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Effects of Adipose Tissue-Derived Stem Cells Transplanted to a Bone Defect after Irradiation

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Abstract: We examined whether the effect of adipose tissue-derived stem cell (ADSC) transplantation improved bone wounds healing irradiation. ADSCs were harvested from F344 rats and the cells cultured until the second passage for transplantation. Before ADSC transplantation, a single dose of 15 Gy irradiation was administered to the head of every rat using ¹³⁷Cs gamma-ray irradiation system, except for the rats of the control group. Two weeks after the irradiation, ADSCs were seeded on a carrier (collagen sponge) and transplanted into the bone defects formed on the rat parietal bones (the irradiation transplant group). This group was compared with two other groups: the carrier only group (without ADSCs) implanted after irradiation (the irradiation group) and; the group in which the carrier was implanted without irradiation (the control group). The results were obtained by histological, immunohistochemistry and ultrastructural observation. We examined the effects of ADSC transplantation on the delay of bone wound healing after irradiation. The new bone formation area of the irradiation group was significantly suppressed ($p < 0.05$) as compared with the other groups. The vascular density at the site of new bone formation in the irradiation group decreased as compared with the other groups. In the irradiation transplant group, the BrdU positive cells were arranged like osteoblasts at the newly formed bone area and were also seen in the vascular wall and the interstitial tissue at the bone defect site. In the irradiation transplant group, VEGF positive cells appeared in blood vessels and the interstitial tissue in the bone defect area, whereas in the other groups, they were hardly found. ADSCs improved bone wound healing after irradiation by direct differentiation of bone forming cells and vascular endothelial cells. In addition, ADSCs have a paracrine effect which induces cell differentiation into VEGF positive cells which acts on angiogenesis.

Key words: Adipose tissue-derived stem cells, Bone regeneration, Radiation injury

Introduction

Radiation therapy is a treatment options for cancer therapy and is especially effective for oral and maxillofacial cancers. Since the irradiation field contains normal tissues around the focus, microcirculatory disturbance takes place, as well as granulation tissue abnormality, retardation of bone renewal and the delay of coating of the epithelium in normal tissues after irradiation. In an irradiated oral lesion, the retardation of wound healing occurs as a side effect of irradiation. It leads to osteonecrosis of the alveolar bone after tooth extraction and extensive jaw osteonecrosis, and the quality of life of the patient deteriorates significantly.

It is known that retardation of wound healing of the bone after irradiation is connected to damages to bone cells and angiogenesis disorders due to the effects of radiation¹⁾. In 1983, Marx et al.²⁾ reported that radiation osteonecrosis occurred due to the reduction of blood vessels and cellular components of the bone tissue, resulting in the tissue becoming hypoxic.

Mesenchymal stem cells (MSCs) are widely used in the study of damaged tissue repair because MSCs have a self-renewal, a differentiating and a proliferative potential^{3, 4)}. Deshpande et al.⁵⁾ reported that the transplantation of bone marrow-derived mesenchymal stem cells (BMSCs) was useful for improving wound healing after irradiation in a dis-

traction osteogenesis study. Until now, most research on regenerative medicine using BMSC has been conducted conventionally. Adipose tissue-derived mesenchymal stem cells (ADSCs) has a high proliferation ability and pluripotency as do BMSCs, and also ADSC harvesting is minimally invasive⁶⁾. ADSCs are superior for clinical applications in comparison with BMSCs. It is reported that ADSCs transplanted in bone wounds are related to bone tissue repair and the promotion of angiogenesis with the ability to differentiate into osteoblasts, fibroblasts and vascular endothelial cells, etc⁶⁾.

In this study, we aimed to clarify the effect and the usefulness of bone wound healing in ADSC transplantation after irradiation.

Materials and Methods

All experimental protocols were guided and approved by Institution Animal Care Center and the ethics committee of Asahikawa Medical University (protocol number 16096).

