

COMP-Ang1, Angiopoietin-1 Variant Protects Radiation-Induced Bone Marrow Damage in C57BL/6 Mice

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Angiopoietin 1/Bone marrow protection/COMP-Ang1.

Angiopoietin-1 (Ang1) is a vasculogenic factor which is signaled through the endothelial and bone marrow cell-specific, Tie2 receptor tyrosine kinase and has potential therapeutic applications for the induction of angiogenesis, enhancing endothelial cell survival, and preventing vascular leakage. In this study, we examined whether Ang1 directly exhibits bone marrow protection after ionizing radiation (IR) using an adenoviral vector of COMP-Ang1 (Ad-COMP-Ang1). This is a variant of Ang1 by replacement of the N-terminal portion of Ang1 with short coiled-coil domains of cartilage oligomeric matrix protein-Angiopoietin 1 (COMP-Ang1) which are, long enough for oligomerization but short enough to avoid problems of aggregation and insolubility. A spleen colony assay after 4.5 Gy whole body radiation, indicated that COMP-Ang1 significantly increased the mean colony numbers. Both the decrease in bone marrow cellularity and increased TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) positive cells produced by radiation in bone marrow were significantly inhibited by COMP-Ang1 transfer. The expression of the ligands of Ang1 and Tie2 receptors were increased by radiation and, the COMP-Ang1 transfer potentiated this protein expression. Pre-treatment of Ang1 could be beneficial in protecting bone marrow from damage by radiation and COMP-Ang1 may be an effective alternative to native Ang1 for therapeutic purposes.

INTRODUCTION

Myelosuppression is one of the major side effects of radiotherapy and chemotherapy in the treatment of solid tumors. In the early years of research, a variety of immune-modulating agents (e.g. *Corynebacterium parvum*) were shown to be capable of stimulating hematopoiesis at the stem and committed progenitor cell levels. However, adverse side effects have been associated with these agents due to their potentially infectious capabilities, antigenic properties and undefined chemical constituents.^{1–3)} In subsequent years, many similar agents were tested and found to be effective radioprotectors, including glucans and amifostine (WR2721).^{4,5)} Amifostine is perhaps the most effective agents, but the toxicities such as hypotension, nausea, vom-

iting, and allergic reactions were reported.^{6,7)} Moreover, it has limitations in its use as a protector of bone marrow (BM) damage.^{8,9)} Therefore, the search for newer and more effective radioprotective agents remains an inevitable necessity.

Angiopoietin-1 (Ang1) and Ang2 have been identified as secreted protein ligands of the endothelial cell-specific epidermal growth factor homology domains (Tie) receptor, which are the receptor tyrosine kinases (RTKs).^{9–13)} *In vivo* analysis by targeted gene inactivation reveals that, Ang1 recruits and sustains periendothelial support cells,¹⁴⁾ whereas, transgenic overexpression¹⁵⁾ or gene transfer¹⁶⁾ of Ang1 increases vascularization *in vivo*. *In vitro* experiments have shown that Ang1 has specific effects on endothelial cells and has little effect on proliferation; However, it potentially induces sprouting,¹⁷⁾ chemotactic response,¹⁸⁾ and network formation.¹⁹⁾ Also, Ang1 is a significant factor in the survival of endothelial cells,^{19–21)} since Ang1 does not cause proliferation in endothelial cells.^{12,17)} As a result Ang1 could be a clinically useful protective factor.

The Ang-Tie ligand-receptor system consists of two receptor tyrosine kinases, Tie1 and Tie2. The Tie receptors are almost exclusively expressed by endothelial cells and hematopoietic stem cells (HSC).^{22–27)} Tie1 and Tie2 share a similar overall structure consisting of an extracellular

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domain with 33% similarity and an intracellular tyrosine kinase domain with 76% similarity.²⁶⁾ The angiopoietins were originally identified as ligands for Tie2, however, no specific ligands have been identified for Tie1.²⁸⁾ Tie1-deficient cells, expressing normal levels of Tie2, contribute to hematopoiesis,^{29–31)} suggesting that Tie2 alone is either not required or plays a role redundant to that of Tie2 in the formation and differentiation of HSC. Thus, a reduced contribution of Tie receptor-deficient cells to adult BM hematopoiesis may be attributed to the loss of Tie2 in HSC. The quiescence of stem cells is of critical biological importance in protecting the stem cell compartments.³²⁾ When stem cells enter the cell cycle and proliferate, this activity results in differentiation to replace damaged and senescent cells.

