Stem cell, cytokine and plastic surgical management for radiation injuries

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Abstract. Increasing concern on systemic and local radiation injuries caused by nuclear power plant accident, therapeutic irradiation or nuclear terrorism should be treated and prevented properly for life-saving and improved wound management.

We therefore reviewed our therapeutic regimens and for local radiation injuries and propose surgical methods reflecting the importance of the systemic and general conditions.

For local radiation injuries, after careful and complete debridement, sequential surgeries with local flap, arterialized or perforator flap and to free flap are used when the patients' general conditions allow. Occasionally, undetermined wound margins in acute emergency radiation injuries and the regenerative surgical modalities should be attempted with temporal artificial dermis impregnated and sprayed with angiogenic factor such as basic fibroblast growth factor (bFGF) and secondary reconstruction can be a candidate for demarcation and saving the donor morbidity.

Human mesenchymal stem cells (hMSCs) and adipose-derived stem cells (ADSCs), together with angiogenic and mitogenic factor of basic fibroblast growth factor (bFGF) and an artificial dermis were applied over the excised irradiated skin defect are tested for differentiation and local stimulation effects in the radiation-exposed wounds.

The perforator flap and artificial dermal template with growth factor were successful for reconstruction in patients who are suffering from complex underlying disease. Patients were uneventfully treated with minimal morbidities.

The hMSCs are strongly proliferative even after 20Gy irradiation in vitro. Immediate artificial dermis application impregnated with hMSCs and bFGF over the 20 Gy irradiated skin and soft tissues demonstrated the significantly improved fat angiogenesis, architected dermal reconstitution and less inflammatory epidermal recovery.

Even though emergent cases are more often experienced, detailed understanding of underlying diseases and rational reconstructive procedures brings about good outcomes for difficult irradiated wound healing. Also, ADSCs are also implicated in the limited local injuries for short cell harvesting and processing time in the same subject.

KEYWORDS: local flap, angiogenesis, basic fibroblast growth factor, artificial dermis, stem cells

1. Introduction

There are increasing worried on both systemic and local radiation injuries probably caused by nuclear power plant (NPP) accidents, therapeutic irradiation for malignancy, interventional radiology (IRV) of unexpectedly prolonged fluoroscopic procedures for cardiovascular diseases such as arrhythmia, ischemic heart diseases or nuclear medicine of over-dose intake of the radioactive for nuclear medicine of internal radiation therapy. These conditions should be treated and prevented properly for sake of life-saving and improvement of local wound healing [1]. However, holistic clinical analysis and supporting experimental basis were not founded yet. Nagasaki University of authors' group is selected as global strategic center for radiation health risk control by the Japan's Ministry of Education, Culture, Sports and Technology and now exploring to establish therapeutic regimens, prevention the radiation injuries and possible regeneration medical therapy for radiation injuries.

Often seen chronic radiation injuries are well handled by sufficient enough blood supply to the radiated tissues, especially in the cartilage, bare bone, and hardened scar tissues. For this purpose, local, distant and microsurgical vascularized flap are applied. Recent development of micro-vasculature of the

skin and soft tissues including the connective tissues play major roles in attributing to accelerate local wound healing. Also, externally administered angiogenic growth factor such as basic fibroblast growth factor (bFGF) together with temporal wound coverage artificial skin substitute is very effective for those patients with severely injured and their basic co-morbidities are intolerant to the extensive and long surgeries [2]. In contrast, acute phase radiation injury demonstrates very fluctuated response to the medication and surgery. Also, systemic exposure of radiation often ameliorates the body immune response, cellular proliferation, differentiation capacity in total body, thus early administration of cells, preferably radiation-resistant, cell-renewal and of high differentiation capacity, in this context, stem cells from the bone marrows or adipose cells are recommended. In order to elucidate efficacy of the stem cells, both in vitro and in vivo experiments are undertaken.

2. Materials, patients and methods

2.1 Chronic local radiation injuries

Often experienced in radiation therapy for malignancy, cardiovascular modalities, these should be categorized as difficult wounding with poor vasculature.

