Bone Marrow–derived Cell Therapy for Oral Mucosal Repair after Irradiation

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Abstract

Oral mucositis (ulcer) is a serious and painful side effect for patients with head and neck cancer following radiation therapy. However, current clinical strategies cannot efficiently prevent the occurrence of oral mucositis. In this study, we investigated whether bone marrow–derived cells (BMDCs) prevented the occurrence and/or decreased the severity of radiationinduced oral mucositis. Fresh concentrated BMDCs from male C3H mice were transplanted intravenously into female mice after tongue irradiation. For 14 days postirradiation, the changes of body weight and the time courses of ulceration were observed. Until the ulcer reached maximum size (7 days postirradiation), macroscopic and histologic analyses of harvested tongues were performed to detect the behavior of donor BMDCs. Between 2 and 5 days postirradiation, BMDCs-transplanted mice showed more expression of stem cell markers (c-Kit, Sca-1) and EGFR and fewer apoptotic cells when compared with nontransplanted control mice (irradiation group). On day 7, there were fewer and smaller ulcers observed in the BMDCstransplanted group. Tongues of these mice had preserved their epithelial thickness, and regenerative activities (blood vessels formation, cell proliferation) were higher than they were in the irradiation group. Fluorescently labeled BMDCs were not detected in tongue epithelium but rather in

connective tissue (dermis) just below the basal cell layer. These findings suggest that exogenous BMDCs behave to reduce radiogenic oral mucositis in a paracrine manner.

KEY WORDS: cell transplantaion, stem cells, radiation injury, epithelial tissue, paracrine effect, mucosal regeneration.

Introduction

Oral mucositis (ulcer) is one of the severe side effects in patients receiving radiation ± chemotherapy treatment for head and neck cancer. Oral mucositis has harmful effects on nutritional intake, speaking, quality of life, and limiting the ability of patients to tolerate cancer therapies. Clinical treatment of oral mucositis mainly focuses on symptomatic measures, such as pain control, nutritional intake, and keeping good oral hygiene (Lalla *et al*., 2008). These treatments are insufficient, and unfortunately, there is currently no effective therapy for this side effect. Therefore, more effective treatment should be urgently developed.

Regenerative therapies using tissue stem cells have recently been tested. In particular, the effectiveness of cell therapy based on bone marrow–derived cells (BMDCs) has been

demonstrated in the regeneration of various tissues, such as cardiac muscle, liver, brain, or bone (Hao *et al*., 2011; Loffredo *et al*., 2011; Ali and Masoud, 2012; Zhong *et al*., 2012). Similarly, there are reports that BMDCs contributed to skin and mucosal wound healing through cell differentiation or release of paracrine factors (Wu *et al*., 2010). For instance, in rat experimental colitis, BMDCs mobilized from bone marrow homed to the injured tissue and differentiated into mucosal and submucosal interstitial cells to regenerate the colon (Komori *et al*., 2005). Moreover, several studies have shown that BMDCs contributed to not only fibroblast-shaped cells in the dermis but also to cells in the epidermis, such as keratinocytes (Wu *et al*., 2010). In line with these reports, our group recently demonstrated that BMDCs rescued radiation-damaged salivary glands through mechanisms involving a paracrine effect as well as a direct differentiation of BMDCs into salivary epithelial cells (Sumita *et al*., 2011).

Several studies have shown the effectiveness of cell therapy based on mesenchymal stem cells (MSCs) or endothelial progenitor cells (both these cell populations are components of BMDCs) in skin and mucosal healing. MSCs can differentiate into multiple cell types as well as display paracrine functions, such as suppressing cell apoptosis, promoting angiogenesis, and activating stem cells at injured sites, including

normal wound healing (Duffy *et al*., 2011; Boomsma and Geenen, 2012; Sémont *et al*., 2013). Transplanted MSCs inhibited TGF-β-induced apoptosis and suppressed activation of NF-κB signaling pathway at injured sites (Moodley *et al*., 2013; Zhang *et al*., 2013). Interestingly, formation of radiogenic oral mucositis was reported to be suppressed on Smad7-overexpressed transgenic mice (Han *et al*., 2013). Smad7 can block both TGF-β-induced apoptosis and NF-κB-activated inflammation. Therefore, exogenous MSCs may suppress the formation of oral mucositis via similar mechanisms. Meanwhile, endothelial progenitor cells favor angiogenesis and vasculogenesis by their proliferation and differentiation abilities (Asahara *et al*., 1997; Masuda *et al*., 2011). Cell therapy through endothelial progenitor cells promotes the healing of diabetic ischemic foot ulcers by paracrine effects, such as stimulation of angiogenesis (Barcelos *et al*., 2009). Taking these data together leads us to hypothesize that transplanted BMDCs (which contain both MSC and endothelial progenitor cell subpopulations) could mobilize to radiogenic oral mucosal wound sites and decrease ulceration (*i.e.*, ameliorate wound healing).

