Conditioned media from human dental pulp stem cells prevent radiation-induced skin injury

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Key words

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Running title

Dental pulp stem cell factors for treating RISR

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 media (SHED-CM) on radiation-induced ulceration. 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]) which 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. 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 media (CM). CMs from various 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 neuroectodermal 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

SHED (stem cells from human exfoliated deciduous tooth) 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 3mg/ml collagenase type I and 4mg/ml dispase for 1 hour at 37°C. 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. SHED at 70-80% confluency was washed with PBS, and the culture media was changed to serum-free DMEM. After 48 hours culture, the medium was collected and centrifuged for 10 minutes at 2000g and the supernatant were used as SHED-CM.

Animals

Female, ICR mice aged 7 weeks (25g - 28g) were purchased from Carles river (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 (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 0.75mg/kg + midazolam 2mg/kg + butorphanol 2.5mg/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.

Radiation-induced skin reaction (RISRs) were scored by Common Terminology Criteria for Adverse Events 5.0 published from NIH at November 27, 2017, as described in introduction.

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 (Thermo Fisher Scientific, Tokyo, Japan). The sections were stained with hematoxylin and eosin (Sakura finite, Tokyo Japan).

Finally, the sections were photographed using a BZ-9000(Keyence, Osaka Japan). Five different fields within the wound tissue were randomly selected from each section, and five number of inflammatory cells per field was counted. In addition, these sections were measured for dermal thickness length.

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, untreated irradiated mice (control) 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 control groups was approximately 28.95 mm^2. In contrast, in the SHED-CM group, the area was approximately 7.46 mm^2 (Figure 2B). The evaluation of the lesion based on Common Terminology Criteria for adverse Events v5.0 showed that phenotypic score of treated mice (1.23 ± 0.43) was significantly lower than for untreated mice (3.75 ± 0.43) (Figure 2C).

SHED-CM protects the cutaneous structure from RISR

Histological examination revealed that the tissues surrounding the ulceration of the control DMEM-group showed thickening of dermal layer (204.83 ± 7.88). In contrast, irradiated skin from SHED-CM treated showed just modest dermal thinking and epithelial hyperplasia (155.87 ± 12.90, $p < 0.01$: Figure 3A-B). The infiltrating mononuclear immune cells in dermal layer was significantly reduced in SHED-CM treatment group (346.1 ± 48.4) when compared with the control group (498.3 ± 74.8, $p < 0.05$; Figure 3C).

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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

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healing [29]. Stem cell factor (SCF), angiogenin and vascular endothelial growth factor (VEGF) promote neo-vascularization [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 induces massive necrosis/necroptosis and apoptosis and activate pro-inflammatory responses [1, 34, 35]. We found that SHED-CM treatment inhibited radiation-induced dermal thinking 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

References

1   Wei J, Meng L, Hou X et al. Radiation-induced skin reactions: mechanism and treatment. Cancer Manag Res 2019;11:167-177.

2   Haber AH, Rothstein BE. Radiosensitivity and rate of cell division: "law of Bergonie and Tribondeau". Science 1969;163:1338-1339.

3   Laverdet B, Micallef L, Lebreton C et al. Use of mesenchymal stem cells for cutaneous repair and skin substitute elaboration. Pathol Biol (Paris) 2014;62:108-117.

4   Nicolay NH, Lopez Perez R, Debus J et al. Mesenchymal stem cells - A new hope for radiotherapy-induced tissue damage? Cancer Lett 2015;366:133-140.

5   Motegi SI, Ishikawa O. Mesenchymal stem cells: The roles and functions in cutaneous wound healing and tumor growth. J Dermatol Sci 2017;86:83-89.

6   Chapel A, Bertho JM, Bensidhoum M et al. Mesenchymal stem cells home to injured tissues when co-infused with hematopoietic cells to treat a radiation-induced multi-organ failure syndrome. J Gene Med 2003;5:1028-1038.

7   Francois S, Bensidhoum M, Mouiseddine M et al. Local irradiation not only induces homing of human mesenchymal stem cells at exposed sites but promotes their widespread engraftment to multiple organs: a study of their quantitative distribution after irradiation damage. Stem Cells 2006;24:1020-1029.

8   Francois S, Mouiseddine M, Mathieu N et al. Human mesenchymal stem cells favour healing of the cutaneous radiation syndrome in a xenogenic transplant model. Ann Hematol 2007;86:1-8.

9   Agay D, Scherthan H, Forcheron F et al. Multipotent mesenchymal stem cell grafting to treat cutaneous radiation syndrome: development of a new minipig model. Exp Hematol

2010;38:945-956.