Cells

The inguinal adipose tissue harvested from male F344 rats (Sankyo Lab Service, Co. Tokyo, Japan) was thoroughly irrigated with 0.01 M phosphate buffered saline (PBS), according to the method described by Zuk et al.⁶⁾. After the tissue was minced, it was shaken at 37°C for 30 minutes in a 0.1% solution of collagenase type I (Roche Diagnostic, Co. Ltd. Tokyo, Japan) to digest the tissue. After the ADSCs were isolated by the enzyme treatment, they were cultured in Dulbecco's modified

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Figure 1. Method for ADSC transplantation. The inguinal adipose tissue harvested from male F344 rats. In the irradiation transplant group, 3×10^5 ADSCs disseminated to collagen sponge and transplanted into the bone defects.

Eagle's medium. We then added 2.5% fetal calf serum (SIGMA-ALDRICH, St. Louis, MO, USA) at 37°C with 5% carbon dioxide (CO_2) for 2 weeks. After the first culture was achieved 80% confluence, the cells were adjusted to 1×10^6 cells/ml with the addition of 0.2% trypsin, and then the cells were cultured for the first passage. We marked the first passage cells with 5-2'-deoxyuridine (BrdU; Sigma Chemical Co., St. Louis, MO, USA) for the tracer experiment to investigate transplanted cell movement. In the cell labeling method, BrdU in the culture solution was adjusted to 10 μM and allowed to stand for 2 hours.

The cells marked by BrdU were cultured in Dulbecco's modified Eagle's medium until the second passage, and then the cells were transplanted to the bone defect made at the parietal bone of the lab animals.

Method for ADSC transplantation

Irradiation

We used 120 eight-week-old male F344 rats (Sankyo Lab Service, Co. Tokyo, Japan). For the radiation method, a single dose of 15 Gy irradiation using the ^{137}Cs gamma-ray irradiation system (Gammacell 40: Best Theratronics Co. Ltd. Canada) was performed to the head region of each rat. Two weeks after the irradiation, we created a 4 mm-diameter bone defect on either side of the parietal bone of the rats using dental micro-motor with a 4 mm-diameter trephine bur under peritoneal anesthesia with pentobarbital (Nembutal® 100 $\mu\text{l}/100$ g: Abott Co Ltd. North Chicago, IL, USA) (Fig. 1).

ADSCs Transplantation to Rat Parietal Bone Defect

We had 3×10^5 ADSCs disseminated to a 4 mm-diameter collagen sponge (Terudermis® TERUMO, Tokyo, Japan) and they were transplanted into the bone defects. In this study, the animals were divided into three groups as follow.

Irradiation transplant group

The group in which irradiation was performed and then collagen sponge with ADSCs was transplanted. 42 rats were used for the group.

Irradiation group

In this group irradiation was performed and a collagen sponge was

Figure 2. Measurement of new bone formation area. The area of the host bone (HB) shown in red gradually decreased by new bone formation. We measured the area of the red part.

transplanted without ADSCs. 42 rats were used for the group.

Control group

For a control, 36 rats were used. We used the collagen sponge without disseminating ADSCs and there was no irradiation.

At the first, second and fourth weeks after the transplantation, samples were produced according to each of the evaluation methods. Every three to five rats were used for each evaluation method in every group.

Sampling

We administered deep peritoneal anesthesia on the rats with Nembutal® at the first, second and fourth weeks after surgery. A continuous perfusion fixation with 4% paraformaldehyde (Merck KGaA, Darmstadt, Germany) was used. Then samples were prepared for the ultra-microstructural, the histological and the immunohistochemical observations, respectively.

Assessment of bone formation

Ultra-microstructural observation

Attached soft tissue on the bone samples for the ultrastructural observation were removed by tweezers under a stereomicroscope. Following the soft tissue removal, they were immersed in a 5% sodium hypochlorite solution at room temperature to remove organic matter. After the samples were irrigated with 0.01M PBS, post fixation was performed with 1% osmium PBS. Then, they were dehydrated in an ethanol rise column, substituted by tertiary butyl alcohol, and the samples were freeze-dried (ID-2 Eiko Engineering Co. Ltd., Mito, Japan). After platinum-gold vapor deposition, the morphological conditions of the new bone formation and the bone resorption on the surface of the host and new bone and vascular cavities were observed with a scanning electron microscope (S-4100, Hitachi, Ltd.).