The Ang1 structure consists of a C-terminal fibrinogen-like domain, which is responsible for receptor binding, a central coiled-coil domain which oligomerizes these fibrinogen-like domains, and a short N-terminal domain which separates these oligomers into variable sized multimers.^{12,33,34)} Despite this, the central coiled-coil domain and N-terminal superclustering domain responsible for the modular and multimeric structure of Ang1 results in protein aggregation and insolubility. Therefore, we modified Ang1 containing short coiled-coil domains of cartilage oligomeric matrix protein to a minimal coiled-coil domain (COMP-Ang1), long enough for oligomerization but short enough to avoid problems of aggregation and insolubility was developed.³⁵⁾

Previously, since COMP-Ang1 inhibited radiation-induced apoptosis in endothelial cells of mouse intestines,³⁶⁾ we sought to demonstrate that adenoviral transfection of COMP-Ang1 protects against radiation-induced damage of BM, another target organ of Tie2.

MATERIALS AND METHODS

Construction of recombinant viral vectors

The plasmid vector was used for the coiled-coil domain of rat cartilage oligomeric matrix protein (COMP) (DLAPQMLRELQETNAALQDVRELLRQQVKEITFLK-NTVMEC-DACG) fused to the fibrinogen-like domain of Ang1 (COMP-Ang1).³⁵⁾ To make viral vectors, the sequence in COMP-Ang1 was inserted into the pShuttleCMV vector. The construct was recombined with pAdEAsy-1 to make recombinant viral vector which contained COMP-Ang1. As a control, we used a recombinant adenovirus which expressed luciferase for the convenient detection of expression in the cell (Ad-LacZ). Typical virus titers were 10^9 – 10^{10} pfu/mL.³⁷⁾

Animals

Female and male C57BL/6 mice were obtained from Japan SLC Inc. (Hamamatsu, Japan) at 7 weeks of ages (average body weight, 18.2 ± 2.1 g) and held for 1 week prior to experiments. Animals were kept under conventional

conditions of free access to water and food. The studies were carried out under the guidelines for the use and care of laboratory animals and were approved by the Institutional Animal Care and Use Committee of the Korea Institute Radiological and Medical Sciences (KIRAMS).

Irradiation and virus infection

The C57BL/6 mice were randomly assigned into three groups (6 mice/group): 1) normal control group, 2) control adenoviral vector transferred and irradiated group (Ad-LacZ), 3) Ad-COMP-Ang1 transferred and irradiated group. At 24 hrs after the intravenous injection of Ad-COMP-Ang1 (1×10^8 pfu /head), whole body irradiation was administered using a ^{137}Cs γ -ray source (Atomic Energy of Canada, Ltd., Ontario, Canada) at a dose rate of 3.51 Gy/min.

Sample analysis

Samples from femurs were obtained at 1, 4, 7, 14, and 21 days following radiaton. Femurs were fixed by formalin, decalcified with CaciClear™Rapid (National Diagnostics Inc., Atlanta, USA), embedded in paraffin, and sectioned at 3–4 μm . The sections were stained with hematoxylin-eosin, examined for evidence of pathological changes, and measured for bone marrow cellularity by using an image analyzer.

Immunohistochemistry

Sections were taken at 3 μm , dewaxed and then rehydrated. For immunoperoxidase labeling, endogenous peroxidase was blocked with 0.3% H_2O_2 in absolute methanol for 15 min at room temperature. For antigen retrieval, sections were placed in a citric buffer (pH 6.0) which was heated in a microwave oven for 10 min. Non-specific immunoglobulin binding was prevented by incubating sections in blocking solution (Cap-plusTM detection kit, Zymed Laboratories, San Francisco, U.S.A.) for 20 min. Sections were incubated overnight at 4°C with diluted Tie2 antibody (1:200) (Santa Cruz, USA), and washed with PBS containing 0.05% Triton X-100 (3 × 5 min). Incubation with the corresponding secondary anti-rabbit immunoglobulin G and the peroxidase-antiperoxidase (PAP) complex was carried out for 30 min at 22°C. The immunoreactive sites were visualized using 3,3'-diaminobenzidine (0.1%), and hydrogen peroxide solution (0.03%).