The aim of this study was to assess the preventive/regenerative capacity of BMDCs for radiogenic oral mucositis by their direct transplantations through intravenous injections. This study is a prerequisite step for future clinical trials aiming at developing

cell-based therapies for radiation-induced oral mucositis, salivary gland atrophy, and osteonecrosis. We believe that using concentrated BMDCs, without an *in vitro* culture system, is a simple and direct approach for transplantation and that this strategy should be investigated as a priority to regenerate oral mucositis.

Materials & Methods

Tongue Irradiation and BMDCs Transplantation

All experiments were approved by the Nagasaki University Ethics Committee. Recipient mice were eight-week-old female and six-week-old male mice (C3H/HeJ; CLEA, Tokyo, Japan), according to a sex-mismatched bone marrow transplantation strategy.

We developed a model of radiogenic oral mucositis by exposing the tongue of female mice to 16 Gy using the X-ray ISOVOLT TITAN 320 (General Electric, Tokyo, Japan). To select the optimal dosage for tongue irradiation/mucositis, we performed preliminary experiments. Detailed methods for irradiation are described in the Appendix Methods and in Appendix Figure 1.

BMDCs were isolated from male mice and labeled with PHK26 (Sigma, St. Louis,

MO, USA), and the characteristics of donor BMDCs are shown in Appendix Figure 2. For transplantation, mice were randomly divided into 3 groups (6 mice per group) in a blinded fashion: irradiation plus BMDCs transplantation (BM group), irradiation and no cell transplant (IR group), and no irradiation and no cell transplant (control group). Immediately following irradiation, mice of the BM group received an injection of BMDCs via the tail vein. For further confirmation, cultured dermal fibroblasts were prepared and injected to mice after irradiation, as a negative control (dermal fibroblast group). Detailed methods were described in the Appendix Methods.

Macroscopic and Histologic Observations

At 2, 5, 7, 11, and 14 d postirradiation, excised tongues were stained with 0.05% toluidine blue to visualize the ulceration. Then, the paraffin and frozen sections were prepared for histologic and immunohistologic observations. The stainings for hematoxylin and eosin, cell apoptosis, cell proliferation (PCNA), stem cell markers (c-Kit and Sca-1), blood vessel formation (vWF), and epithelial restoration (EGFR) were performed in this study as described in the Appendix Methods in detail.

Sry mRNA Expression

To confirm the presence of donor cells (male) in recipient tissues (female), reverse transcription polymerase chain reaction was performed to detect *Sry* mRNA (Y chromosome signal) in harvested tongues as described in the Appendix Methods.

Statistical Analysis

Means were analyzed via 1-way analysis of variance. The Dunnett multiple-comparison *t* test was used to detect any significant differences within each group. Experimental values were presented as mean ± SD; $p < .05$ was considered statistically significant.

Results

Macroscopic Findings after Irradiation

In mice that received irradiation to their tongues, their body weights decreased for the first 9 d (Figure 1A). Thereafter, their weights gradually recovered until 14 d. Maximum ulcer formations (maximum size of ulcers) in tongues were found at 7 d postirradiation. Then, their epithelizations were recognized by 14 d. The changes of body weights did not differ in irradiated/ nontransplanted mice (IR group) and irradiated/BMDCstransplanted

mice (BM group). However, the body weights in the BM group were recovered more quickly than those in the IR group after 11 d.

During the maximum ulceration period (day 7 postirradiation), mucositis area (including ulcer) in tongues was recognized by toluidine blue staining (Figure 1B and Appendix Figures 3, 4A). When the mucositis area was quantified, its surface area, including ulcers in tongues of the BM group (9.4%), was significantly lower than that of the IR group (56.7%) and the dermal fibroblast group (62.5%; Figure 1B, 1C and Appendix Figure 4B). Furthermore, the area of only ulcers in tongues of the BM group (6.8%) was also markedly lower when compared with that of the IR group (23.1%) and the dermal fibroblast group (18.3%; Figure 1B, 1D and Appendix Figure 4C).

Apoptotic and Stem Cell Activity before Ulceration

On day 2 and 5 postirradiation, a number of apoptotic cells were detected in the basal, spinous, and granular layers of the epithelium in the mucositis area of the IR group (Figure 2A, 2C, and Appendix Figure 5B), while apoptotic cells were mainly detected in the granular layer of mice in the BM group and the control (nonirradiated) group (Figure 2A). When the apoptotic cells in the basal layer were quantified, the positive cells were

detectable significantly in the IR group (Figure 2D, Appendix Figure 5C). In contrast, c-Kit-positive cells (stem cell marker) were found sparsely in the epithelium of the IR group, while more were seen in the BM group (Figure 2B, 2E, and Appendix Figure 5D). Furthermore, on day 5, more expression of Sca-1-positive cells (stem cell marker) was found in the basal layer of the BM group as compared to the IR and control groups (Figures 2F, 2G).