Measurement of new bone formation area

New bone formation in the defect of the skull occurred at the periosteum of the host bone distant from the defect, and is growth toward the defect. On a SEM (scanning electron microscope) photograph, the area without new bone formation between the bone defect and the newly formed bone area is shown in red (Fig. 2). The area of the host bone

Figure 3. Scanning electron micrographs. At the first week after surgery of control (3a, 3b). Second week after surgery of control (3c). Fourth week after surgery (3d). At the first week after surgery of irradiation group (3e). Second week after surgery of irradiation group (3f). Fourth week after surgery of irradiation group (3g). The first week after surgery of irradiation transplant group (3h). Second week after surgery of irradiation transplant group (3i). Fourth week after surgery of irradiation transplant group (3j). Newly formed bone (NB), Host bone (HB), Created bone defect (D), Vascular space (V).

(HB) shown in red gradually decreased new bone formation. We measured the area of the red part using Image J (National institute of Health, USA) to evaluate the extent of the new bone formation by observing the changes of the red colored area. The measured values were statistically processed by unpaired t-tests between two groups using Stat View (HULINKS INC. Leesburg, VA, and USA).

Fine distribution of blood vessels

For the investigation of angiogenesis where the ADSCs were transplanted, the rats in the first and second weeks after surgery were intravascularly injected with a vascular template infusing agent (Acrylic resin: Mercor[®]; DIC Corporation, Tokyo, Japan) after blood removal with a physiological saline solution under deep peritoneal anesthesia with Nembutal[®]. After curing the resin, the parietal bone from each rat was collected and the soft tissue was removed. Then, the samples were prepared by the same method as the ultrastructural observation, and observed with a scanning electron microscope.

Histological observation

The samples were collected after a perfusion fixation, and were washed three times with 0.01 M PBS. They were decalcified with a 10% EDTA solution at room temperature for 7 days. Then they were dehydrated with an ethanol rise column, and embedded in paraffin. The par-

affin blocks were cut into 6 μ m slices and each slice was observed with a light microscope after being stained with hematoxylin and eosin.

Immunohistochemical observation

Immunohistochemical staining was performed by an indirect enzyme antibody method using monoclonal anti-BrdU antibody (BrdU) (Becton Dickinson and Co, Franklin Lakes, NJ, USA) and polyclonal anti-VEGFR2 antibody (VEGF) (GeneTex Co. Los Angeles, CA, USA). The samples were decalcified with 10% EDTA solution and embedded in paraffin, the method used for the histological observation, and were cut into 6 μ m slices. Following the conventional method, each slice was deparaffinized and treated with endogenous peroxidase inactivation by immersion in a methanol solution containing 0.3% hydrogen peroxide (H_2O_2). DNA denaturation of the sample with immunostaining using anti-BrdU antibody was carried out by neutralization with boric acid and borax buffer after treatment with 4 N hydrochloric acid.

Thereafter, the primary antibodies of anti-VEGF antibody and anti-BrdU antibody were reacted at room temperature. Then, the samples were reacted with a secondary antibody (anti-mouse IgG) by the ABC method (VECTASTAIN[®] ABC KIT, Vector Laboratories, Inc. Burlingame, CA, USA). Immunohistochemical staining was carried out using DAB $\cdot$ H_2O_2 as a substrate, and the localization of the anti-VEGF antibody and anti-BrdU antibody positive cells in the bone defects were ob-

served with a light microscope.

Results

Ultra microstructural findings

Control

Scanning electron microscope (SEM): first week after surgery

The low magnification findings of SEM revealed that spongy-like new bone with pores of various sizes had been formed at the side of the cerebral dura mater around the created bone defects, but uncovered parts of new bone were also observed (Fig. 3a).