TUNEL assay

The apoptotic cells were detected, using In Situ Cell Death Detection kits (Roche Diagnostic GmbH, Mannheim Germany), by the indirect TUNEL method. In addition, the paraffin-embedded tissue sections were hydrated and incubated with the TUNEL reaction mixture containing TdT and fluorescein-dUTP without proteinase K pre-treatment. Moreover, the reactions were terminated by washing 3x with PBS. Anti-fluorescein-peroxidase antibody was then

applied, and the reactions were visualized using 3,3'-diaminobenzidine. The sections were counterstained with auto-hematoxylin, and negative control sections were incubated with distilled water in the absence of TdT.

Histologic image acquisition and analysis

Images of BM sections were taken with a digital camera mounted on a microscope (Leica DM IRBE, Leica Microsystems GmbH, Wetzlar, Germany). The quantification was performed using image analyzing software (Leica QWin, Leica Microsystems, Wetzlar, Germany). The labeling incidences of each animal were obtained by averaging the percentages of five or 10 fields, followed by the determination of the mean and standard deviation (SD) at each time interval for 6 experimental animals. We compared the experimental and control data at each time interval using a one-way analysis of variance (ANOVA) followed by the Student's *t*-test. Statistical significance was set *a priori* at $p < 0.05$.

Endogenous spleen colony forming assay

Seven-week-old male C57BL/6 mice (average body weight, weighing 21.3 ± 2.2 g) were used this experiment. Enumeration of the spleen colonies forming Ad-COMP-Ang1 (1×10^8 pfu/head) involved intravenous transfer via the lateral tail vein followed by irradiation. The mice were euthanasia at 9 days after administering of 4.5 Gy gamma-ray irradiation, and the mouse spleens were fixed in Bouin's solution. Individual surface colonies, each comprising the progeny of a single stem cell, were counted and the numbers of colonies per mouse were calculated by gross examination and p values (evaluated by Student's *t*-test, statistical significance set *a priori* at $p < 0.001$).

RESULTS

COMP-Ang1 exhibits colony stimulating activity

Previous data indicate that COMP-Ang1 increased mice survival after lethal dose of radiation.³⁸ To test whether COMP-Ang1 had colony stimulating activity or could modulate proliferation of HSC, we administered Ad-COMP-Ang1 intravenously to mice preceding the 4.5 Gy radiation. No alteration of spleen colonies was evident in either the control or mice treated with Ad-COMP-Ang1 only (data not shown). Conversely, spleen colony numbers were significantly greater in mice which were treated by radiation and Ad-COMP-Ang1 (17.8 ± 8.58), compared to radiation alone (6.2 ± 3.83) (Fig. 1), suggesting that Ad-COMP-Ang1 showed a greater recovery potential in HSC from radiation injury.

COMP-Ang1 inhibits radiation-induced myelosuppression

We found that radiation-induced myelosuppression led to

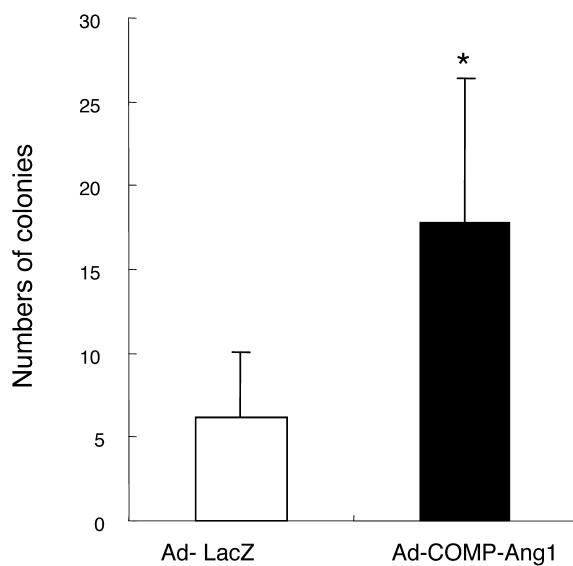


Fig. 1. Increase of colonies in the spleen after the gene transfer of Ad-COMP-Ang1. Ad-LacZ or Ad-COMP-Ang1 was transferred to mice (10 mice per group) at 24 hrs before being irradiated with 4.5 Gy. Colonies (the round or oval, gray in color, and embedded in the spleen), were counted using a macroscope at the 9th day of radiation. The number of spleen colonies were higher in mice treated with Ad-COMP-Ang1 as 17.8 ± 8.58 ($p < 0.001$) than in mice treated with control Ad-LacZ as 6.2 ± 3.83 .

