Life-Prolonging Effects of Adipose Tissue-Derived Stem Cell Transplantation into Mice Exposed to a Lethal Dose of X-Rays

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***Abstract* :** In the event of a high-dose radiation exposure accident, adipose-derived stem cell (ADSC) transplantation might be used as an emergency medical treatment to compensate for bone marrow failure. To investigate the possible course of that treatment, we examined whether transplantation of ADSCs into whole-body X-ray irradiated mice would provide resistance to radiation damage. ADSCs were obtained from a primary culture of adipocytes from adipose tissue of syngeneic mice. The ADSCs were transplanted via an intravenous (i.v.) route after whole-body irradiation (6 Gy, X-rays) of the ICR mice. Fifty days after transplantation, the survival rate of the transplanted group was 40% higher than the control group, and the difference in survival rates was maintained in the following 200 days. After 400 days, however, the difference in survival rates became smaller and disappeared after 650 days. The results indicate that ADSC transplantation may reduce lethality from acute radiation bone marrow injury for several hundred days.

***Keywords* :** adipose-derived stem cell, radiation injury, transplantation, survival.

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Introduction

The accident at Fukushima Daiichi Nuclear Power Station will require nuclear fuel processing and dismantling to decommission the unit. Other nuclear power plants will also undergo decommissioning. Workers engaged in such activities are always at risk of exposure to high-dose radiation. If a high-dose exposure accident occurs, hematopoietic stem cell transplantation is a treatment option of radiation emergency medicine. While the need for hematopoietic stem cell transplantation would be urgent, it is difficult to obtain Human leukocyte antigen (HLA) compatible donors rapidly. In previous radiation-related accidents, the matching of HLA was insufficient and no clear trans-

plant effect was obtained [1, 2].

Pluripotent stem cells, which were recently discovered in adipose tissue, are capable of developing into various mesenchymal cells, including fat, bone, muscle, cartilage, and blood vessels [3, 4]. These cells have been experimentally used in a variety of ways, including (1) orthopedic applications such as vascular regeneration and skeletal muscle regeneration, (2) treatment of nephro-urinary disorders such as acute kidney injury and defibrillation smooth muscle regeneration [7, 8], (3) hematological disorders such as myocardial infarction [9] and limb revascularization; and, (4) gastrointestinal disorders such as enteric fistula regeneration, mitigation of intestinal inflammation, and hepatocyte regeneration. Actual clinical applica-

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tions are already underway [10, 11]. These cells also secrete cell growth factors such as Vascular endothelial growth factor (VEGF) and Hepatocyte growth factor (HGF) [5, 6]. A large number of ADSCs can also be harvested for transplantation, and their HLA compatibility is not demanding [12, 13], making it possible to reduce time constraints for finding an allogeneic donor. At present, it does not appear that ADSC can entirely replace bone marrow transplantation, but bone marrow stem cell (BMSC)-level hematopoiesis-supporting ability has been recognized [14]. Thus, it may be possible to increase the time until bone marrow transplantation is used.

The dose limit was prescribed strictly by regulations to protect radiation workers from health hazards caused by radiation exposure, although in high-dose work environments such as decommissioning work even a small operational error may lead to a high dose exposure above the limit. It is necessary to always be prepared for an emergency response, but at present, as mentioned above, there have been HLA compatibility problems and unfavorable results in past accidents. ADSC transplantation, which has a potential advantage in stem cell transplantation therapies, could be important in the field of occupational health.

This study explored the concept that transplantation of ADSCs could enhance radio-resistance and thus increase life span of mice exposed to whole body lethal radiation.

Materials and Methods

Animals

Ten-week-old Slc: ICR Female mice (Japan SLC, Inc. Japan) were used as donors and recipients. The mice were maintained on laboratory food (MF, Oriental Yeast Co., Ltd., Japan) and water ad libitum and kept according to the Guiding Principles for the Care and Use of Animal approved in 2007 at the Faculty meeting of the University of Occupational and Environmental Health, Japan. An experimental permit was obtained from the Animal Experiment Committee of the University of Occupational and Environmental Health, Japan. The approval number is AE14–020.