From January 1990 to April 2007, 10 (8 females and 2 male) patients who demonstrated radiation injuries such as telangiectasia, xerosis, epidermal atrophy karatoses, and fibrosis as well as deep ulcers in the costal ribs and sternum by adjuvant radiation therapy post-mastectomy and prolonged fluoroscopic procedures for cardiovascular diseases, were surgically treated and included in this investigation.

Other selective clinical cases were used angiogenic growth factor namely human recombinant basic fibroblast growth factor (rh-bFGF), which is clinically approved and widely used for clinical wounds in Japan with skin substitutes, which are also clinically available not only in Japan but many other nations including USA, majority of EU nations and several Asian counties, and the effectiveness of using the artificial skin substitutes in the chronic radiation injuries is temporal coverage and sustainability of both internal and external cells and growth factors. Therefore, combined use of bFGF and artificial skin substitute leads to improved quality of wounds (scars) as well as facilitated wound healing [3].

2.2 Acute local radiation injuries

Theoretically when the radiation does not affect harvesting donor-sites such as abdomen, thighs, buttock and arms, adipose-derived stem cells (ADSC), or adipose-derived regenerative cells (ADRC) are often of choice of immediate regeneration for radiation-exposed wounds, since the lipoaspirated fat cells are easily processed within a few hours in a closed circuit of the processing machine only used for each specific patient.

Internal Review Board (IRB) of ethic committee of Nagasaki University allowed us to proceed the radiation injured wound healing.

2.3 Acute systemic radiation injuries

Extensive both in vitro and in vivo studies are explored using human mesenchymal stem cells, since these cells are readily available in frozen cell stockpiles and thus will be potential therapeutic regimens for unscheduled radiation injuries.

2.3.1 An in vitro stem cell biology and analysis

Human mesenchymal stem cells from a single human bone-marrow donor were isolated by density gradient centrifugation and strictly sorted as positive for markers such as CD105, CD166, CD29 and CD44, and negative for cell surface makers such as CD14, CD34 and CD45. Human mesenchymal stem cells (hMSCs) were purchased from BioWhittaker, Inc. (Cat # PT-2501, Walkersville, MD, USA) and the cryopreserved cells were thawed immediately according to the manufacturer's instructions. Two different donor-derived hMSCs, whose gender were female and whose racial backgrounds were a Caucasian (Lot # 1F0658 and 1F1061), were used in the experiments. The cells

were cultured in “basic medium” of Dulbecco’s modified Eagle Medium (DMEM) containing low glucose supplemented with 10% fetal bovine serum (FBS, heat-inactivated, cat # 16000-044, GIBCO, Invitrogen™, Life Technologies, Japan K.K.), 200mM L-glutamine, and penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in 95% humidified air and 5% CO_2. The medium was exchanged every 3 days until the cells were confluent, and then the cells were passaged up to 4 times. The growth characteristics during the four passages in FBS were indistinguishable. The cells were washed using 10 ml of phosphate-buffered saline (PBS) and then liberated by exposure to 0.25% trypsin/1mM EDTA (GIBCO, cat # 25200-056) for three minutes at 37°C, followed by tapping of the dishes and the addition of 5 ml of culture medium. The cells were centrifuged at 400*g*, and then re-suspended in basic medium for the following in vitro examinations. The other cells were stored at –70°C until use in a solution containing 5% human serum albumin (IS Japan, Co., Ltd., Saitama, Japan, Cat # 9988) and 10% dimethylsulfoxide (DMSO, Sigma-Aldrich Japan K.K., Tokyo, Japan, Cat # 41641) according to the manufacturer’s instruction. The hMSCs were cell counted by a Beckman Coulter® cell and particle counter (Beckman Coulter KK., Tokyo, Japan). After cell counting, the pellets were dissolved in total amount of 100 μl of in culture medium 30 min before in vivo use.