both the depletion of hematopoietic cells, and the induced regression of the majority of BM sinusoidal vasculature. This resulted in profound hypocellularity and disruption of BM cytoarchitecture with diffuse intracavitory hemorrhaging and concomitant peripheral pancytopenia (Fig. 2A). Also, a rapid proliferation of hematopoietic cells with simultaneous regeneration of sinusoidal vasculature in the BM was observed 7 days after radiation. In contrast, treatment with Ad-COMP-Ang1 before administering 4.5 Gy of whole body radiation inhibited hypocellularity and disruption of BM by radiation and demonstrated rapid recovery of hematopoietic cells. The quantification of bone marrow cellularity using the image analyzer also revealed that Ad-COMP-Ang1 significantly reduced radiation-induced hypocellularity (Fig. 2B).

COMP-Ang1 inhibits radiation-induced apoptosis in bone marrow

We found that radiation (4.5 Gy) caused apoptosis in BM, as substantiated by TUNEL positive cells and the BM of normal controls and Ad-COMP-Ang1 individually, did not induce any apoptotic cells. However, the peak induction of TUNEL positive cells was 22% which was observed 24 hrs after radiation; however, this value did not change for an additional 21 days of radiation administering. The Ad-COMP-Ang1 transfer reduced radiation-induced apoptosis in BM by approximately 70% after 24 hrs of radiation (as