Irradiation and transplantation

ICR mice in the transplant group received ADSCs from syngeneic mice immediately after whole-body irradiation (6 Gy X-rays) (MBR-1520R-3, Hitachi Engineering & Services Co. Ltd., Japan). Here, we used 6 Gy within the bone marrow death dose range to see the effect on bone marrow damage by irradiation. Specifically, the mice received 1×10^6 primary cultured adipocytes /500 μl via the saphenous vein. ICR mice in the control group received whole-body irradiation (6 Gy X-rays) only. The survival of both experimental groups was checked and scored daily through the lifetime of the mice. Five experiments were conducted in this study, and data were obtained from 56 animals in the control group and 42 animals in the transplant group.

Isolation of adipose-derived stem cells

Subcutaneous adipose tissue was excised from five ICR mice per experiment. The tissue was minced and incubated in a 0.15% collagenase solution (FUJIFILM Wako Pure Chemical Co., Ltd.) with shaking for 1 h at 37°C, and then the cell suspension was passed through 100 mesh filters (Steriflip Filter Unit. NY1H, Merck-MILIPORE Corp. Germany). The cells were seeded into 100 mm dishes containing DMEM (Dulbecco's Modified Eagle Medium, FUJIFILM Wako Pure Chemical Corp. Japan) with 10% FBS (Fetal Bovine Serum) and cultured in a 5% CO2 incubator at 37°C. Medium changes were performed on the third day. Cells were passaged with trypsin (FUJIFILM Wako Pure Chemical Corp. Japan) on the sixth and twelfth day. ADSCs were prepared 14 days after the start of culture for transplantation or flow cytometric (FCM) analysis. ADSCs for transplantation were prepared for each experiment.

Identification of ADSCs in isolated primary cultured adipocytes

There are various theories regarding the expression pattern of surface epitopes of mouse ADSC. Here, we used the surface markers expressed by commercial ADSCs [C57BL/6 mouse adipose-derived mesenchymal stem cell Catalog# MUBMD-01001] from Cyagen (Cyagen Biosciences Inc., USA). The characteristics and identity of the cells are defined as CD29(+) CD44(+) Sca-1(+) CD117(−).
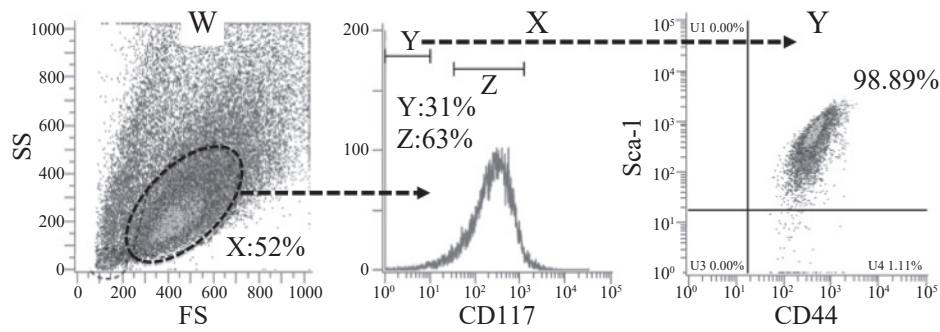
Figure 1. The proportion of CD44(+) Sca-1(+) CD117(−) cells in primary cultured adipocytes isolated from ICR mice. W: The density plot of primary cultured adipocytes. Gate X identifies cells containing ADSCs. X: The histogram that evaluated CD117. Y: The quadrant region that determined the CD44(+) Sca-1(+) plot from gate Y. FS: Forward Scatter, SS: Side Scatter.

The isolated primary cultured adipocytes from the ICR mice were stained with three color fluorescent antibodies (CD44-FITC, Sca-1-PE, CD117-APC, Miltenyi Biotec. Com. Japan). CD44(+) Sca-1(+) CD117(−) cells were analyzed with an EC800 Flow Cytometry Analyzer (Sony Biotechnology Inc. Tokyo).

Statistical analyses

The survival rates of both groups were calculated by the Kaplan-Meier method. The statistical analysis was performed using a log-rank test and generalized Wilcoxon test. A confidence level of $P < 0.01$ was considered significant.

Results

We investigated the proportion of ADSCs in the primary cultured adipocytes isolated from ICR mice. We assumed that cells with an immunophenotype of CD44(+) Sca-1(+) CD117(−) were ADSCs. The relative expression of CD117 was evaluated in a histogram (Figure 1. X) for cells within the adipocyte gate defined by gate X in the density plot (Figure 1. W), and CD117(−) cells were identified by gate Y. The CD44(+) Sca-1(+) double-positive cells in gate Y were determined in the quadrant region of the bivariate plot (Figure 1. Y). The fraction of ADSCs in the extracted adipocytes was calculated to be about 16%.