Morphologic Observation of the Ulcerated Area in Tongues

Although maximum ulcer formations were observed on the tongues of irradiated mice (IR group and BM group) after 7 d, the ulceration was smaller (Figure 1B) and less deep (Figure 3A, 3B) on the lingual surfaces of BM group mice when compared with IR group mice. The epithelial thickness of the ulcerated area was 23.8 μm (average) in the BM group while 16.2 μm in the IR group ($p < .01$; Figure 3C). Numerous inflammatory cells were noted in the dermis underlying the thin epidermis of the ulcerated area in IR group mice (Figure 3B).

Tissue Regenerative Activity in Mucositis Area

PCNA-positive cells were abundantly detected in the epithelial basal layer of nonirradiated control mice. There was a smaller number of PCNA-positive cells at the ulcer area of the IR and BM groups at 7 d postirradiation (Figure 4A). However, basal cells in the BM group had high PCNA activity, although there was no significant difference compared with those cells in the IR group ($p = .105$; Figure 4A, 4B). Moreover, blood vessel formation was more abundant in the dermis below the basement membrane adjacent to the ulcer area of the BM group when compared with that of the control and IR groups (Figure 4C, 4D).

To further evaluate tissue regenerative activity, expression of EGFR (which has crucial roles for epithelium restoration) in the epithelium adjacent to ulcer area was assessed. In the BM group, EGFR expression was observed from 2 to 5 d postirradiation (Figure 4E and Appendix Figure 5E). However, this expression almost disappeared at 7 d. EGFR expression was detected later in the IR group (7 d postirradiation). These data suggest that epithelium restoration was favored in the BM group and delayed in the IR group.

Detection of Donor BMDCs in the Mucositis Area

At day 7 postirradiation, Y chromosome signal (*Sry* gene derived from donor male cells) was detected in harvested tongues of the BM group as well as in control male mice (Figure 4F). However, this signal was not found in female mice of the IR group. Meanwhile, PHK26- labeled (red fluorescent) donor cells were detected mainly in dermis just below the basement membrane adjacent to the ulcer area of tongues in the BM group (Figure 4F).

Discussion

Our results demonstrated that cell therapy based on BMDCs reduced the severity of radiogenic oral mucositis. Our successful treatment outcomes were as follows: (1) Transplanted BMDCs reliably decreased radiogenic ulceration of the tongue and (2) BMDCs displayed paracrine effects, including vasculogenesis without transdifferentiation to epithelial cells, to reduce ulcer formation.

Regarding the first outcome, ulceration size was reduced to approximately 30% of irradiated/nontransplanted mice when BMDCs were administered. Furthermore, epithelial thickness of the ulcer area was more preserved by BMDCs administration. These phenomena reliably indicate that exogenous BMDCs can reduce the occurrence of

oral mucositis. Proposed experimental treatments of radiogenic oral mucositis via Tempol or keratinocyte growth factor (KGF) have recently been reported (Zheng *et al*., 2009; Cotrim *et al*., 2012). Tempol is a stable nitroxide that protects cells against superoxide and hydrogen peroxide–mediated oxidative stress induced by radiation (Mitchell *et al*., 1991; Cotrim *et al*., 2012). Meanwhile, KGF protein can bind to its receptor and lead to the proliferation of epithelial cells (*i.e.*, injured epithelium retains the ability to respond to KGF; Pinakini and Bairy, 2005). Additionally, KGF reduces reactive oxygen species caused by irradiation (Geiger *et al*., 2005; Zheng *et al*., 2009). Overall, at damaged oral mucosa, Tempol behaves as a radioprotector, and KGF stimulates cell proliferation and modulates cellular response to radiogenic stress. In the current study, it was not obvious whether exogenous BMDCs have such similar radioprotective effects. However, we showed that BMDCs inhibited cell apoptosis and activated cell proliferation after irradiation. More recently, MSCs were shown to increase wound closure and reduce reactive oxygen species production at injured brain in a paracrine manner (Torrente *et al*., 2013). Our transplanted BMDCs may be able to regulate oxidative stress after irradiation, which leads to decreased radiogenic oral mucositis.

With regard to paracrine functions of BMDCs, our data suggested transplanted

BMDCs activated stem cells (c-Kit- or Sca- 1-positive cells) and EGFR in lingual epithelium and induced blood vessel formation in dermis. Several studies have reported that c-Kit- and/or Sca-1-positive stem cells were mobilized from the bone marrow to injured sites for wound healing (Takemoto *et al*., 2012); then, these recruited stem cells produced various growth factors, such as EGF or VEGF, to accelerate wound healing. In this study, activation of stem cells and EGFR was observed at early stages (2-5 d) following irradiation; then, abundant blood vessel formation in the dermis was detected just below the basement membrane at 7 d. In view of these phenomena, paracrine factors of transplanted BMDCs may function to induce endogenous stem cells to the site of tongue mucositis. Actually, we could not find transdifferentiation of transplanted BMDCs into tongue epithelial cells throughout the observation period. This fact may also support our hypothesis that transplanted BMDCs behave in a paracrine manner at injured sites. Meanwhile, Zhang *et al.* (2012) demonstrated that 3-dimensional spheroid gingival-derived MSCs contributed to the treatment of chemogenic oral mucositis due to their enhanced transdifferentiation capacity. Gingival MSCs from 3-dimensional spheroids were capable of increased homing and transdifferentiation into epithelial cells, and they exhibited a more immature stem cell phenotype when compared with gingival MSCs from