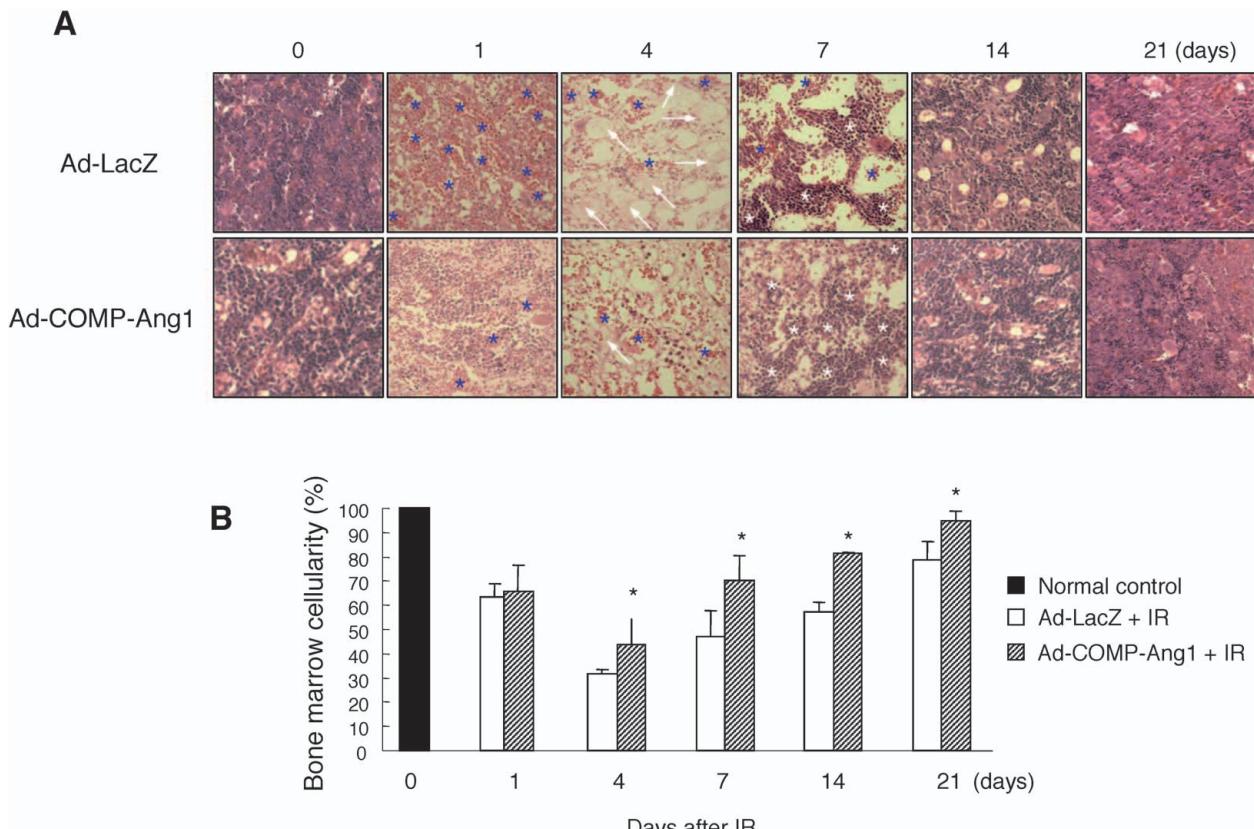


Fig. 2. Ad-COMP-Ang1 enhanced the reconstitution of BM after radiation-induced myelosuppression. **A.** Paraffin sections of BM were stained by H&E and the representative sections at steady state after 1, 4, 7, 14 and 21 days were shown. Radiation (4.5 Gy) induced the disruption of HSC and vasculature in BM. There was severe hypocellularity in bone marrow at 4 to 7 days as well as hemorrhage (blue stars), plasma leakage (white arrows) and regeneration of bone marrow with proliferating cells (white stars) within 14 days after radiation. Ad-COMP-Ang1 transferred mice were observed to have a higher cellularity of HSC in BM during post-irradiation days compared with the Ad-LacZ group (Original magnification was 100 \times). **B.** Quantification of BM cellularity using an image analyzer. The graph indicates the percentage (mean \pm SD) of HSC against the unit area (200 $\mu\text{m} \times 200 \mu\text{m}$, at a magnification of 200 \times) of BM compared with Ad-LacZ transferred group. Each group consisted of 6 mice, and five fields were obtained from each animal with both right and left femurs. Bone marrow cellularity of Ad-COMP-Ang1 treated mice indicated a significantly quicker recovery than Ad-LacZ treated mice ($p < 0.05$).

measured by the number of TUNEL positive cells), and almost disappeared after 4th day of radiation (Fig. 3). TUNEL positive cells usually found in HSC, however, some positive cells were seen in vasculated cells.

COMP-Ang1 potentiates radiation-induced Tie2 expression in bone marrow

Since Tie2 is reported to be a ligand of Ang1,¹⁸⁾ we examined Tie2 expression in bone marrow by immunohistochemistry. Without irradiation (day 0), Ad-COMP-Ang1 alone treatment did not show any significant difference in Tie2 expression, when compared to normal BM. However, radiation of 4.5 Gy alone increased Tie2 expression after 4 days of radiation treatment and remained the same till the end of the experiment. Moreover, the Ad-COMP-Ang1 transfer before radiation treatment demonstrated potentiation of Tie2 protein expression which correlates with the protective effect

of Ad-COMP-Ang1 in BM (Fig. 4).

DISCUSSION

In this study, we demonstrated that COMP-Ang1, which is a variant of Ang1, by replacing the N-terminal portion of Ang1 with the short coiled-coil domain of cartilage oligomeric matrix protein (COMP), is a potential candidate for radioprotection with regards to radiation-induced bone marrow damage. Previously, we were aware of the ability of COMP-Ang1 to protect against radiation-induced endothelial cell apoptosis in small intestinal villi; however, this has not been observed in non-endothelial cells.³⁸⁾ Since Tie2 was reported to be a receptor of COMP-Ang1 and Tie2 is mainly expressed in endothelial and HSC,^{22–25)} we examined if COMP-Ang1 also protects against radiation-induced BM damage.

