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Original Article

Conditioned Media from Human Dental Pulp Stem Cells Prevent Radiation-induced Skin Injury

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Abstract: Radiation-induced skin ulceration is a frequent complication of radiotherapy for cancer treatment. Stem cells from human exfoliated deciduous teeth (SHEDs) can regenerate various tissues. In this study, we investigated the impact of SHED-conditioned medium (SHED-CM) on radiation-induced skin injury. Mouse necks were locally irradiated with a single dose of 15 Gy of radiation. A week after the irradiation, most of the wild-type mice generated ulcer surrounded by severe erythema. Intra-venous administration of SHED-CM effectively inhibited the ulcer formation. Histological examination revealed that SHED-CM treatment inhibited radiation-induced dermal thickness and epithelial hyperplasia. SHED-CM could be a useful treatment option for radiation-induced skin ulceration.

Introduction

Radiotherapy is a tissue-perseverative treatment for many cancers; however, it damages healthy tissues surrounding cancer as well. One of the most common side effects of the radiation is an acute skin reaction (radiation-induced skin reaction; RISR[1]) Radiotherapy is a treatment that takes advantage of the differences in sensitivity and responsiveness of normal and tumor tissues to radiation[2].

The keratinocytes in basal cell layer of skin epidermis, exhibiting the higher the frequency and number of cell division and undifferentiated properties, are highly sensitive to radiation. During early RISR, basal cells die and induce an inflammatory response, which recruits and activates various type of immune cells, such as eosinophils and neutrophils, leadings to self-perpetuating tissue damage and loss of protective barriers[1]. RISR can be distressing patients and can lead to treatment interruption. Currently, no satisfying therapeutic strategies for severe RISR has been developed.

Stem cell therapy holds great promise for the establishment of effective treatments for RISR[1,3-5]. It has been shown that the transplantation or infusion of adult mesenchymal stromal cells (MSCs) isolated from bone marrow (BMSCs) improved tissue destruction and fibrosis of RISR in animal model[6-10] and clinical studies[11,12], primarily through paracrine mechanisms. However, for clinical applications, stem cell therapies must overcome serious hurdles, including tumorigenesis and strong immune reactions, as well as cost and time to prepare enough cell numbers. Stem cells secrete a broad repertoire of trophic and immuno-modulatory factors, which can be collected as serum-free conditioned medium (CM). CMs from various

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stem cell types have been shown to exhibit considerable potential in treating myriad intractable diseases[13]. However, the therapeutic benefits of CMs derived from stem cell for RISR treatment are largely unknown.

Human adult dental pulp stem cells (DPSCs) and stem cells from human exfoliated deciduous teeth (SHEDs) are self-renewing MSCs residing within the perivascular niche of the dental pulp. These cells are thought to originate from the cranial neural crest, which expresses markers for both mesenchymal and neuro-ectodermal stem cells, as well many genes encoding extracellular and cell-surface proteins[14]. Studies of engrafted SHEDs or DPSCs in various animal models of disease, including myocardial infarction[15], ischemic brain injury[16], spinal cord injury[14], liver fibrosis[17], fulminant liver failure[18], bleomycin-induced fibrotic lung injury[19], autoimmune encephalomyelitis[20], type I diabetes[21], rheumatoid arthritis[22] indicate that these cells can promote significant recovery through the activation of endogenous tissue-repairing activities. However, the therapeutic potential of tooth-derived stem cells for the treatment of RISR has not been examined. In the present study, we investigated the therapeutic effects of SHED-CM administration in a mouse RISR model.

Material and methods

Preparation of SHED-CM

SHEDs were isolated as described previously[14]. In brief, deciduous teeth from 6- to 12-year-old individuals were collected at Nagoya and Tokushima University Hospital. This study was approved by the Institutional Ethical Committee of Nagoya and Tokushima University Hospital and performed according to the principles of Helsinki Declaration (Permit No H-73 and No: 3268 for Nagoya and Tokushima University, respectively). After separation of the crown and root, the dental pulp was isolated and treated with 3 mg/ml collagenase type I and 4 mg/ml dispase for 1 hour at 37℃. Single-cell suspensions (1-2 × 10^4 cells/ml) were plated and cultured in Dulbecco's modified Eagle's Medium (DMEM, Sigma-Aldrich, Japan) with 10 % FBS. SHEDs at 70-80 % confluency was washed with PBS, and the culture medium was changed to serum-free DMEM. After 48 hours culture, the medium was collected and centrifuged for 10 minutes at 2000 g and the supernatant were used as SHED-CM.