adherent cultures. To induce transdifferentiation of exogenous BMDCs into epithelial cells, it may be essential to isolate and concentrate more immature stem cells. However, because of the plasticity of stem cells, transplantation with stem/progenitor cells has been reported to activate tumor proliferation, angiogenesis, and metastasis in several studies (De Boeck *et al*., 2010; Waterman *et al*., 2012), whereas other studies showed that stem/progenitor cells suppressed tumor activity (Klopp *et al*., 2011; Waterman *et al*., 2012). In any case, the fact that transplanted BMDCs could not transdifferentiate into tongue epithelial cells leads us to hypothesize that BMDCs have not much risk of contributing to tumor growth. However, a recent clinical study reported that esophageal squamous cell carcinomas derived from donor BMDCs were observed as secondary solid tumors after BMDCs transplantation, although oral and tongue squamous cell carcinomas derived from donor BMDCs were not observed (Kano

et al., 2014). Therefore, the applicability of our current strategy should be restricted to patients who receive the concurrent chemoradiotherapy, such as the superselective intra-arterial infusion chemotherapy concurrent with radiotherapy, for the early stage of solid tumors or after surgical removal. Further analyses for identifying the safe cell population in BMDCs must be essential to widen the applicability of our strategy.

In conclusion, our findings demonstrated the capacity of BMDCs for reducing the severity of radiogenic oral mucositis by their direct transplantation through intravenous injection. Although the exact mechanisms of transplanted BMDCs that lead to the amelioration of damaged tissue remain unclear in this study, we found that they acted in a paracrine manner. We recently demonstrated that BMDCs and their cell extract (BM soup) could rescue the functions of radiation-damaged salivary glands, which led to saliva production, including EGF secretion (Sumita *et al*., 2011; Tran *et al*., 2013). Meanwhile, a previous study demonstrated that KGF gene transfer to salivary glands could prevent radiogenic oral mucositis by secreting KGF into saliva or serum of mice (Zheng *et al*., 2009). These findings proposed that cell therapy with BMDCs may comprehensively ameliorate radiogenic injuries encountered in the oral/maxillofacial area, such as oral mucositis and xerostomia. However, for future clinical applications, additional investigations are needed to understand the exact mechanisms of tissue restoration by transplanted BMDCs. In fact, this study, based on single-dose irradiation, does not correlate with the current therapeutic regimens. Therefore, future investigation should be carried out by using more clinical models given the fractionated-dose irradiation with chemotherapy. Moreover, we consider our strategy of stem cell therapy (*e.g.*, using fresh

concentrated BMDCs) without the need for an *in vitro* culture system a simple, safe, and effective approach. To materialize this strategy in the clinic, further investigations will be needed for the identification of efficient and safe cell populations in BMDCs. Developing a simple technique for the harvesting of such cell populations is crucial as well.

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Figure legends

Figure 1. Changes in body weights for 14 d after tongue irradiation and gross appearances of tongue ulcer at day 7 postirradiation. (A) Changes of body weight of mice at 0, 7, 9, 11, and 14 d postirradiation. At 9 d, the body weights of both irradiated groups (IR and BM) decreased significantly compared with normal mice (Contl). Asterisk represents statistical significance ($p < .05$). (B) Goss appearance and toluidine blue

staining of harvested tongues at 7 d postirradiation. The areas of mucositis with (yellow arrows) and without ulcer (red arrows) were stained blue. White arrows: staining at the site of incision to remove tongue. Mucositis and ulcer due to irradiation are on the dorsal surface of tongue. (C) Area of mucositis per tongue (mucositis plus ulcer/whole surface area) in both irradiated groups. (D) Area of ulcer per tongue (ulcer/whole surface area) in both irradiated groups. Asterisk represents statistical significance ($p < .01$) ($n = 6$ mice per group) (mean value ± SD). Contl, control group; IR, irradiation + no cell transplant group; BM, irradiation + bone marrow–derived cells transplantation group.