For cell proliferation and characteristic analysis, sub-confluent cultured hMSCs were radiated by an X-ray radiation generator (EXS-300-5, Toshiba, 200kV, 15 mA, 0.405 Gy/min) at Atomic bomb Disease Institute, Nagasaki University. For control cells of different species of origins are used. Both human neuroblastoma cells (NG1087-15) and rat pheochromocytoma cells (PC-12) are considered rather resistant to external radiations. Also, ultrastructure of the hSMCs after various doses of radiation was investigated by a transmission electron microscope. For transmission electron microscopy (TEM), basic medium containing 10% FBS or bFGF treated hMSCs was pre-fixed in half-strength Karnovsky fixative (pH 7.2, osmolarity 1,400 mOsm) buffer consisting of 2% paraformaldehyde and 2% glutalaldehyde in 0.1 M cacodylate buffer for 2 hours, post-fixed in 2% osmium tetroxide solution (pH 7.4), dehydrated using a conventional procedure and embedded in epoxy resin. The cells were first centrifuged, and then washed with PBS, and the pellets at the bottom of the tube were subsequently dissolved with the fixative after washing with PBS. Embedded specimens were ultrathin-sectioned and double-stained with uranyl acetate and lead citrate. These sections were observed using a Hitachi H-7100 electron microscope (Hitachi, Tokyo, Japan) at 75 kV accelerating voltage.
Next, specimens of the cell culture insert prepared for scanning electron microscopy (SEM) were dehydrated and dried by critical-point drying apparatus, HCP-2 (Hitachi, Tokyo) for scanning electron microscopy. Specimens in a 35mm tissue culture dish, 3000-035 (IWAKI, Tokyo) were dehydrated through an ethanol series and freeze dried in t-butyl alcohol in a freeze-dryer. The dried specimens were scatter-coated with gold using an ion-coater, IB-2 (Eiko Engineering, Tokyo) and observed with a scanning electron microscope, S-3500N (Hitachi, Tokyo) [4].

2.3.2 An in vivo model and whole body irradiation by an X-ray generator

Animals were aged 10 weeks and weighing 300–350 g, and were used. Animals were obtained from CLEA JAPAN (Tokyo, Japan), housed in the laboratory animal centre for biomedical research, Nagasaki University School of Medicine (Nagasaki, Japan), and the protocol of the animal experiment was approved by the Institutional Animal Care and Use Committee of Nagasaki University, no. 0204080111. They were handled according to the guidelines established for animal care at the centre. Each rat had free access to both sterile water and standard rodent soft chow *ad libitum* [6].
20 Gy whole body irradiation to 10 nude rats (F344/NJCl-rnu), which are deleted T-cell function and thus acute immune rejection to human derived cells is minimized, were performed at Atomic bomb Disease Institute, Nagasaki University, by a X-ray radiation generator (EXS-300-5, Toshiba, 200kV, 15 mA, 0.405 Gy/min). Animals were divided into two groups of five each, control group and hMSCs-with bFGF-treated group and surgical procedures were performed immediately after irradiation.

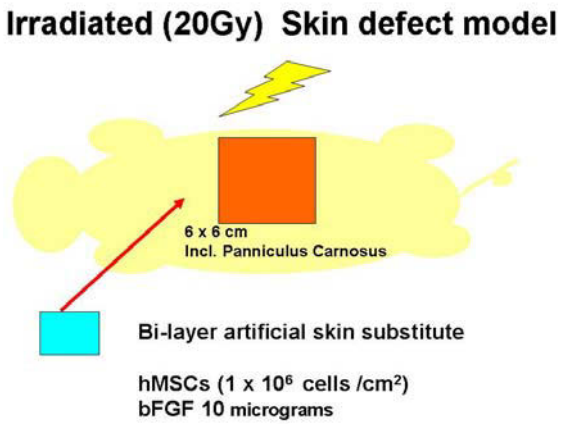
2.3.3 Angiogenic growth factor, basic fibroblast growth factor (bFGF)

Genetically recombinant human bFGF (Fiblast®, Trafermin) was purchased from Kaken Pharmaceutical Co., Inc (Tokyo, Japan). The freeze-dried samples were dissolved in PBS at a concentration of 1 mg/mL and dissolved in culture medium 30 min before experimental use.