Using high magnification, we observed the newly formed bone showing a rough bone trabecula structure with various sized vascular spaces, and rice-grain sized calcified substances were irregularly aligned on the surface of the newly formed bone. This showed common morphology of membranous bone formation containing many vascular cavities in an early stage⁷⁾ (Fig. 3b).

SEM: second week after surgery

Low SEM magnification revealed new bone covering on almost all surfaces of the host bone. The edges of the bone defects were almost covered with newly formed bone. The newly formed bone surface was smoother than that of the first week after the surgery, and the vascular spaces had become more uniform in size and had decreased in number (Fig. 3c).

Under high magnification, we observed irregularly aligned rice-grain like calcified substances on the newly formed bone surface, which were more compact than those of the first week after surgery.

SEM: fourth week after surgery

Under low SEM magnification, the newly formed bone surface was flatter than that of the second week after surgery. The edges of the bone defects were completely covered with the newly formed bone. The newly formed bone had grown toward the inside of the bone defect, so the area of the bone defect was reduced compared with the areas of first and second week after surgery. The number of vascular spaces on the newly formed bone had also decreased (Fig. 3d).

Under high SEM magnification, the newly formed bone surface appeared as regularly aligned rice-grain like calcified substances, showing a mature bone surface with scattered osteoblast lacunae and the bone resorption areas, the so called Howship's lacunae.

Irradiation group

SEM: first week after surgery

Under low SEM magnification, the area of new bone formation was seen on part of the surface of the host bone, and it was clearly smaller compared to the first week of the control group.

New bone formation was not observed on the edge of the bone defects on the host bone (Fig. 3e).

The surface of the newly formed bone had rough surface and the size of vascular space was irregular as compared with those of the first week of the control group.

Under high SEM magnification, the morphology of the newly formed bone was similar to the surface of the early stage of membranous bone formation of the control group.

SEM: second week after surgery

At the second week, the newly formed bone covered a part of the host bone surface, but the area of newly formed bone was small compared with the second week of the control group using low SEM magnification. Also, newly formed bone was not seen in the edge of the bone defects on the host bone as was found in the first week of the irradiation group (Fig. 3f).

Figure 4. The exposed area of host bone surface. The irradiation group had a significantly larger exposed area at the host bone surface compared with the control group and the irradiation transplantation group. At the fourth week after the surgery, the host bone surfaces in all experimental groups were covered with newly formed bone, and the measured value was 0.

Under higher SEM magnification, the surface of the newly formed bone consisted of dense and regularly aligned rice-grain like calcified substances, and many osteoblastic lacunae were observed on the surface.

SEM: fourth week after surgery

Under low SEM magnification, the newly formed bone completely covered the entire surface of the host bone. The surface of the newly formed bone had become smooth, and there were vascular spaces of various sizes on the surface, but the number of the cavities had decreased as compared with the second week of the irradiation group (Fig. 3g). The area of the bone defect on the host bone was smaller than that of the first and second week of the irradiation group.

Under high SEM magnification, the newly formed bone surface appeared to show a mature bone surface with the existence of both osteoblast lacunae and Howship's lacunae.

Irradiation transplant group

SEM: first week after surgery

Under low SEM magnification, new bone was formed at the dura mater side and the newly formed bone covered a larger area of the host bone surface compared to the irradiation group.

The edges of the bone defects were not covered by newly formed bone. The surface of the newly formed bone was rough, and the size of the vascular spaces was irregular as compared with those of the first week of the control group (Fig. 3h).

Under high magnification, the newly formed bone showed the same morphology as in the early stage of membranous bone formation like that of the control group.

SEM: second week after surgery

Under low SEM magnification, almost of all the surface of the host bone was covered by newly formed bone but in a small area the edges of the bone defect were exposed. The morphology at the second week of the irradiation transplant group was similar to the structure of the new bone surface and the size of vascular space as the second week of the control group (Fig. 3i). In comparison with the irradiation group, the surface of host bone was more widely covered by newly formed bone.

Under high SEM magnification, on the new bone surface, we observed the morphology of a mature bone surface with regularly aligned calcified substances like rice-grains and scattered osteoblast lacunae and Howship's lacunae.