Figure 2. Histologic analysis of mucositis area of tongues at days 2 and 5 postirradiation. (A) Detection of apoptotic cells (stained in brown) at 2 d. In the BM group and in nonirradiated control mice, apoptotic cells were detected mainly in the granular layer of the epithelium. In the IR group, apoptotic cells were found from the basal (black arrows) to granular layers (×200). (B) c-Kit immunostaining on day 2 postirradiation. Positive cells (stained in brown; red arrows) were recognized in the epithelium, except the basal layer. Negative controls of this staining are in Appendix Figure 5A (×200). (C) Number of apoptotic cells in the epithelium (basal, spinous, and granular layers) on day

2 postirradiation. (D) Number of apoptotic cells in the basal layer of the epithelium on day 2 postirradiation. (E) Number of c-Kit-positive cells in the epithelium on day 2 postirradiation. (F) Stem cell activities (Sca-1 immunofluorescence staining) on days 2 and 5 postirradiation. Sca-1 protein (stained in red; white arrows) was clearly detectable only in the BM group (×200). (G) Number of Sca-1-positive cells in the epithelium on days 2 and 5 postirradiation. Black arrows, basal layer; blue, DAPI; yellow-dotted line, boundary of the epithelium and dermis (scale bar is 50 μm). Black asterisk represents statistical significance between control and both irradiated groups ($p < .05$). Red asterisk represents statistical significance between IR and BM groups ($p < .05$) (mean value ± SD). Contl, control group; IR, irradiation + no cell transplant group; BM, irradiation + bone marrow–derived cells transplantation group.

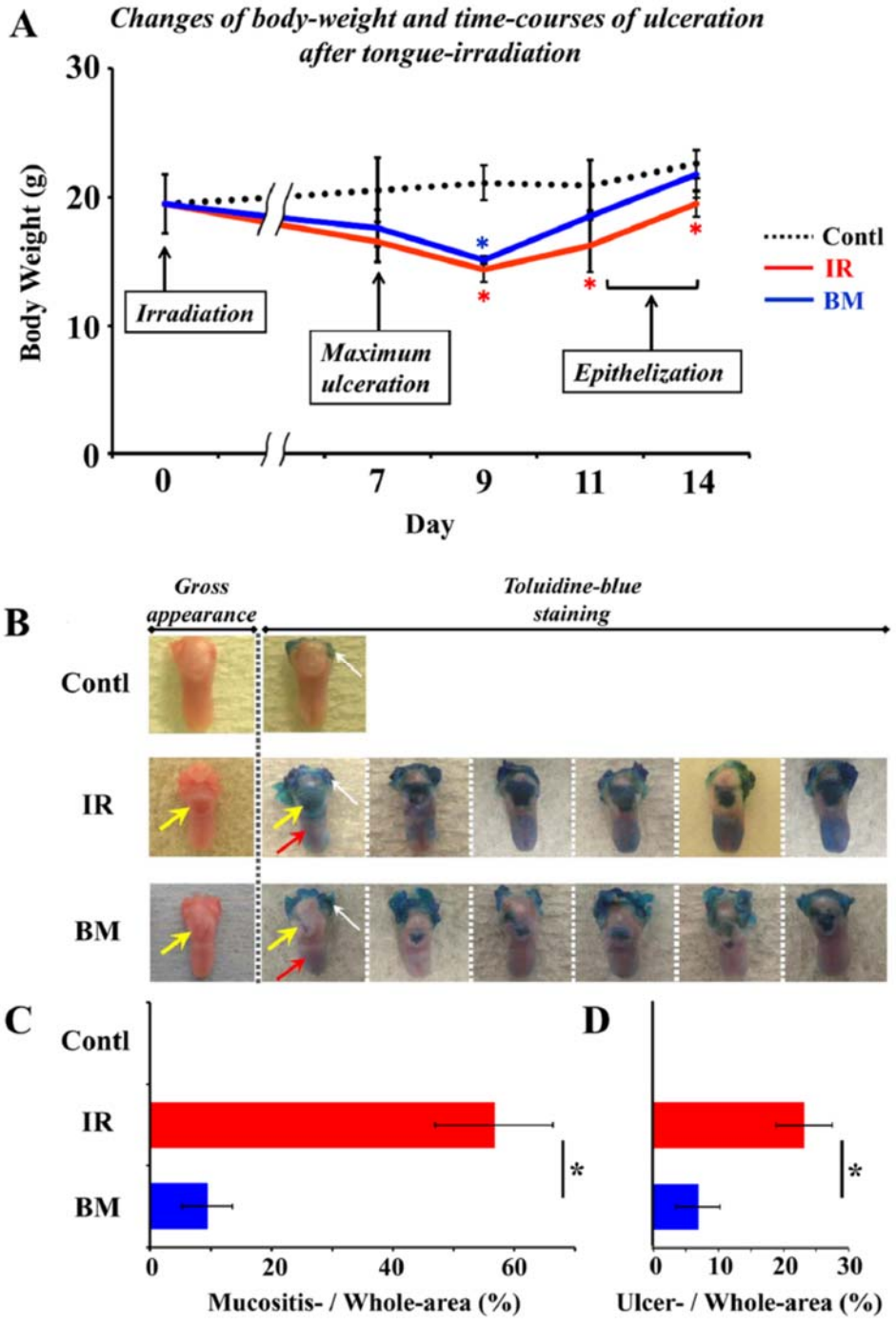
Figure 3. Histologic analysis of mucositis ulcer areas (in a sagittal view) at day 7 postirradiation. (A) Hematoxylin and eosin staining of the ulcerated areas seen at low magnification (scale bar is 100 μm) (×100) and (B) at higher magnification (scale bar is 50 μm) to assess epithelial preservation. The 3 blue boxes in the upper panels (×100) are shown in higher magnification in the lower panels (×200). (C) Epithelial thickness of

ulcer area on day 7 (mean value ± SD). The asterisk represents statistical significance ($p < .01$). Contl, control group; IR, irradiation + no cell transplant group; BM, irradiation + bone marrow–derived cells transplantation group.