Animals

Female, ICR mice aged 7 weeks (25 g-28 g) were purchased from Charles River Laboratories (Yokohama, Japan). The mice were maintained under specific pathogen free conditions and fed ad libitum food pellets, CE-2 (CLEA Japan, Shizuoka, Japan) and sterile water. The animals were kept on a 12-12 h light-dark cycle. They were randomly divided into 4 groups (n = 19), including sham group (n = 5), irradiation group (RT group, n = 5), irradiation plus DMEM group (n = 4), irradiation plus SHED-CM group (n = 5). Under general anesthesia with intraperitoneal injection of three types of mixed anesthetic agents (medetomidine 0.75 mg/kg + midazolam 2 mg/kg + butorphanol 2.5 mg/kg). Necks were locally irradiated with a single dose of 15 Gy of radiation, at a rate of 4 Gy/min and a distance of 150 mm, using a MBR (Hitachi medical, Kashiwa, Japan). Before irradiation, the mice were shaved and protected except for the irradiated area by 2 mm filter of lead. The mice were housed individually to prevent gnawing of ulcers and other potentially damaging interactions. The irradiated mice were injected daily with SHED-CM (10 μl/g) into the tail vein immediately post irradiations to day 6. This study was approved by the Tokushima University Animal Care and Use Committee (Permission No. T28-92).

Measurement of the dermatitis score and ulcer area

Radiation-induced skin reaction (RISRs) were scored by Common Terminology Criteria for Adverse Events 5.0 published from NIH at November 27, 2017. The damage of skin is classified on scales 1-5 based on the Common Terminology Criteria for Adverse Events v5.0. Grade 1 is a faint edema with dry desquamation; Grade 2 is an edema with restricted moist desquamation in skin fold; Grade 3 is an extensive moist desquamation; Grade 4 is a skin necrosis or ulceration of full thickness dermis with spontaneous bleeding; Grade 5 is death by radiation. Photographs of the ulcer area were taken and was used to trace the ulcer margin and measure the ulcer area with Image J, v. 1.52 (NIH, Bethesda, MD, USA).

Histological analysis

The mice were sacrificed with intraperitoneal injection by pentobarbital inhalation at 7 days post irradiation, and then the wounds were harvested with the surrounding tissue. Skin were fixed in 10 % formaldehyde in PBS at 4 C for 24 hours, embedded in paraffin and 5 μm sections were generated with a HM 450 Sliding Microtome (Therrmo Fisher Scientific, Walldorf, Germany). The sections were stained with hematoxylin and eosin (Sakura finite, Tokyo Japan) and photographed using a BZ-9000 (Keyence, Osaka, Japan). Five different fields within the wound tissue were randomly selected from each section and counted. In addition, these sections were measured for dermal thickness length by BZ analyzer.

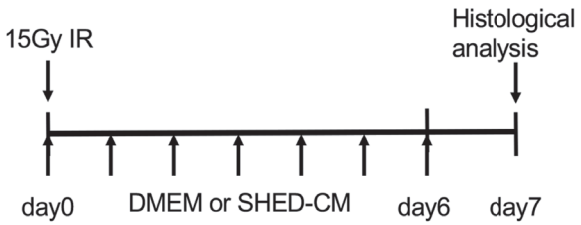

Fig. 1 Experiment design
The time points are indicated as follows: at day 0, mice were irradiated with a single dose of 15 Gy of radiation and received daily intravenous administration of DMEM or SHED-CM for six consecutive days after irradiation. At day 7, the mice were sacrificed for analysis.