Approximately 20 days after transplantation, survival dropped sharply in both groups; to 30% in the control group and 71% in the transplant group. The difference

in survival rates between the two groups remained 40% for the next 180 days. The difference in survivals began to decrease until 400 days, at which point the difference suddenly narrowed at around 450 days. The survival of both groups fell to zero after 600 days (Figure 2). The statistical difference in survival between the two groups, calculated by the Kaplan-Meier method, was significant, with $P < 0.05$ for the log-rank test and $P < 0.01$ for the generalized Wilcoxon test. Transplantation of ADSCs into mice irradiated with lethal doses of X-rays proved to be life-prolonging.
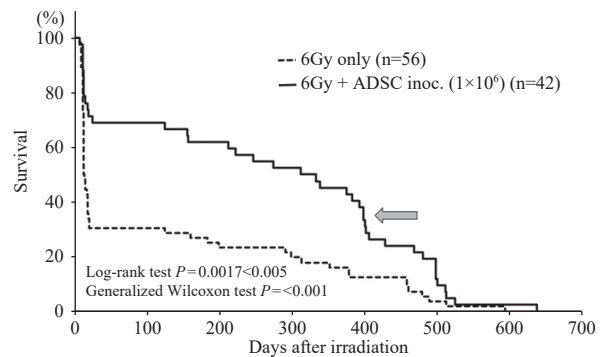

Figure 2. Life-prolonging effect of ADSC transplantation in 6 Gy whole-body irradiated ICR mice. The survival rate of both groups was calculated by the Kaplan-Meier method. The transplantation of ADSCs led to a 40% higher survival rate for 400 days in the transplant group compared to the control group, demonstrating a clear life-prolonging benefit, but the survival rate of the transplanted group began to decline rapidly 400 days post-transplantation (arrow). ADSCs: adipose-derived stem cell, inoc.: inoculation.

Discussion

Adipose tissue is actively involved in the regulation of energy homeostasis via metabolism and the release of various bioactive molecules called adipokines [15]. There are two types of adipose tissue: brown adipose tissue (BAT) and white adipose tissue (WAT), and the WAT itself can be further divided into subcutaneous and internal fat tissues. All of these tissues have been shown to exhibit remarkable tissue plasticity, but internal and subcutaneous WAT show some discrete differences in the phenotypes of their cell populations [16, 17]. The inguinal WAT is considered to be the most plastic adipose tissue [18, 19] and represents a potent and suitable source of stem cells that is easy to sample and has a major advantage in cell therapy.

Although there are many theories about the percentage of ADSC cells that can be extracted from adipose tissue, Bone Marrow-Derived Stem Cells (BMSCs) have been reported to account for approximately 0.05−0.001% of bone marrow, whereas ADSCs account for approximately 0.5−1% of primary cultured cells extracted from adipose tissue [4, 20−24]. The percentage of ADSCs obtained is likely to vary depending on the animal species, adipose tissue site and extraction method. In our FCM analyses of cells isolated from subcutaneous adipose tissue, surface antigen analysis showed that CD44(+) Sca-1(+) CD117(−) constituted about 16% of the population and were likely to be ADSCs (Figure 1). This result indicates that the ADSC extraction method is simpler than that of BMSC and that ADSCs are available in large quantities as stem cells. This conclusion supports the idea that this method is very advantageous for applications in regenerative medicine.

In 2003, Cousin *et al* transplanted a stroma-vascular fraction (SVF) extracted from inguinal adipose tissue into 10 Gy whole-body irradiated C57BL/6J mice. The mice received 1×10^6 cells via an i.v. route or 1×10^7 cells via an intraperitoneal (i.p.) route. The transplant group maintained a survival rate > 60% whereas no control animal survived after 30 weeks [25]. All the SVF i.v. transplant group, however, died immediately after i.v. due to heart and/or brain failure. Cousin *et al* stated that the reason for the increased survival of the transplant group in this experiment may have been