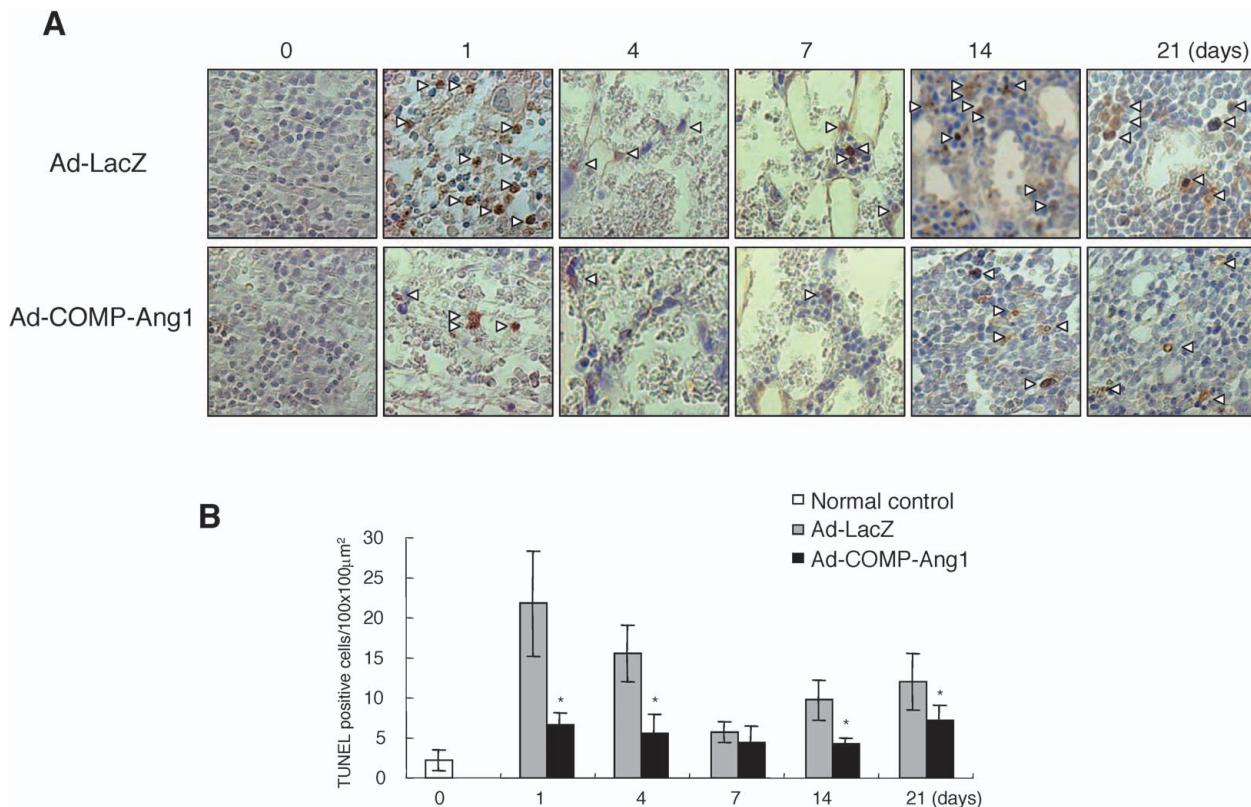


Fig. 3. Ad-COMP-Ang1 reduced apoptosis of BM during myelosuppression. **A.** Mice were killed before irradiation and 1, 4, 7, 14 and 21 days after irradiation. Femurs were collected and prepared for TUNEL assay for the detection of apoptotic hematopoietic cells. Apoptotic positive cells were presented as brown in color (arrow head) in $400\times$ magnification. **B.** An apoptotic positive cell index demonstrated that Ad-COMP-Ang1 treatment significantly reduced cell death by irradiation during radiation-induced myelosuppression periods when compared to the control group ($p < 0.05$). The positive signals were counted under a light microscope in the same area of each field. We counted 10 fields in each femur (field size was $60 \mu\text{m} \times 50 \mu\text{m}$) at an original magnification of $1000\times$.