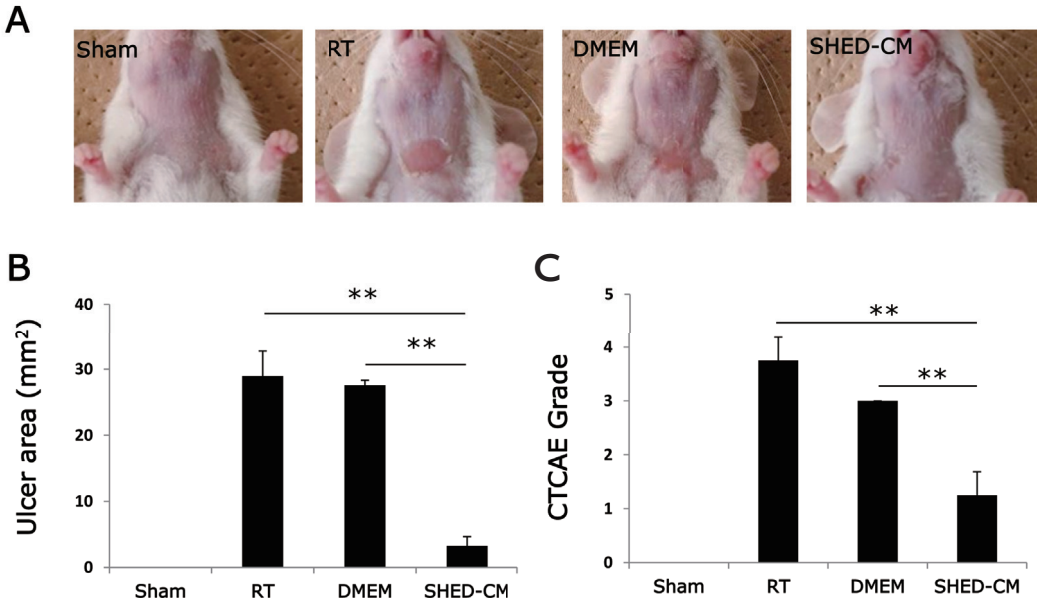

Fig. 2 Effects of SHED-CM on RISR
(A) Representative images of RISR in Sham group (n = 5), RT group (n = 5), DMEM group (n = 4) and SHED-CM group (n = 5). (B, C) Statistical analysis of CTCAE Grade (scored 1-5, maximum score: 5), $^{**}p < 0.01$.

Statistical Analysis

An unpaired two-tailed Student's *t* test was used to compare two groups. A p value < 0.05 was considered to be statistically significant.

Results

Intravenous injection of SHED-CM ameliorates the clinical symptoms of RISR

To examine the therapeutic effects of SHED-CM for radiotherapy-induced adverse events, we used a mouse RISR model induced by a selective irradiation to neck. The irradiated mice were injected daily with SHED-CM into the tail vein immediately post irradiations to day 6 (Figure 1). After seven days from irradiation, RT group and DMEM group formed a large cutaneous ulceration surrounded by severe erythema, whereas mice received SHED-CM showed faint erythema or dry desquamation (Figure 2A). Seven days after the irradiation, the mean ulcer area of the RT group was 28.95 ± 3.83 mm^2 and DMEM group was 27.57 ± 0.76 mm^2. In contrast, in the SHED-CM group, the area was 3.25 ± 1.41 mm^2 (Figure 2B). The evaluation of the injury based on Common Terminology Criteria for adverse Events v5.0 showed that phenotypic score of SHED-CM group (1.23 ± 0.43) was significantly lower than RT group (3.75 ± 0.43) and DMEM group (3.00 ± 0.00) (Figure 2C).