Figure 4. Tissue regenerative activity and detection of donor cells at day 7 postirradiation. The photomicrograph on the upper left corner shows a hematoxylin and eosin staining of a tongue. The red box represents the area of the ulcer and is shown in higher magnification; the blue box represents the area of the mucositis adjacent to the ulcer and is shown in higher magnification (×15). (A) Cell proliferation (PCNA) immunostaining of ulcerated area at day 7 postirradiation. Three blue boxes in the upper panels (×100) are shown in higher magnification in the lower panels (scale bar is 50 μm) (×200). PCNA-positive cells (stained in brown) were mainly detected in the basal layer of the epithelium. (B) Number of PCNA-positive cells in epithelium (mean value ± SD). (C) Blood vessel area in dermis just below the basement membrane (mean value ± SD). (D) vWF immunofluorescence staining of the mucositis area adjacent to ulcer at day 7. Three white boxes in the upper panels (×100) are shows in higher magnification in the lower panels (scale bar is 50 μm) (×200). Abundant blood vessel formation (stained in

red) is shown under the basement membrane of the BM group. (E) EGFR immunofluorescence staining (red) of the lingual epithelium in mucositis area adjacent to ulcer area on 2, 5, and 7 d postirradiation (scale bar is 25 μm) (×400). (F) Detection of donor BMDCs in tongues at day 7 postirradiation. Expression of *sry* mRNA (on Y chromosome) in female mice (reverse transcription polymerase chain reaction) and detection of PKH26-labeled cells (red) were assessed. *Sry* gene expression was not detected in the female mice of the IR group. *Sry* gene was detected in female mice transplanted with male BMDCs. PKH26-labeled cells were detected right under the basement membrane adjacent to the ulcer area in the BM group (scale bar is 50 μm) (×200). IV, intravenous injection; BM group; blue, DAPI; yellow-dotted line, boundary of the epithelium and dermis. Red: PKH26-labeled cells. Black asterisk represents statistical significance between control and both irradiated groups ($p < .05$). Red asterisk represents statistical significance between IR and BM groups ($p < .05$) (mean ± SD). BMDC, bone marrow–derived cell; Contl, control group; IR, irradiation + no cell transplant group; BM, irradiation + bone marrow–derived cells transplantation group.

Figure 1.