2.3.4 Surgery

In an aseptic surgical room and with sterile surgical instruments, skin tissue of 6 × 6 cm^2, including the panniculus carnosus, was excised from F344/NJCl-rnu nude rats, and subsequently from artificial dermal substitute impregnated with 10 μg bFGF with or without the hMSCs and, after anaesthetizing with a 40-mg kg^{-1} intra-peritoneal injection of pentobarbital sodium, U.S.P. (Nembutal; Abbot Laboratories, North Chicago, IL, U.S.A.). Five microliters of DMEM containing hMSCs and bFGF were added to the inner layer of the same-sized (6 × 6 cm^2) skin substitute for 30 s at room temperature. The wound was covered with the impregnated artificial dermis and sutured using 5-0 nylon. Each animal was then housed in its own cage to avoid damaging the dermis.
The control group (medium and bFGF) received 5 μl of DMEM. The hMSC treatment group received 5μl of DMEM. The experimental treatment group received 5μl of DMEM containing 5 × 10^6 hMSCs and 10μg of bFGF at 30 min before transplantation for each wound defect (Fig. 1) [5].

Figure 1: Nude rat irradiation model. After 20Gy whole body irradiation, immediate surgical removal of the affected tissues including panniculus carnosus and replacement with artificial skin substitute containing angiogenic growth factor (bFGF) and hMSCs



3. Results

3.1 Chronic local radiation injuries

All surgeries were uneventfully performed with patients' autologous well-vascularized tissues such as muscular flap, myocutaneous (muscle and skin) flap and fascial (vascularized connective tissue) flap. The mean postoperative follow-up was 11 years and 3 months (3 year to 16 years). The average age was 67 years (53 to 78 years). Eight female patients received fractioned radiation therapy in post-mastectomy, ranging from 35 Gy to 50 Gy (mean 46.1 Gy). Two male patients underwent percutaneous transluminal coronary angioplasty (PTCA) under fluoroscopy, which was unexpectedly prolonged. The time to reconstructive surgery averaged 21 years and 8 months (6 to 32 years). Some patients undertook multiple surgeries, and the average number of the surgical procedures was 1.8 (1 to 4 surgeries). Rectus abdominis myocutaneous flaps were used for 4 cases and one of which had a free vascularized flap. Latissimus dorsi myocutaneous flaps were used for 4 cases. One of the latissimus dorsi myocutaneous cases was reconstructed with an osteo-musculo-cutaneous flap. Other reconstruction modalities include the trapezius muscle flap, abdominal wall flap, deltopectoral flap, and free vascularized groin flap. One case required three major flaps (rectus abdominis myocutaneous flap and trapezius muscle flap, latissimus dorsi flap, and free vascularized groin flap) for extra-ordinary larger irradiated tissue coverage. Five patients suffered from heart diseases such as angina, aortic regurgitation and heart failure. Two cases experienced PTCAs, one case suffered from an old myocardial infarction, another case suffered from an angina pectoris and developed heart failure, and one other case demonstrated of hypertension as well as of aortic regurgitation (Fig. 2a-2c).

Figure 2a: A 43 year-old woman. A 50 Gy-fractionated radiation in a post-mastectomy demonstrated chronic chest wall ulcers. The arrow indicates exposed costal rib.

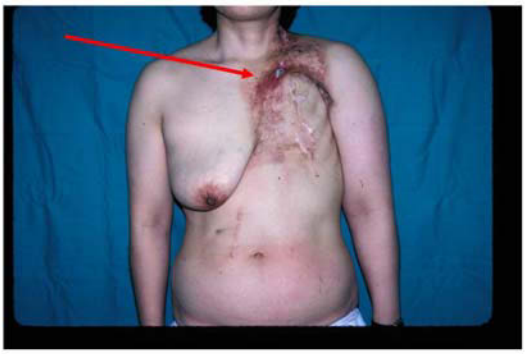


Figure 2b: Three staged surgeries including rectus abdominis myocutaneous flap, latissimus dorsi myocutaneous and free vascularized groin flap were used for chest wall reconstruction and defect coverage after affected tissues were removed.