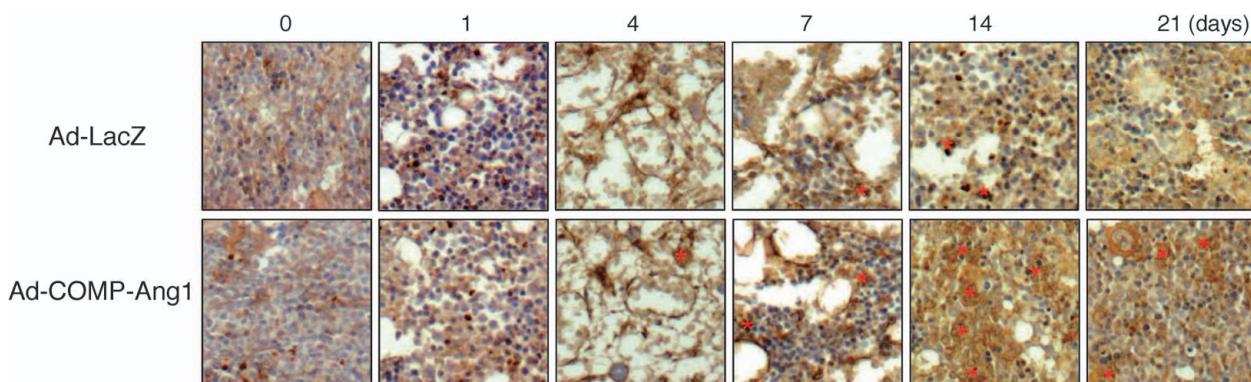


Fig. 4. Radiation induced Tie2 expression was augmented by the gene transfer of Ad-COMP-Ang1. Tie2 expression (red stars) was detected by immunohistochemistry and representative sections of femur at 1, 4, 7, 14 and 21 days after irradiation and the expression pattern of Tie2 in BM were shown at a $400\times$ magnification. There was no difference of Tie2 expression at Ad-LacZ or Ad-COMP-Ang1 transferred BM without IR (at 0 day). Tie2 expression was significantly increased in BM of Ad-COMP-Ang1 administered mice at 14 to 21 days after irradiation when compared to Ad-LacZ treated mice.

The effects of whole body irradiation are primarily attributed to the damage of the BM progenitor cells, which are critically important for survival. Whole body irradiation

drastically impairs normal physiological processes through the damage to such rapidly proliferating cells. Previous data suggested that COMP-Ang1 prolonged survival in mice irra-

diated with 12 or 15 Gy, which was mediated by protection of radiation-induced endothelial cell injury.³⁵⁾ However, because Tie2 is also expressed in HSC and HSC is a major target of radiation injury, here, we examined the effect of COMP-Ang1 in radiation-induced HCS damage. To elucidate the effects of COMP-Ang1 against BM damage, we first assayed for colony stimulating activity by treating with a sublethal dose of radiation (4.5 Gy). We found that colonies prior treatment with Ad-COMP-Ang1 before radiation generated a significantly greater number of colonies, which substantiates the protective effect of COMP-Ang1 on HSC (Fig. 1). Upon examination of bone marrow cellularity, we found that injection of COMP-Ang1 prior to radiation treatment partially restored radiation-induced depletion of hematopoietic cells and regression of the majority of BM sinusoidal vasculature (Fig. 2). Moreover, the induction of radiation-induced apoptosis in BM also was significantly inhibited by pre-treatment with COMP-Ang1 (Fig. 3). Previously, Ang1 was reported as having the ability to maintain the repopulation of HSC by the prevention of cell division.³⁹⁾ In addition, Ang1 promotes the quiescence of HSC *in vivo*, resulting in the protection of HSC from various stresses.^{40,41)} The quiescence of stem cells is of critical biological importance in protecting the stem cell compartment,³²⁾ and protection of stem cells from physiological stress is required for an organism's survival, suggesting that common basic mechanisms contribute to regulation of the cell cycle and cell death in stem cells. Therefore, we hypothesized that Ang1 mediated quiescence of HSC reduce the susceptibility to radiation damage, which corresponds to radioprotective effects of COMP-Ang1.

Tie2/Ang1 signaling is known to contribute to features, which regulate HSC in the BM including, quiescence, cell death inhibition, and tight adhesion.^{40,41)} It is also known that Tie2 is expressed in quiescent HSC of BM.⁴²⁾ Our data suggests that radiation increased Tie2 expression and the gene transfer of COMP-Ang1 potentiated this phenomenon (Fig. 4). Since a report exists, stating that Tie2 expression is up-regulated by the regenerating BM after myelosuppression⁴³⁾ and our data also indicated that Tie2 expression was dominant in HSC (however, some positive cells were found in vasculated cells), we may conclude that regeneration after radiation damage increased Tie2 expression and this regeneration was augmented by COMP-Ang1. Indeed, since apoptotic TUNEL positive cells did not correlated with Tie2 expression (data not shown), we hypothesized that Tie2 positive cells rescued from the apoptotic cell death.

In conclusion, we have shown that COMP-Ang1 can be a possible protective protein against radiation-induced BM damage *in vivo*.

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