A
Changes of body-weight and time-courses of ulceration after tongue-irradiation
Body Weight (g)
30
20
10
0
Irradiation
Maximum ulceration
Epithelization
Contl
IR
BM
0
7
9
11
14
Day
B
Gross appearance
Toluidine-blue staining
Contl
IR
BM
C
Contl
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Mucositis- / Whole-area (%)
D
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20
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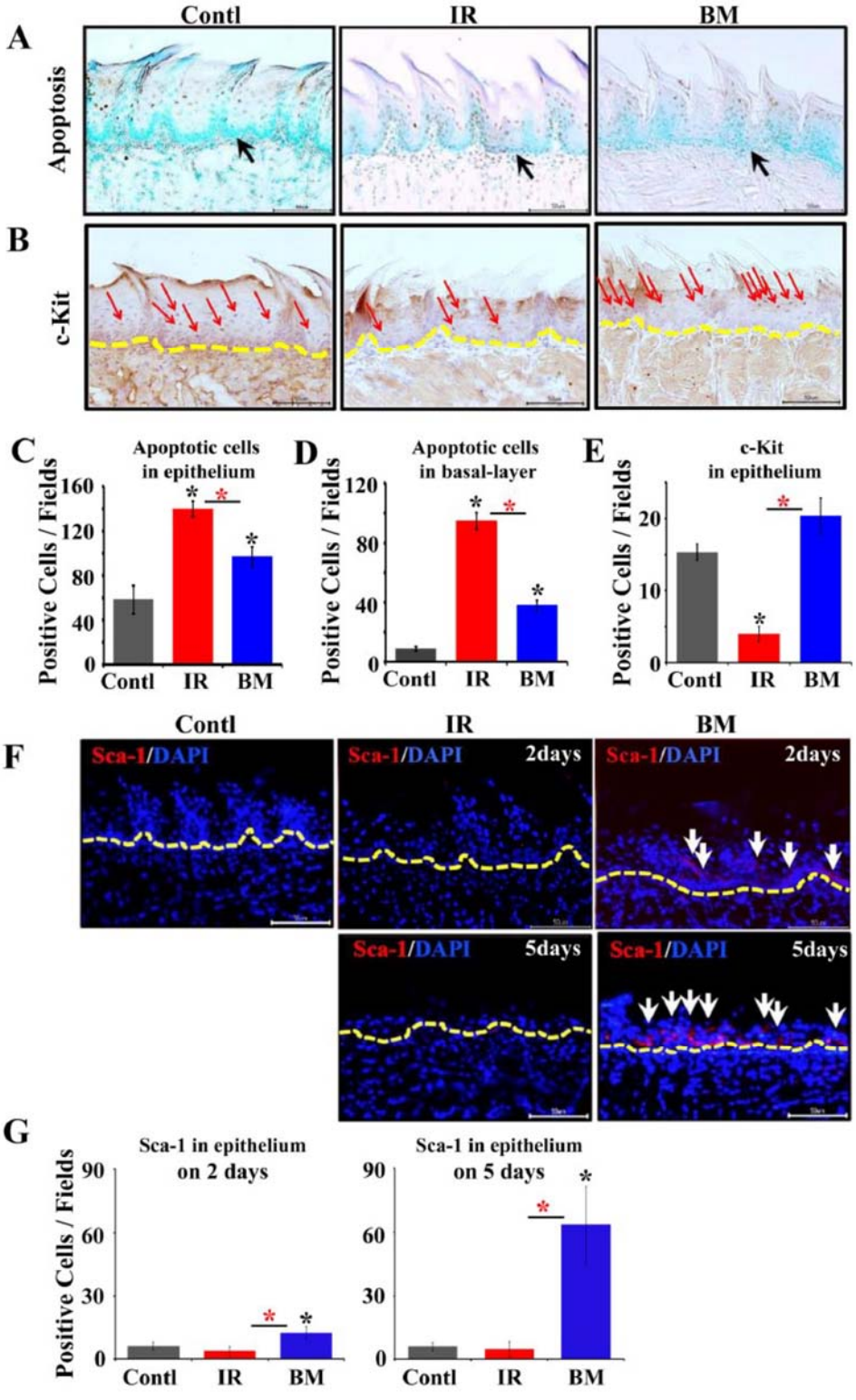
Ulcer- / Whole-area (%)

Figure 2.


A
Contl
IR
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160
120
80
40
0
Contl
IR
BM
D
Apoptotic cells in basal-layer
120
80
40
0
E
c-Kit in epithelium
20
10
0
F
Sca-1/DAPI
2days
5days
G
Sca-1 in epithelium on 2 days
Sca-1 in epithelium on 5 days
90
60
30

Figure 3.