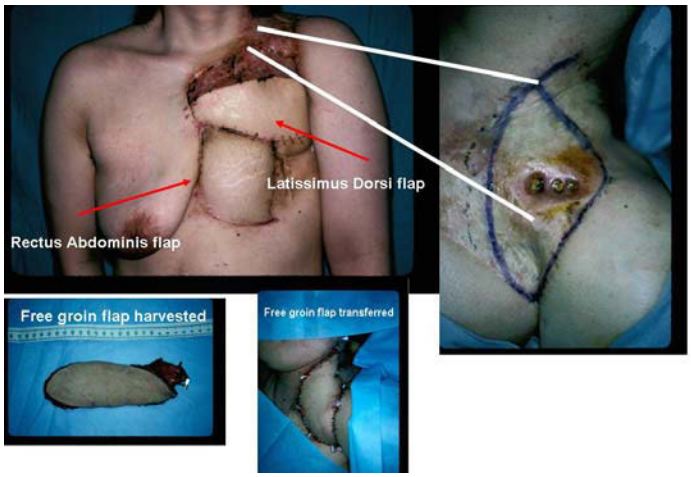
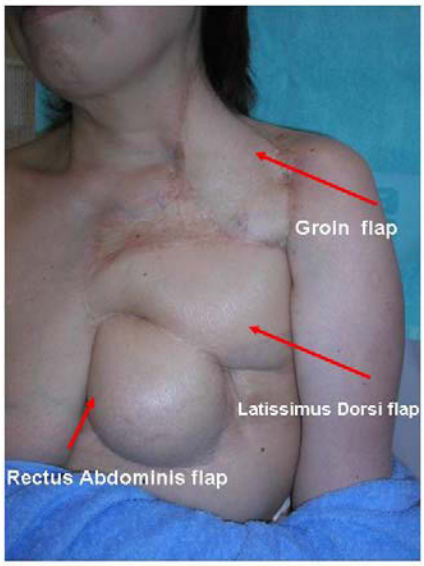


Figure 2c: Five years after last surgery. There was no ulcer recurrence.



3.2 Acute local radiation injuries

Autologous adipose derived stem cells (or “regenerative” cells), which are distant from radiation sites, are most favourable for regeneration source and conditioning for the pre-reconstruction procedures. When the radiation safely enough distant from the adipose cell donor sites such as lower abdomens, tights, buttocks and arms, then liposuction of the adipose cells are started. Approval for treatment for the radiation injuries is now obtained through Internal Review Board (IRB) of Nagasaki University and the adipose cell processing in the operation room within 2 hours is underway using a Celution™ system with a collaboration of Cytori Therapeutics, Inc. (San Diego, USA). Preliminary study of the cell characteristics of the adipose-derived stem cells (ADSC), or adipose-derived regenerative cells (ADRC) are very comparable to those of the human mesenchymal stem cells (hMSCs) and thus experimental data with hMSCs may be applicable to the ADSC/ADRC in regeneration and wound healing. Among notable characteristics, multi-lineage differentiation mechanisms promote the complex difficult wound healing in regards to epithelialisation, neo-vascularization and matrix deposition by fibroblast production [6].

3.3 Acute systemic radiation injuries

3.3.1 An in vitro stem cell biology and analysis

In order to investigate human mesenchymal stem cell proliferation, sub-confluent cultured hMSCs were used and irradiated by an X-ray radiation generator. The cells were immediately transferred to the incubators after irradiation. For control cells of different species of origins are used. Both human neuroblastoma cellls (NG1087-15) and rat pheochromocytoma cells (PC-12) are used. Cell proliferation was consistent in three cell groups in the normal condition (no radiation and normal medium), however, 20Gy irradiation caused cell death in groups of NG1087-15 and PC-12 in 48 hours, in contrast, the hMSCs survived up to next 96 hours (Fig 3a).

In an electron microscopy, irradiated hMSCs demonstrated surface microvilli all over the cells, however the hMSCs still survived after 60 Gy irradiation, of which dose is considered medium and induce the significant intestinal bleeding (Fig. 3b).

In next, signaling pathway of 20Gy and 60Gy irradiated hMSCs were investigated for ERK, JUNK, RSK, CREB, PCNA, Elk-1, Bad, Akt and Tyr. With increase of radiation dose from 20 Gy to 60 Gy, phosphorylated ERK were gradually increased, on the other hand, phosphorylation of JUNK was not altered. Phosphorylation of the downstream protein of ERK inversely decreased with increase of radiation. The cellular expressions of PCNA, VEGF, and fibronectin decreased after irradiation while there was no phosphorylation after irradiation in CREB. Further analysis in terms of inhibition of apoptosis, the BAD pathway was further studied. Phosphorylation of BAD (Ser 112) and Akt (Ser 473) was down-regulated. Therefore, irradiated hMSCs may survive through BAD-Akt (Ser) pathway (Fig. 3c).