Figure 5. Scanning electron micrographs of fine distribution of blood vessels. AT the first week after surgery of control (5a). Second week after surgery of control (5b). At the first week after surgery of irradiation group (5c). Second week after surgery of irradiation group (5d). At the first week after surgery of irradiation transplant group (5e). Second week after surgery of irradiation transplant group (5f). Newly formed bone (NB), Host bone (HB), Created bone defect (D), Blood vessels (V).

SEM: fourth week after surgery

Under low SEM magnification, the newly formed bone completely covered the host bone surface and the edge of the bone defect. The newly formed bone surface was observed to be smooth, and various sizes of vascular spaces existed on the surface, and the number of the vascular spaces further decreased as compared with the second week of the same group (Fig. 3j). The newly formed bone had grown toward the inside of the bone defect, so the area of the bone defect was reduced as compared with the second week of the same group.

Under high SEM magnification, the newly formed bone surface appeared as a mature bone surface with many osteoblastic lacunae and Howship's lacunae.

SEM measurement of bone formation area (Fig. 4)

SEM observation showed that new bone was formed on the host bone surface on the dura mater side, so as new bone formation improved the exposed area of the host bone surface became smaller. At the first week after surgery, the newly formed bone area of the control group was

4.6 mm², the irradiation transplanted group was 6.4 mm², and the irradiation group was 12.1 mm². The result of statistical processing by unpaired t-tests showed the irradiation group had a significantly larger exposed area at the host bone surface compared with the control group ($p < 0.0001$) and the irradiation transplantation group ($p < 0.002$).

At the second week after surgery, the control was 1.6 mm², the irradiation transplanted group was 1.39 mm², and the radiation group was 7.09 mm². The same statistical processing showed that the irradiation group had a significantly larger exposed area on the host bone surface compared with the control group ($p < 0.02$) and the irradiation transplantation group ($p < 0.02$).

At the fourth week after the surgery, the host bone surfaces in all experimental groups were covered with newly formed bone, and the measured value was 0.

Observation of fine distribution of blood vessels

Control

At the first week after surgery, numerous capillaries, which existed on the newly formed bone surface, were observed on the new bone surface growing toward the bone defect (Fig. 5a). At the second week after surgery, blood vessels were seen on the new bone surface as in the first week, and were thick and compact (Fig. 5b).

Irradiation group

At the first week after surgery of the irradiation group, capillaries were observed on the new bone surface growing toward the bone defect. However, they were fine, and the number of them was less than those of the first week of the control group and of the irradiation transplant group (Fig. 5c). At the second week in the irradiation group, the capillaries observed around the newly formed bone were more localized than that of the first week and also the number of the blood vessels was less than that of the second week of the control group and the irradiation transplantation group (Fig. 5d).

Irradiation transplant group

At the first week after surgery in the irradiation transplant group, many capillaries were seen on the new bone surface toward the bone defect, and it was more vigorous than the control group. At the second week in the irradiation transplant group, the number of blood vessels was less than that of the first week and a few thick blood vessels were found on the newly formed bone surface (Fig. 5e). The shape of the blood vessels at the second week was thicker and more compact compared to that of the first week, and was similar to those of the control group the second week after surgery (Fig. 5f).

Histological findings by conventional light microscope

Control

At the first week after surgery, histological findings of the control group showed, the newly formed bone was observed only on the host bone surface of the dura mater side, and the new bone around the bone defect was formed toward the bone defect. There were large cells with round-shaped nuclei on the outer side of the newly formed bone, and the trabeculae of the newly formed bone were generally rough with many thick blood vessels found inside of the trabeculae (Fig. 6a). The transplanted collagen sponge retained its structure, and only a few inflammatory cells were found inside, and no blood vessels were seen (Fig. 6b).