SHED-CM protects the cutaneous structure from RISR

Histological examination revealed that the thickness of

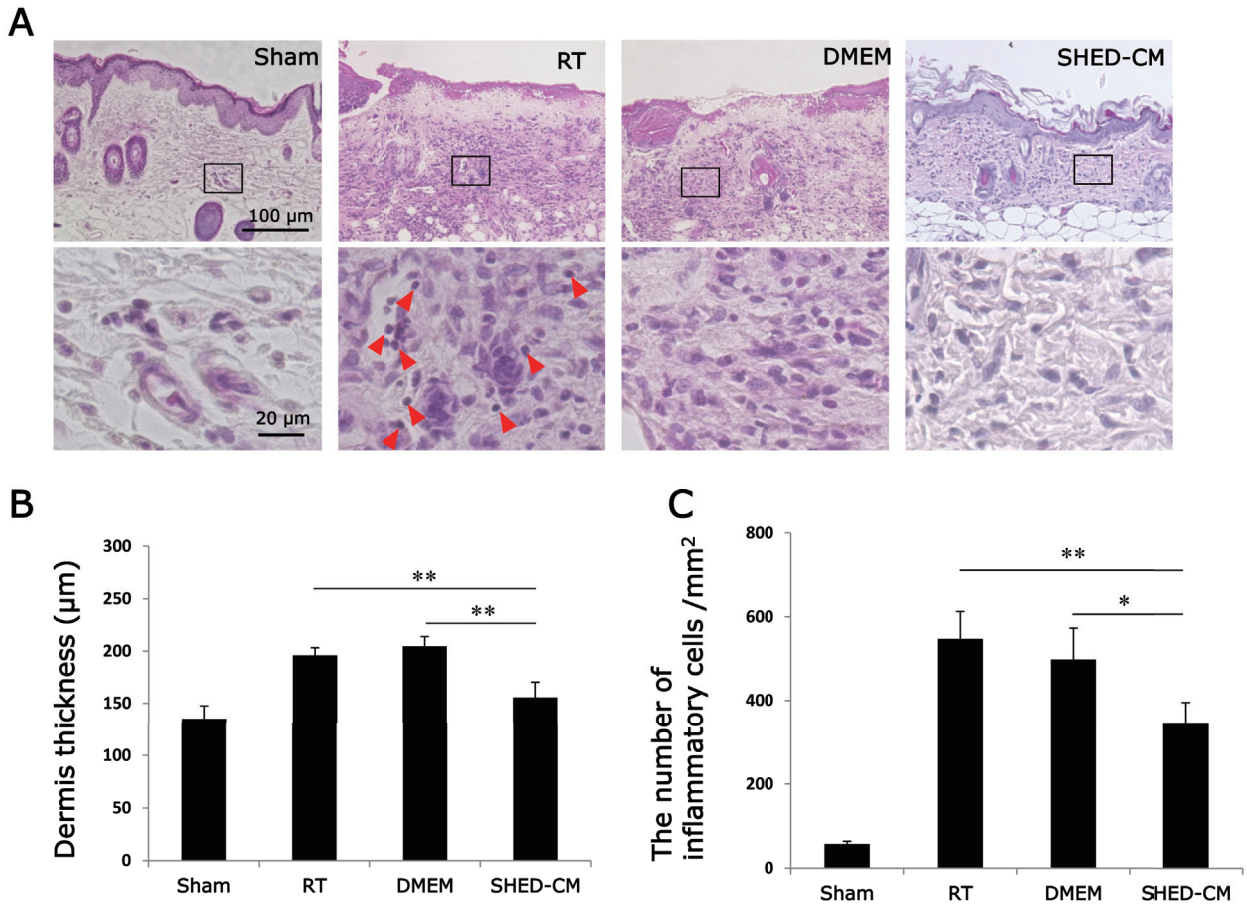

Fig. 3 Hematoxylin and eosin staining of the irradiated skin

(A) Representative images of RISR stained with hematoxylin and eosin in Sham group (n = 5), RT group (n = 5), DMEM group (n = 4) and SHED-CM group. Notably, RT and DMEM group, but not SHED-CM, shows skin ulceration lacking epidermis and thickened dermis. The bottom images of each group are high magnification. Arrowheads indicate inflammatory cells. Scale bar in low and high magnification are 100 and 20 μm, respectively (B, C) Statistical analysis of dermis thickness (B) and the number of inflammatory cells (C). *$p < 0.05$, **$p < 0.01$.

dermal layer of sham, RT and DMEM group, were 135.20 ± 12.5 μm, 196.00 ± 7.16 μm and 204.83 ± 7.88 μm, respectively. Notably irradiated skin of SHED-CM group showed modest thinking and epithelial hyperplasia (155.87 ± 12.90 μm, $p < 0.01$ SHED-CM vs RT or DMEM: Figures 3A-B). The infiltrating mononuclear immune cells in dermal layer was significantly reduced in SHED-CM group (346.1 ± 48.4/mm^2) when compared with the RT group (547.40 ± 65.0/mm^2) and DMEM group (498.3 ± 74.8/mm^2) ($p < 0.01$ SHED-CM vs RT, $p < 0.05$ SHED-CM vs DMEM; Figure 3C).