Figure 3a: Cell proliferation was tested in a 10% FBS after 20 Gy irradiation. Both NG108-15 (human neuroblastoma cell line) and PC 12 (rat pheochromocytoma cell line) demonstrated within 48 hours, while hMSCs survived at least 120 hours post-irradiation.

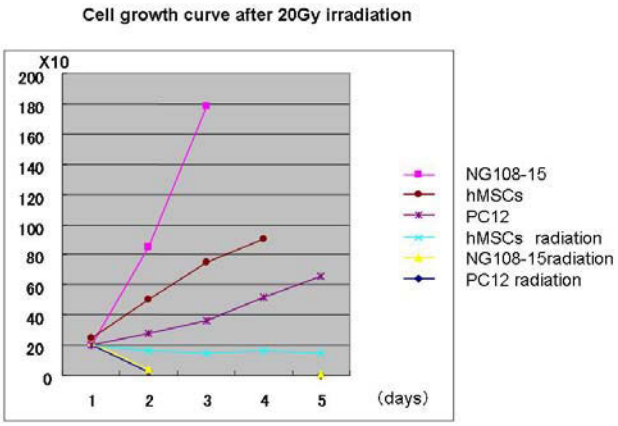


Figure 3b: The hMSCs were irradiated at 60Gy and ultrastructure was analyzed. At day 1, compared to control (left panel), irradiated cells demonstrated the shaggy cell surface of microvilli formation. In a higher magnification, it is densely distributed all over the surface. At day 4, the cell demonstrated less microvilli as compared to day 1.

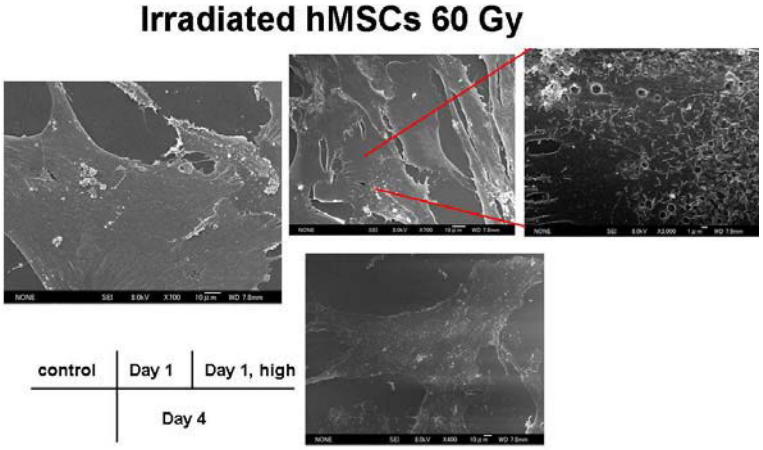
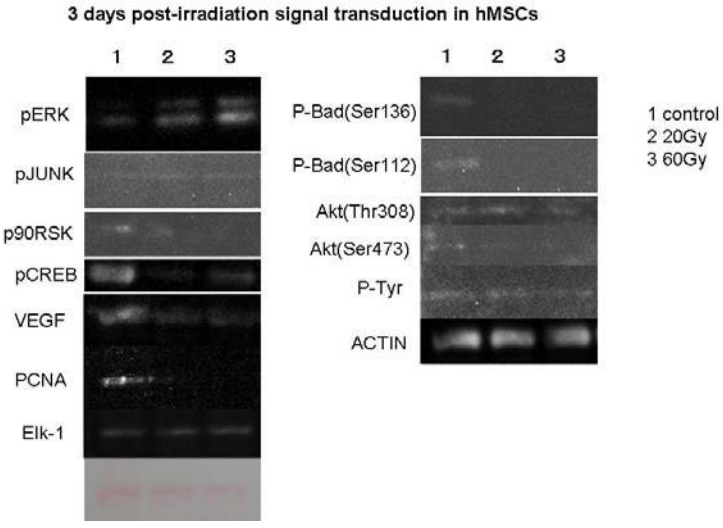