At the second week in the control group, the newly formed bone was observed only on the dura mater side of the host bone: this was same as the result of the first week after surgery. The periphery of the newly

Figure 6. Light micrographs of bone and crated defect area. At the first week after surgery of control of dura side (6a) and bone defect area (6b). Second week after surgery of dura side (6c) and bone defect area (6d). At the first week after surgery of irradiation group of dura side (6e) and bone defect area (6f). Irradiation group at second week after surgery of dura side (6g) and bone defect area (6h). At the first week after surgery of irradiation transplant group of dura side (6i) and bone defect area (6j). Second week after surgery of irradiation transplant group of dura side (6k) and bone defect area (6l). Newly formed bone (NB), Host bone (HB), Transplanted Collagen sponge (C), Blood vessel (V).

Figure 7. Immuno-histochemical findings (BrdU). In the irradiation transplant group, many BrdU-positive cells were revealed in the periosteum around the new bone at the first week (7a) and second week after transplantation (7c). BrdU-positive cells appeared in the vascular wall and the interstitial tissue in the inside of the transplanted collagen sponge at the first week (7b) and second week after transplantation in the irradiation transplant group (7d). Newly formed bone (NB), Host bone (HB), Blood vessel (V).

formed bone was enclosed by cubic osteoblasts, and the trabeculae on the new bone were more compact compared to those of the first week in the control group, and blood vessels were observed inside of new bone (Fig. 6c). The structure of the transplanted collagen sponge remained in the bone defect, and many cells and blood vessels were seen inside the collagen sponge (Fig. 6d).

At the fourth week in the control group, new bone was seen to be covering the bone defect edge. The newly formed bone was enclosed by flat osteoblasts lined up on the surface, and the trabecular bone was denser than that at the second week after surgery, also the blood vessels were fewer than those at the second week after surgery. The histological finding of the transplanted collagen sponge showed a fibrous form that resembled subcutaneous tissue.

Irradiation group

At the first week after surgery in the irradiation group, newly formed bone was slightly observed on the dura mater side of the host bone. The area of the new bone formation was clearly less than that of the control and the irradiated transplant group. The trabecular bone of the newly formed bone was rough and the blood vessels surrounding the bone trabeculae were few compared with those of the control group at the first week (Fig. 6e). The transplanted collagen sponge in the bone defect retained its structure, and there were a small number of the inflammatory cells, but no blood vessels (Fig. 6f).

At the second week after surgery, newly formed bone was observed only on the dura mater side of the host bone. The periphery of the newly

formed bone was enclosed by cubic osteoblasts, and the bone trabeculae on the new bone were coarse compared to that of the control group at the second week after surgery (Fig. 6g). The structure of the collagen sponge was maintained, and the blood vessels and the mesenchymal cells were observed among the structure (Fig. 6h). At the fourth week of the irradiation group, new bone formation was seen covering the bone defect edge. The periphery of the newly formed bone was enclosed by cubic osteoblasts, and the bone trabeculae were compact as we saw in the control group at the fourth week after surgery and also the number of blood vessels in the trabeculae was smaller. The structure of the collagen sponge remained, with a few cells and blood vessels among the structure, but the number of the blood vessels was fewer than the control group.

Irradiation transplant group

In the first week after surgery of the irradiation transplant group, new bone formation was observed only on the dura mater side of the host bone. Large round shaped cells were lined around the newly formed bone. The bone trabeculae formed on the new bone were compact and the blood vessels in the new bone were few compared with those of the control group at the first week after surgery (Fig. 6i). At the bone defect site, mesenchymal cells were found in the inside of the transplanted collagen sponge, and numerous blood vessels were present among the cells. The small circular-shaped inflammatory cells which were observed inside the collagen sponge in the control group at the first week were fewer than in the irradiation transplant group (Fig. 6j).

Figure 8. Immuno-histochemical findings (VEGF). Control groups of surgery after first week (8a) and second week (8b). Irradiation group at first week (8c) and second week (8d). Irradiation transplant group of first week (8e) and second week after transplantation (8f). In the irradiation transplant group, a lot of VEGF positive cells were observed around the blood vessels inside of the transplanted collagen sponge. Transplanted Collagen sponge (C), Blood vessel (V).