10   Horton JA, Hudak KE, Chung EJ et al. Mesenchymal stem cells inhibit cutaneous

        radiation-induced fibrosis by suppressing chronic inflammation. Stem Cells

        2013;31:2231-2241.

11   Lataillade JJ, Doucet C, Bey E et al. New approach to radiation burn treatment by

        dosimetry-guided surgery combined with autologous mesenchymal stem cell therapy.

        Regen Med 2007;2:785-794.

12   Bey E, Prat M, Duhamel P et al. Emerging therapy for improving wound repair of

        severe radiation burns using local bone marrow-derived stem cell administrations.

        Wound Repair Regen 2010;18:50-58.

13   Konala VB, Mamidi MK, Bhonde R et al. The current landscape of the mesenchymal

        stromal cell secretome: A new paradigm for cell-free regeneration. Cytotherapy

        2016;18:13-24.

14   Sakai K, Yamamoto A, Matsubara K et al. Human dental pulp-derived stem cells

        promote locomotor recovery after complete transection of the rat spinal cord by

        multiple neuro-regenerative mechanisms. J Clin Invest 2012;122:80-90.

15   Yamaguchi S, Shibata R, Yamamoto N et al. Dental pulp-derived stem cell conditioned

        media reduces cardiac injury following ischemia-reperfusion. Sci Rep-Uk 2015;5.

16   Yamagata M, Yamamoto A, Kako E et al. Human dental pulp-derived stem cells protect

        against hypoxic-ischemic brain injury in neonatal mice. Stroke 2013;44:551-554.

17   Hirata M, Ishigami M, Matsushita Y et al. Multifaceted Therapeutic Benefits of Factors

        Derived From Dental Pulp Stem Cells for Mouse Liver Fibrosis. Stem Cells Transl Med

        2016;5:1416-1424.

18   Matsushita Y, Ishigami M, Matsubara K et al. Multifaceted therapeutic benefits of

factors derived from stem cells from human exfoliated deciduous teeth for acute liver failure in rats. J Tissue Eng Regen Med 2017;11:1888-1896.

19   Wakayama H, Hashimoto N, Matsushita Y et al. Factors secreted from dental pulp stem cells show multifaceted benefits for treating acute lung injury in mice. Cytotherapy 2015;17:1119-1129.

20   Shimojima C, Takeuchi H, Jin S et al. Conditioned Media from the Stem Cells of Human Exfoliated Deciduous Teeth Ameliorates Experimental Autoimmune Encephalomyelitis. J Immunol 2016;196:4164-4171.

21   Izumoto-Akita T, Tsunekawa S, Yamamoto A et al. Secreted factors from dental pulp stem cells improve glucose intolerance in streptozotocin-induced diabetic mice by increasing pancreatic beta-cell function. BMJ Open Diabetes Res Care 2015;3:e000128.

22   Ishikawa J, Takahashi N, Matsumoto T et al. Factors secreted from dental pulp stem cells show multifaceted benefits for treating experimental rheumatoid arthritis. Bone 2016;83:210-219.

23   Rodgers K, Jadhav SS. The application of mesenchymal stem cells to treat thermal and radiation burns. Adv Drug Deliv Rev 2018;123:75-81.

24   Matsubara K, Matsushita Y, Sakai K et al. Secreted ectodomain of sialic acid-binding Ig-like lectin-9 and monocyte chemoattractant protein-1 promote recovery after rat spinal cord injury by altering macrophage polarity. J Neurosci 2015;35:2452-2464.

25   Marti GP, Mohebi P, Liu L et al. KGF-1 for wound healing in animal models. Methods Mol Biol 2008;423:383-391.

26   Wang X, Tang Y, Shen R et al. Hepatocyte growth factor (HGF) optimizes oral traumatic ulcer healing of mice by reducing inflammation. Cytokine 2017;99:275-280.

27   Akhmetshina A, Palumbo K, Dees C et al. Activation of canonical Wnt signalling is

required for TGF-beta-mediated fibrosis. Nat Commun 2012;3:735.

28   Antsiferova M, Klatte JE, Bodo E et al. Keratinocyte-derived follistatin regulates epidermal homeostasis and wound repair. Lab Invest 2009;89:131-141.

29   Perrault DP, Bramos A, Xu X et al. Local Administration of Interleukin-1 Receptor Antagonist Improves Diabetic Wound Healing. Ann Plast Surg 2018;80:S317-S321.