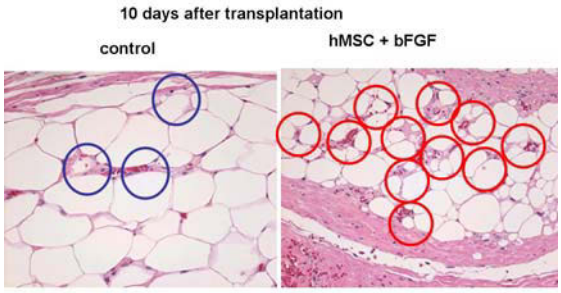
Figure 3c: Signaling pathway at 3 days post-radiation. Cell proliferation, protein expression and apoptosis were analyzed at 20- and 60 Gy.



3.3.2 An in vivo analysis after whole body irradiation

After 20Gy whole body irradiation, the seemingly radiation-affected surfaces were removed surgically including the panniculus carnosus. Immediate resurfacing with skin substitutes impregnated with hMSCs and bFGF facilitated wound healing. At day 10, the histology demonstrated the vascular rich subcutaneous tissues with more interstitial cellularity (Fig. 4).

Figure 4: Subcutaneous tissues after 20 Gy irradiation. The tissues treated with hMSCs and bFGF demonstrated more vasculature than those of control after 10 days. Circles indicate the vascular components of the subcutaneous tissues.



4. Discussion

Management of radiation injuries compose two major parts. One is localized injuries and the other is of systemic injuries. Among localized radiation injuries, chronic injuries are more common in the medical field after caner radiation therapy, IVR such as fluoroscopic procedures and nuclear medicine in the past events. Usually management of these chronic wounds are well-handled by well-vascularized tissue transfers as various plastic surgical procedures have proved [7]. In consideration of each patient general condition and preference, the choice of therapeutic selections should be performed. On the other hand, when the local radiation injuries are encountered in an acute phase, there are high chances for innovative procedures using autologous stem cells. Since human stem cells from bone marrows (hMSCs) are resistant to radiation as demonstrated in vitro cell proliferation curve and also able to produce protein avoiding cell apoptosis [8]. Also, increasing evidences demonstrate that adipose- derived stem cells (ADSC) are similar to hMSCs in cell properties and characteristics both in vitro and in vivo. When localized radiation was distant enough from the donor sites adipose tissues, immediate debridement and regeneration using adipose-derived stem cells, which are available for processing within 2 hours in an simultaneously in a same operation theater without cell culture since adipose tissues (fat tissues) are abundant in adult humans compared to other stem cell sources (Fig. 5).

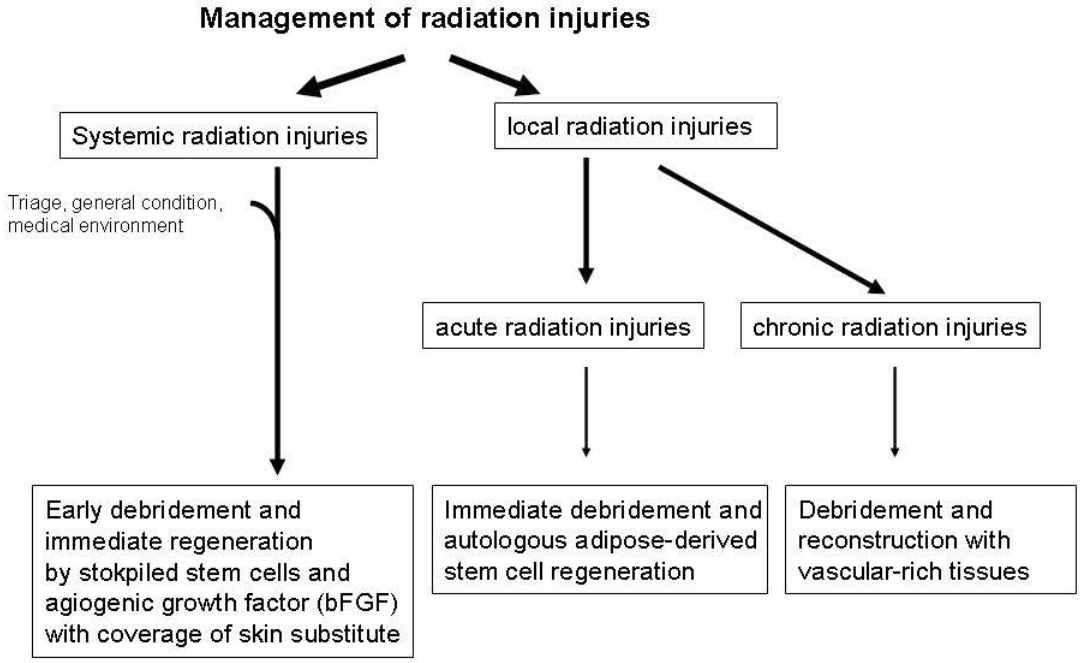
For treatment for systemic radiation injuries, stockpiled stem cells should be globally available through medical assistance network system under WHO-REMPAN (World Health Organization – Radiation Emergency Medical Preparedness and Assistance Network), in which Nagasaki University is highly involved in its activity, or other international frameworks. Early resurfacing of the damaged skin and subcutaneous tissues are as important as hematological and intestinal system resuscitation [9]. Also, therapeutic guidelines for systemic radiation injuries are anticipated from practical and regulatory view points. Highlighting innovative technology and devices such as well as currently existing medicines and devices are expected on behalf of preparing to treat “systemic” radiation injuries most effectively.