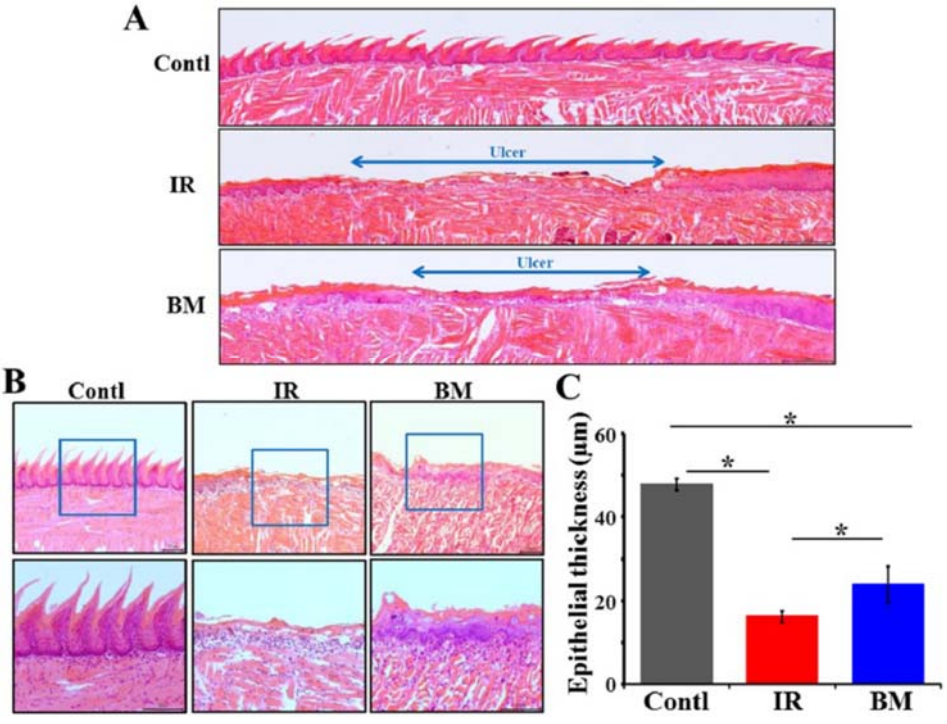
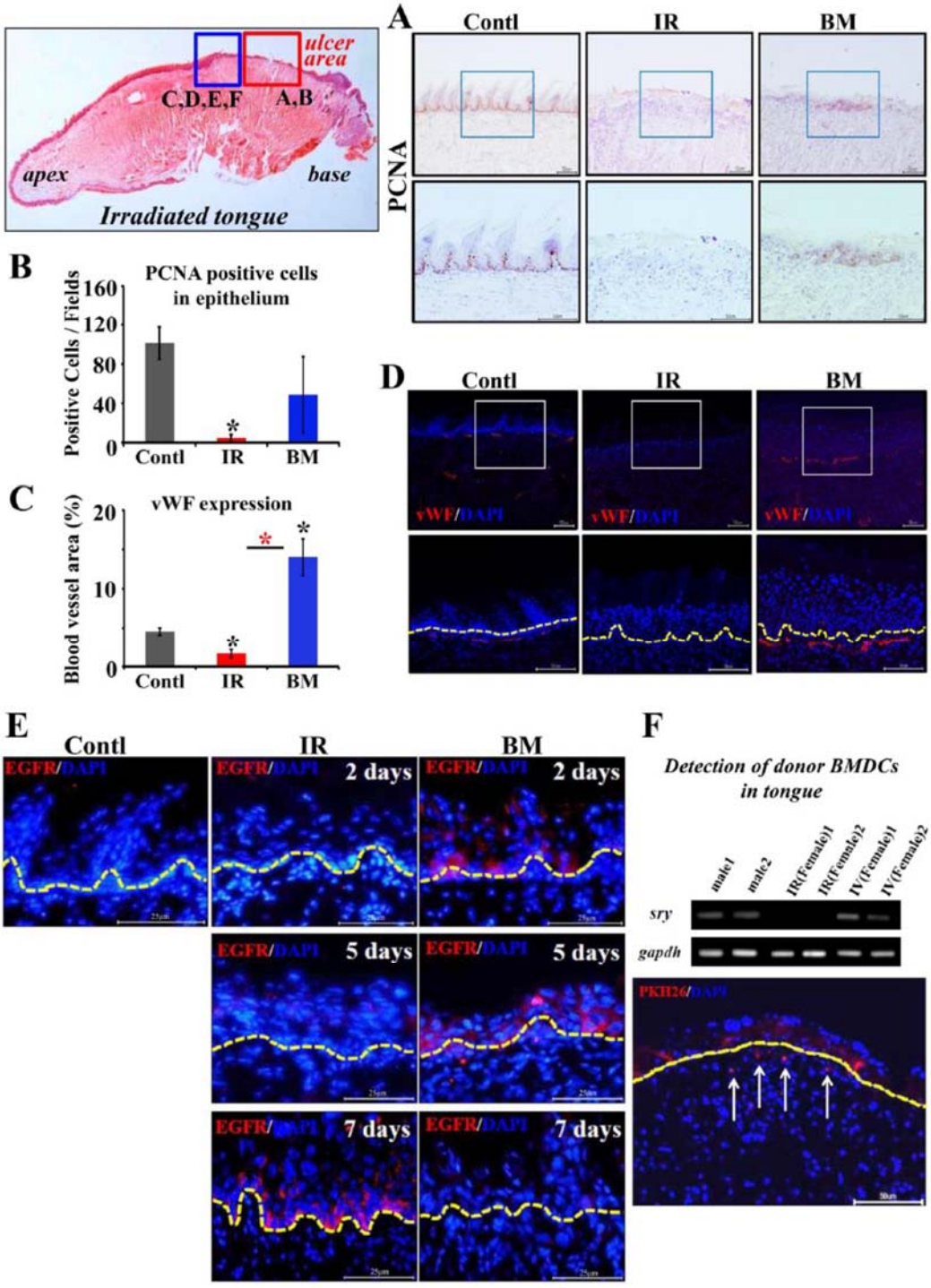

A
Contl
IR
Ulcer
BM
Ulcer
B
Contl
IR
BM
C
Epithelial thickness (μm)
60
40
20
0
*
*
*
Contl
IR
BM

Figure 4.


A
Contl
IR
BM
PCNA
ulcer area
C,D,E,F
A,B
apex
base
Irradiated tongue
B
PCNA positive cells in epithelium
Positive Cells / Fields
160
120
80
40
0
Contl
IR
BM
C
vWF expression
Blood vessel area (%)
20
10
0
Contl
IR
BM
D
Contl
IR
BM
vWF/DAPI
vWF/DAPI
vWF/DAPI
E
Contl
IR
BM
EGFR/DAPI
EGFR/DAPI
2 days
EGFR/DAPI
2 days
EGFR/DAPI
5 days
EGFR/DAPI
5 days
EGFR/DAPI
7 days
EGFR/DAPI
7 days
F
Detection of donor BMDCs in tongue
male1
male2
IR(Female)1
IR(Female)2
IV(Female)1
IV(Female)2
sry
gapdh
PKH26/DAPI