30   Tonnesen MG, Feng X, Clark RA. Angiogenesis in wound healing. J Investig Dermatol Symp Proc 2000;5:40-46.

31   Carmeliet P. Angiogenesis in health and disease. Nat Med 2003;9:653-660.

32   Wood S, Jayaraman V, Huelsmann EJ et al. Pro-inflammatory chemokine CCL2 (MCP-1) promotes healing in diabetic wounds by restoring the macrophage response. PLoS One 2014;9:e91574.

33   Gong F, Zhao F, Cheng SL et al. Effect of insulin-like growth factor-1 on promoting healing of skin ulcers in diabetic rats. J Biol Regul Homeost Agents 2019;33:687-694.

34   Najafi M, Motevaseli E, Shirazi A et al. Mechanisms of inflammatory responses to radiation and normal tissues toxicity: clinical implications. Int J Radiat Biol 2018;94:335-356.

35   Pugin J. How tissue injury alarms the immune system and causes a systemic inflammatory response syndrome. Ann Intensive Care 2012;2:27.

36   Shen Y, Jiang X, Meng L et al. Transplantation of Bone Marrow Mesenchymal Stem Cells Prevents Radiation-Induced Artery Injury by Suppressing Oxidative Stress and Inflammation. Oxid Med Cell Longev 2018;2018:5942916.

37   Motegi SI, Sekiguchi A, Uchiyama A et al. Protective effect of mesenchymal stem cells on the pressure ulcer formation by the regulation of oxidative and endoplasmic reticulum stress. Sci Rep 2017;7:17186.

38   Li X, Michaeloudes C, Zhang Y et al. Mesenchymal stem cells alleviate oxidative stress-induced mitochondrial dysfunction in the airways. J Allergy Clin Immunol 2018;141:1634-1645 e1635.

39   de Godoy MA, Saraiva LM, de Carvalho LRP et al. Mesenchymal stem cells and cell-derived extracellular vesicles protect hippocampal neurons from oxidative stress and synapse damage induced by amyloid-beta oligomers. J Biol Chem 2018;293:1957-1975.

Figure legends

Figure 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 (n=4) or SHED-CM (n=5) for six consecutive days after irradiation. At day 7, the mice were sacrificed for analysis.

Figure 2. Effects of SHED-CM on RISR.

(A) Representative images of RISR in control (radiation alone; left) and SHED-CM treated group (right). (B, C) Statistical analysis of Ulcer area and Dermatitis Radiation Grade (scored 1-5, maximum score: 5), **$p<0.01$.

Figure 3. Hematoxylin and eosin staining of the irradiated skin.

 (A) Representative images of RISR stained with hematoxylin and eosin in control (DMEM-treated) and SHED-CM treated group. Left and right image of control group represents area of skin ulceration lacking epidermis and thickened dermis, respectively. Scale bar = 100 μm. (B, C) Statistical analysis of dermis thickness and the number of inflammatory cells. *$p<0.05$, **$p<0.01$.
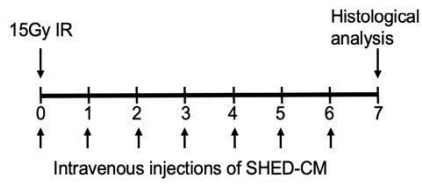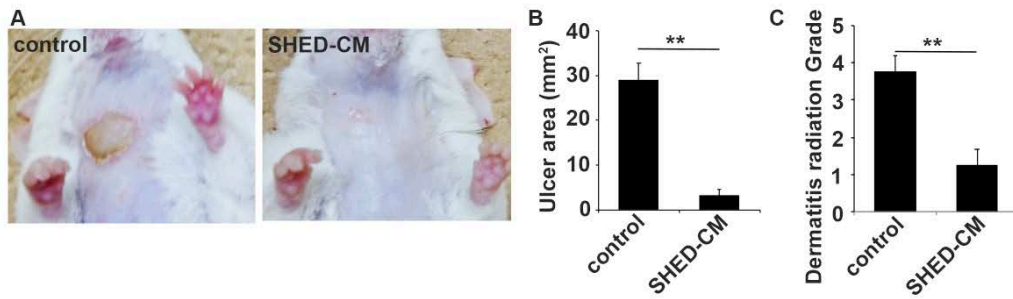
Figure 1

15Gy IR

Histological
analysis

0 1 2 3 4 5 6 7

Intravenous injections of SHED-CM

Figure 2

A
control   SHED-CM

B
Ulcer area (mm²)
**
control   SHED-CM

C
Dermatitis radiation Grade
**
control   SHED-CM

Figure 3

A
Sham   control   control   SHED-CM

B
Dermis thickness (µm)
**
control   SHED-CM

C
The number of inflammatory cells /mm²
*
control   SHED-CM

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