IR (Figure 4I). These results strongly indicate that radiation exposure induces DNA damage in EPCs triggering senescence and functional defects in EPCs. The DNA damage stress responses, which are responsible for the senescence and function defect in EPCs, may be correlated with p53-
dependent alteration in gene expressions such as p21Cip1 and VEGF.

**Functional Defects in IR-Exposed EPCs is Mediated by p53**

In order to examine whether p53 is responsible for the DNA damage stress response in EPCs, senescence and functional defects were determined either with or without p53. To achieve suppression of p53, p53siRNA (siTP53) was introduced to human EPCs and considerable p53 suppression was achieved (Figure 5A). In the absence of p53, reduced proliferation potential of cultured EPCs and senescence onset that were both caused by IR was reversed (Figure 5B and 5C). Additionally, the tubule-forming capability of EPCs (determined by both the number of tubule junctions and the average length of the tubules) that was strongly repressed by IR (Figure 4D) was significantly reversed by suppressing p53 (Figure 5D). In a similar fashion, the migration capability of EPCs that was inhibited by IR (Figure 4E) was also remarkably recovered by p53 suppression under IR (Figure 5E).

Because upregulation of p21Cip1 and downregulation of VEGF expression, which both occur in a p53-dependent manner, were found in IR-exposed EPCs along with decreased proliferation potential and senescence onset as well as functional defects in vascularization, the restoration of function of cultured EPCs with p53 suppression may result from altered p21Cip1 and VEGF expression. As shown in
Figure 5F, levels of p21Cip1 mRNA (left panel) and protein (right panel), which are upregulated by IR, were markedly reduced in p53-suppressed EPCs (siTP53) compared to the control (siNC) after IR exposure. In the case of VEGF expression, p53 suppression markedly increased basal levels of VEGF expression even in the control. Considering the negative effect of p21Cip1 expression on the functional regulation of EPCs, a significant loss of p21Cip1 expression by the knock-down of p53 (Figure 5G, top panel) may be linked to higher expression of VEGF in either control or irradiated EPCs (Figure 5H). These results clearly imply that increased p53 transcriptional activity and altered gene expression by radiation exposure induce stress responses in EPCs such as cell growth arrest, senescence, and vascularization. Such stress responses occurring in IR-exposed EPCs in a p53-dependent manner would be responsible for defective vascular regeneration.

Discussion

As stem/progenitor cells are responsible for regenerating damaged or senescent somatic mature cells, maintenance of an adequate pool of stem/progenitor cells would be critically important for retaining tissue homeostasis. Thus, abnormalities in stem/progenitor cells are considered to be a cause of several serious diseases such as cancers, degenerative disease, and even aging. Therefore, stress induced by external or internal factors such as IR, oxidative damage, lack of nutrients, or exposure to carcinogens would dramatically influence the pool of stem/progenitor cells. Consequently, both oxidative stress and IR have a significant effect on hematopoietic stem cell lifespan. Because IR has a serious negative impact on hematopoietic stem cells leading to bone marrow failure or leukemia, functional defects of stem cells should be closely associated with diverse physiological responses. Therefore, we attempted to explore the pathophysiological effects on EPCs by IR because cardiac or circulatory disease has been shown to be caused by both acute and chronic IR exposure, and EPCs, which play crucial roles in maintaining the circulatory system, are susceptible to a variety of stresses.

The present study demonstrated that growth arrest or senescence onset and functional defects in EPCs by IR exposure occurred along with retarded vascular regeneration. We also showed evidence that the stress response of EPCs after IR exposure is mediated by p53 activation because suppression of p53 reversed the effects of IR on EPCs, growth arrest, and functional defects (Figure 5). These results are consistent with previous studies demonstrating the important roles of p53 in the functional regulation of EPCs under various stress conditions. For example, circulating EPCs are decreased and undergo senescence-like growth arrest, which are both dependent on p53 activation, and follow p21Cip1 expression in a diabetes-induced stress model. Likewise, p21Cip1, a downstream target of p53, which induces cell-cycle arrest and senescence onset under various stress conditions, has been shown to contribute to the turnover rate of mature EPCs. Lack of p21Cip1 not only increases the proliferation and survival of EPCs but also promotes vascular regeneration. Because ECs reportedly play an important role in radiation-induced vascular injury by increasing proliferation, migration, and fibrinogenic phenotype, the possibility of crosstalk between ECs and smooth muscle cells may be an important factor in retarded vascular regeneration phenotype and/or endothelial apoptosis by IR exposure. Considering the increased expression of transforming growth factor-β, subsequently activated Smad signaling by IR, and its strong correlation to apoptosis, it would be interesting to study the possible role of transforming growth factor-β signaling in ECs by IR during radiation-induced vascular injury and retarded vasculogenesis. In addition, we also provided evidence that EPCs appeared to be more sensitive to the DNA damage stress than ECs (Supplemental Figure IX). Thus, we surmised that defects in EPCs by IR-induced DNA damage stress, would be more closely associated with reduced vascular regeneration by IR exposure. Of note, vascular damage by IR exposure can be ameliorated by cell types other than EPCs. For example, irradiated tissue may be rescued by adipose tissue-derived stromal cells, suggesting that cell therapy is a possible therapeutic modality for vascular damage by irradiation.

These studies strongly imply that p53 and/or p21Cip1 are important for regulating EPC growth and function following radiation exposure. Furthermore, we provided evidence that VEGF, a crucial growth factor for EPC function and survival, is suppressed by p53, which has also been demonstrated in diverse cancer cell models. Thus, suppression of VEGF by activated p53 due to radiation exposure is likely to be responsible for functional defects in EPCs, as shown by tubule formation and migration (Figure 4). This notion is well supported by results showing that increased VEGF by p53 suppression, even under exposure, occurred with the restoration of EPC function (Figure 5). Further mechanistic studies of VEGF suppression by p53 transcriptional activation are currently under active investigation. Considering that cardiac and circulatory diseases are serious side effects of radiation exposure, understanding the molecular mechanism of IR-mediated circulatory diseases would be important for minimizing risk. Accordingly, we suggest that transient inhibition of p53 transcriptional activity by a pharmacological inhibitor may minimize the damage to EPCs by IR and possibly reduce circulatory disease caused by radiation exposure. This interesting idea is being actively investigated. Taken together, we demonstrated that radiation exposure caused cell senescence and functional defects in EPCs, which may be responsible for inhibited vascular regeneration. Such radiation-related effects were reduced by p53 suppression, lowering p21Cip1 induction and retaining VEGF expression, suggesting that p53 transcriptional activation and the resultant gene response would contribute to the stress response of EPCs.

Sources of Funding

This work was supported by the National Research Foundation of Korea (2011006300) and Basic Science Research Program (M-2011-A0403-00080) (H.J.C.), and by a National Research Foundation grant funded by the Korean government (2010-0020260) (S.M.K.).