Discussion

Many studies have demonstrated that local or systemic administration of MSCs enhanced cutaneous wound healing after RISR. These preclinical studies claimed that engrafted stem cells promote tissue repair through both cell-autonomous/cell-replacement and paracrine/trophic effects. In contrast, our current study demonstrated that systemic administrations of paracrine factors secreted from SHED, without SHED transplantation, exerted remarkable anti-RISR activities, suggesting that most of the tissue repairing activity of SHED could be mediated by a paracrine mechanism. Several mechanisms of the acceleration of wound healing by MSCs have been identified, including the enhancement of angiogenesis by secretion of pro-angiogenic factors and the differentiation into endothelial cells and/or pericytes, M2 macrophages polarization, the recruitment of endogenous stem/progenitor cells, extracellular matrix production and remodeling, and immunosuppressive effects[5, 23].

We have been characterized trophic factors of SHED-CM, which contained 79 of the array proteins at levels that were more than 1.5-fold greater than those in the control

DMEM sample[24]. Here, we carried out a cluster analysis of them and identified 11 of these proteins, which are known to exhibit functional properties that may be beneficial in treating RISR. Keratinocyte growth factor (KGF) suppresses apoptosis and promotes the proliferation of epithelial cells[25]. Hepatocyte growth factor (HGF) optimizes oral traumatic ulcer healing of mice by reducing inflammation[26]. Follistatin, and dickkopf (DKK) inhibit the tumor growth factor (TGF)-β-induced differentiation of fibroblasts into myofibroblasts[27] and regulates epidermal homeostasis and wound repair[28]. IL-1 receptor antagonist (IL-1Ra) improves diabetic wound healing[29]. Stem cell factor (SCF), angiogenin and vascular endothelial growth factor (VEGF) promote neovascularization[30, 31]. Monocyte chemoattractant protein-1 (MCP-1) promotes healing in diabetic wounds by restoring the macrophage response[32]. Insulin-like growth factor-1 on promotes healing of skin ulcers in diabetic rats[33]. Secreted ectodomain of sialic acid-binding Ig-like lectin-9 and MCP-1 promote recovery after rat spinal cord injury by altering macrophage polarity[24]. The concentrations of these factors in SHED-CM may be quite low; however, we believe that combinatorial effects of these factors in SHED-CM may provide therapeutic benefits for treating RISR.

Most of the tissue damages induced by the X rays for cancer therapy is due to radiolysis of water leading to the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). During acute phase of RISR, ROS and RNS damages DNA, lipids and proteins, which induce massive necrosis/necroptosis and apoptosis and activate pro-inflammatory responses[1, 34, 35]. We found that SHED-CM treatment inhibited radiation-induced dermal thickening and epithelial hyperplasia and preserved cutaneous architecture similar to sham operated group. It has been reported that systemically administrated MSC into the mouse radiation-induced damage model inhibited the expressions of oxidative stress indicators through the upregulation of antioxidant enzymes (hemeoxygenase-1 and catalase) and suppressed radiation-induced injury[36]. Local injections of MSC prevent cutaneous ulcer induced by ischemia-reperfusion through the inhibition of the ROS generation[37]. MSC alleviate oxidative stress-induced mitochondrial dysfunction in the airway epithelium[38]. MSC protect hippocampal neurons from oxidative stress and synapse damage induced by amyloid-β oligomers[39]. These previous studies raise the possibility that SHED-CM protect cutaneous structure after radiation through the direct suppression of the ROS as well as pro-inflammatory mediators' production. In future it will be required to clarify the anti-oxidative activity of SHED-CM and factors involved the process.

Conflicts of interest

The authors state that they have no conflicts of interest.

Acknowledgments

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