At the second week after surgery, newly formed bone was observed only on the dura mater side of the host bone. The periphery of the newly formed bone was enclosed by cubic-shaped osteoblasts. The bone trabeculae formed on the newly formed bone was finer than those of the second week in the control group (Fig. 6k). The structure of the collagen sponge that was transplanted to the bone defect site was unclear compared to that of the first week, and there was numerous blood vessels and the mesenchymal cells in line with the histological finding of the subcutaneous tissue (Fig. 6l).

At the fourth week after surgery, new bone was formed to cover the edge of the bone defect. The newly formed bone was enclosed by cubic-shaped osteoblasts, and the bone trabeculae was compact like those

of the control group at the fourth week after surgery. The histological finding of the transplanted collagen sponge showed fibrous tissue similar to subcutaneous tissue.

Immuno-histochemical findings

Anti-BrdU antibody staining

In the irradiation transplant group, many BrdU-positive cells were revealed in the osteocyte within the newly formed bone and the periosteum around the new bone at the first and second week after transplantation (Fig. 7 a, c). Also BrdU-positive cells appeared in the vascular wall and the interstitial tissue in the inside of the transplanted collagen sponge at the first and second week after transplantation in the irradiation

tion transplant group (Fig. 7b, d). No BrdU-positive cells were found in the control group and the irradiation group.

Anti-VEGF staining

Control

At the first week after surgery of the control group, a large number of VEGF-positive cells were found in the interstitial tissue and around the blood vessels of the subcutaneous tissue. A small number of VEGF-positive cells were found at the interface between the transplanted collagen sponge and the subcutaneous tissue (Fig. 8a).

At the second week after surgery, VEGF-positive cells were seen in the subcutaneous tissue and around the blood vessels, but less in comparison with that of the first week after surgery. A few VEGF-positive cells were found in interstitial tissue inside of the collagen sponge (Fig. 8b).

Irradiation group

VEGF-positive cells were found around the blood vessels of the subcutaneous tissue at the first week after the transplantation in the irradiation group, but less than the control group (Fig. 8c). There were no blood vessels in the inside of the transplanted collagen sponge, and also no VEGF positive cells were found.

At the second week after surgery, VEGF-positive cells were found around the blood vessels in the subcutaneous tissue, the same as the result of the second week of the control group. VEGF-positive cells were scattered in the inside of the collagen sponge (Fig. 8d).

Irradiation transplant group

At the first week after surgery of the irradiation transplant group, numerous VEGF-positive cells were found in the interstitial tissue and the perivascular area of the subcutaneous tissue. VEGF positive cells were revealed around the blood vessels inside of the transplanted collagen sponge (Fig. 8e).

At the second week, VEGF positive cells existed around the blood vessels in the subcutaneous tissue, but there were fewer compared to the first week after surgery. Many VEGF positive cells were present around blood vessels that were abundantly developed at the transplanted site of the collagen sponge (Fig. 8f).

Discussion

This study showed that ADSC transplantation prevented a delay in wound healing that occurs after irradiation. From morphological findings and SEM measurements of newly formed bone areas, we found that the initial bone formation area of the irradiation group was clearly suppressed compared with the control group. We have shown the efficacy of ADSC transplantation in that it enhances bone wound healing after irradiation. Angiogenesis suppressed by irradiation was restored by ADSC transplantation.

The section on SEM observation in this study referenced a report by Boide⁷⁾ which recorded detail of the morphology of membranous bone development. The histological findings from the first week after surgery showed that osteogenic cells and blood vessels found in the bone defect area of the irradiation group was suppressed compared with the control group. The result from the irradiation group in this study was the same as Takekawa *et al.*¹⁾ who created a bone wound after irradiation and observed its healing process using SEM and a light microscope. They reported that the effects of radiation on bone cells and angiogenesis disorders were related to the delay of bone wound healing¹⁾. Generally, the reasons for the retardation of bone wound healing in an irradiated bone

are thought to be as follows: (1) a disruption of osteogenic cell differentiation, (2) an impairment of revascularization, and (3) a failure of micro-circulation in the irradiated area. The importance of blood supply in osteogenesis has also been pointed out previously^{2, 8)}, and osteogenic cells and blood flow are closely related^{2, 9)}. As to the impairment of revascularization, it is reported that vascular endothelial cells are damaged by irradiation and angiogenesis is reduced¹⁰⁾. From the results of vascular corrosion casts, in the irradiated group, the extent of angiogenesis was clearly smaller than that of the control group, indicating that angiogenesis was suppressed by irradiation, and at the same time, new bone formation was delayed.