the result of the ADSC-mediated reconstitution of lymphoid and myeloid tissue, but they indicated that ADSCs did not differentiate into hematopoietic stem cells (HSCs) and may instead have activate HSCs. Further research is needed to resolve these issues. In that study, mice were observed for only 30 weeks after transplantation, and the researchers were unable to define trends in survival in the transplant group after that. Our lifelong observation (650 days) showed that the survival rate dropped sharply 20 days after transplantation in both groups, to 30% in the control group and 71% in the transplant group, and that the survival rate of the transplant group began to decline rapidly 400 days post-transplantation. The final extent of survival was the same as in the control group, suggesting that ADSCs could not differentiate into HSCs as reported by Nakao *et al* [14], but the transplantation of ADSCs resulted in a 40% higher survival rate for 400 days in the transplant group compared to the control group, demonstrating a clear life-prolonging benefit (Figure 2). In the trials by Cousin *et al* all i.v. transplantation experiments with ADSCs failed. Even following successful i.p. transplantation, the migratory route taken by the ADSCs was unclear and the number of ADSCs reaching the bone marrow was not known. Those considerations make it difficult to compare the results of our experiments with theirs, but since ADSCs secrete various groups of growth factors [5, 6], it is possible that those factors may be involved in the activation of HSCs, rather than in the differentiation of ADSCs themselves, which would have contributed to the maintenance of survival in the transplanted group during the first few hundred days after transplantation. Incidentally, we also performed i.p. transplantation experiments under similar conditions to Cousin's, but there was no increase in the survival rate in the transplant group (data not shown).

In a different study, mice that had been exposed to a lethal radiation dose (gamma-rays, 9.5 Gy) were transplanted via i.v. with extracellular vesicles (EVs) recovered from a BMSC-conditioned medium, and more than 60% of the irradiated mice survived for 7 months following transplantation [26]. EVs were reported a quarter of a century ago as shed vesicles that carry information between cells [27, 28]. Recently, the existence of shed vesicles of various sizes (30−1,000

nm) has been confirmed. The particles consist of a phospholipid bilayer membrane surrounding cytoplasmic proteins, lipids, DNA and RNA. Cells secrete EVs not only in vitro but also in vivo [29]. EVs have specific surface molecules and are believed to control the physiological state of the target cells [30].

Schoefinius *et al* showed in in vitro experiments that immature HSCs were the target of EVs derived from a BMSC-conditioned medium, suggesting that the action of EVs on a more immature HSC population in vivo may be one of the mechanisms underlying the long-term survival of exposed mice [26].

Bone marrow cell transplantation is life-prolonging for irradiated animals [31, 32], and it has also been used for humans following radiation exposure accidents, but the therapeutic effects have not been clear in all cases [1, 2, 33, 34]. Nevertheless, bone marrow cell transplantation is now an essential medical treatment for leukemia and other diseases [35]. In humans, various methods of avoiding graft-versus-host disease (GVHD) have been explored [36, 37]. Therefore, the discovery that transplantation of BMSC-derived EVs alone in mice had the same effects as transplantation of BMSCs alone suggests that activation of HSCs by EVs alone may lead to long-term survival without difficulties caused by major histocompatibility complex (MCH) incompatibility.

In this study, a drastic decrease in survival was observed about 450 days after ADSC transplantation, which was likely due to the inability of ADSCs to differentiate into HSCs and maintain hematopoiesis. It is possible, however, that ADSC-derived EVs may have been involved in the activation of HSCs and maintained high survival rates.

Among the transplantation conditions that affect general mesenchymal stem cell transplantation regenerative therapy, the following could be applied to our experimental conditions: the variations in the transplantation of ADSCs after irradiation exposure, such as the number of cells administered (a few million to several hundred million), the route of cell administration (intrathecal, i.v. and i.p.), the number of cell administration (single and multiple), and the timing of cell administration (immediately after irradiation or not), are thought to affect the long-term survival of exposed mice. Multiple transplantations, in particular,

may be effective in delivering multiple fresh cells that can regulate the regeneration of the damaged environment, as the transplanted cells are thought to survive for a limited period of time in the exposed mice [38–40]. Unfortunately, we were not able to try many of these conditions in this study. Perhaps a combination of optimal conditions could see further increases in survival rate and long-term survival.

In the present study, the percentage of ADSCs present in cells isolated and cultured from subcutaneous adipose tissue in mice was calculated from FCM data. Those cultured cells (1×10^6) were transplanted via an i.v. route into whole body irradiated mice. We observed an increase in survival of approximately 40% for approximately 400 days. This phenomenon appears to represent cellular rescue from radiation-induced death rather than bone marrow reconstitution. We are currently analyzing the mechanism of increased survival assisted by ADSC in lethal radiation exposure. These studies might contribute to long-term rescue following radiation exposure and contribute to further developments in the field of regenerative medicine.

Conflict of Interest

I state that I have no conflicts of interest.

Acknowledgments

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