Therapeutic regimens of radiation injuries used to be dependent on each sub-specialty in the medical filed such as internal medicine, radiology and surgery.

Recent establishment of wound care specialty, mostly led by plastic surgeons but other supporting specialists such as nurses, dermatologists and gastrointestinal physicians and surgeons, may be

practically handle these rare but of significant impact “radiation injuries” as a inter-disciplinary approaches [10]. For further more specialized for “radiation injuries” may be required.

Figure 5: Flow chart of management of radiation injuries. Each patient condition should be carefully monitored first. In systemic radiation injuries, after taking in account of triage with consideration of the patients’ general conditions and medical environment, stem cell therapy initiated with cytokine augmentation, while local injures are sub-divided into “acute” and “chronic”. Each sub-divided group can be excellently handles by experts.



REFERENCES

[1] FARNCOIS S, MOUISEDDINE M, et al.. Human mesenchymal stem cells favour healing of the cutaneous radiation syndrome in a xenogenic transplasnt model. 86 (2007) 1.

[2] AKITA S, TANAKA K, HIRANO A. Lower extremity reconstruction after necrotizing fasciitis and necrotic skin lesions using a porcine-derived skin substitute. J Plast Reconstr Aesthet Surg. 59 (2006) 759.

[3] AKITA S, AKINO K, TANAKA K, et al., A basic fibroblast growth factor improves lower extremity wound healing with a porcine-derived skin substitute. J Trauma. 64 (2008) 809.

[4] AKINO K, MINEDA T, AKITA S, et al., Attenuation of cysteinyl leukotrienes induces human mesenchymal stem cells differentiation. Wound Repair Regen. 14 (2006) 343.

[5] AKITA S, et al., “Reconstruction for local radiation injuries and proposed regeneration therapy for acute radiation injuries”, Radiation risk and perspectives (Proc. Second Nagasaki Symp. Nagasaki, 2006), (Shibata Y, Namba H, Suzuki K and Tomonaga M. Ed.), Elsevier, Netherland (2007) 196-202.

[6] ZUK PA, ZHU M, ASHJIAN P, et al., Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell. 13 (2002) 4279.

[7] DORMAND EL, BANWELL PE, GOODACRE TE. Radiotherapy and wound healing. Int Wound J. 2 (2005) 112.

[8] CHEN MF, LIN CT, et al., The sensitivity of human mesenchymal stem cells to ionizing radiation. Int J Radiat Oncol Biol Phys. 66 (2006) 244.
[9] WEINSTOCK DM, CASE C JR, et al., Radiologic and nuclear events: contingency planning for haematologists/oncologists. Blood. 111 (2008) 5440.
[10] GOTTRUP F. A specialized wound-healing center concept: importance of a multidisciplinary department structure and surgical treatment facilities in the treatment of chronic wounds. Am J Surg. 187 (2004) 38S.