As well as facilitating bone wound healing, ADSCs transplanted into bone wounds promote angiogenesis with the ability to differentiate into osteoblasts, fibroblasts and vascular endothelial cells, etc. In this study, ADSC's were transplanted to a bone defect made after irradiation to investigate the effect ADSC's have on bone wound healing. As a result, a large number of cells were present in the bone defect region where ADSCs were transplanted. New bone formation at one week and two weeks after transplantation was significantly improved. There was no difference in new bone formation after ADSC transplantation in the irradiation group and the non-irradiated control group. BrdU-labeled ADSCs were found in the newly formed bone area and showed an osteoblast-like arrangement, although there were few BrdU positive cells in subcutaneous tissues. The results suggest the transplanted ADSC differentiated to osteoblasts and accumulated in the bone wound area. On the other hand, in the corrosion cast findings angiogenesis in the irradiation transplant group was similar to that of the control, while angiogenesis in the irradiated group at the first and second week was clearly suppressed and new bone formation was also reduced. The VEGF-labeled ADSC's were found in the interstitium and around blood vessels, particularly VEGF positive cells in the collagen sponge, at the first week of the irradiation transplant group and were distributed inside the sponge and the perivascular. Hattori *et al.*¹¹⁾ and Miyazaki *et al.*¹²⁾ reported that ADSCs have an enhancing effect on angiogenesis in the bone formation process. It has been reported that ADSCs increase the production of VEGF under hypoxic culture conditions^{13, 14)}. It has also been reported that the ADSCs produce VEGF and angiogenesis is induced by the peripheral mesenchymal stem cells with the support of other cytokines¹⁵⁻²⁰⁾. The results of this study support the reports mentioned above and suggest that ADSC transplantation improves bone wound healing after irradiation.

Hao *et al.*²¹⁾ reported that delayed wound healing after irradiation caused the suppression of synthesis and the secretion of the factors for wound healing because the induction of cells for wound healing was suppressed. There were many reports that the effects of the ADSCs on wound healing were due to direct differentiation of the administered cells to the cells involved in tissue repair, and the action of secreting paracrine factor^{13, 19, 22, 23)}. Thangarajan *et al.*²⁴⁾ suggested that the paracrine effect of the ADSCs on the wound healing process has the effect of inducing the endogenous stem cells in the injured region to initiate the healing process. Furthermore, It was suggested that the ADSC transplantation had the effect of reducing the inflammatory response²⁵⁻²⁷⁾. It has been reported that ADSCs increase the production of VEGF under hypoxic culture conditions^{13, 14)}. In addition, it was reported that the ADSCs increase not only the production of VEGF but also the production of various factors associated with wound healing under hypoxic conditions^{28, 29)}. This shows that ADSC transplantation in a local hypoxic region caused by irradiation enhances vascularization and activates various factors for wound healing in the irradiated tissue.

It is clear that the transplanted ADSCs not only differentiated to vas-

cular endothelial cells and osteoblasts, but also have an important function in wound healing by way of proteins and various cytokine secretions by ADSCs. In recent years, there have been some reports that the paracrine effect of the transplanted mesenchymal stem cells was involved with angiogenesis reversing the DNA damage to cells caused by irradiation³⁰. Future studies are needed to elucidate the kinds of substances ADSCs secrete and what sorts of cells are influenced by them. Further studies will be needed to accumulate evidence in support of the ADSC transplantation method. We hope the present paper will constitute a step toward further development of this therapeutic method.

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Conflict of Interest

The authors have no COI exists.

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