

Advances in Stem Cell Therapy: Specific Applications in the Treatment of Cutaneous Radiation Syndrome

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Abstract

The emergence of stem cell therapy and cellular engineering during the last 10 years has allowed new therapeutic strategies to be considered for the treatment of many wound repair disorders in bones, muscles and skin. The cutaneous radiation syndrome that is often associated with radiation burn may also take advantage of these scientific advances. This syndrome, characterized by inflammatory waves, incomplete wound healing and poor revascularization is the dramatic consequence of local irradiation exposure (above 15Gy). Until the late 90's, the treatment schedule which exhibited poor efficacy consisted in excision followed by transient coverage of the wound bed and then by autologous skin grafts. Recently the local injection of autologous bone marrow mesenchymal stem cells, which favor wound healing and reduce pain, has achieved a major advance. However this strategy hampered by culture delays and requiring non-irradiated areas to harvest stem cells remains to be optimized. Autologous or allogeneic adipose tissue-derived stem cells, easy to collect and expand, may represent a valuable therapeutic alternative especially through pro-angiogenic and anti-inflammatory properties. Other proposed strategies including stem cell manipulation to produce trophic factors (transient gene therapy), bone marrow mesenchymal stem cells or adipose tissue derived stem cells culture media injection appear as valuable alternatives.

In this review we report the latest scientific progresses in pre-clinical and clinical studies concerning stem cell therapy for cutaneous radiation syndrome.

Keywords: Stem cells therapy; Bone-marrow Stem Cell; Adipose tissue derived Stem Cell; Conditioned media; Transient gene therapy; Cutaneous Radiation Syndrome

Introduction

Radiologic Accidents (RA) are tragic events, which may result in mass casualties. For the last few years there has been a higher number of RA resulting from the increased use of ionizing radiation in several activities (mostly industrial and medical). A list of RA identified from 1896 to 2013 compiled by Robert Johnston shows a sharp increase in the last 4 years with a mean of 3 (RA)/year (Figure 1A) [1]. As shown in Figure 1B, the vulnerability of nuclear power plants are responsible for 50% of the total number of serious radiological accidents (for example Fukushima Daiichi disaster, 2011) [2], followed by the industrial sector and the storage of nuclear waste. Four past reactor accidents have each resulted in irreparable damages to the power plant and in substantial radiation exposures involving $\geq 1,000$ people as a consequence of the release of radioactive materials into the environment [3]. Accidental overexposures of persons, in either the occupational or public field, have caused deaths and severe injuries and complications [4]. Many high dose exposures have been reported these last 15 years, such as accidental exposure to radioactive sources in which two victims (accident of Chile, 2006 and accident of Senegal, 2007) were accidentally exposed to an iridium gammagraphy radioactive source, or overexposure to radiotherapy. Importantly these exposures are generally heterogeneous or localized body irradiations. They can induce numerous tissue damages depending on many parameters, the main ones being: (1) the level of absorbed dose, (2) the size of irradiated tissue volumes and the organs exposed, (3) the nature of the radiation.

Medical treatment of irradiated patients is complex depending on the nature or severity of the lesions. For example, a whole body acute irradiation requires hematological reanimation and also treatment for each specific radiosensitive organ or failing system. Radiation skin injury defined as Cutaneous Radiation Syndrome, (CRS) (also called

radiation burn) may need reconstructive surgery acts related to the burn treatment. Assessments of hematopoietic, gastrointestinal and cutaneous syndromes have improved in recent years, but their treatment options remain limited [5]. Importantly, both acute radiation injuries and delayed effects such as cutaneous effects and impaired wound repair are related to angiogenesis deficiency with vascular regression. Vascular damage influences nutrient and oxygen availability to skin tissue as well as epithelial cell viability [6]. The progressive damage of tissues after radiation exposure is the result of the presence of long-lived free radicals, reactive oxygen species, and pro-inflammatory cytokines/chemokines. Normal wound repair process consists of 3 stages (inflammation, proliferation and remodeling) that occur in a predictable series of cellular and biochemical events. The deposition and remodeling of extra-cellular matrix (ECM) are major processes in cutaneous healing and ECM is recognized to play critical roles in regulating progenitors and reparative behaviors such as migration, differentiation, proliferation and survival [7].

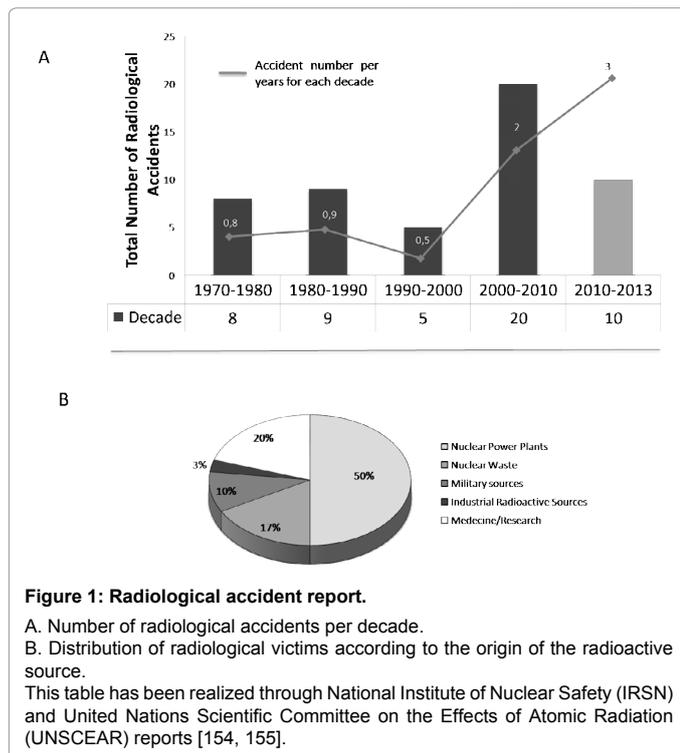
Regarding CRS, the skin is a complex radiosensitive tissue which is exposed to significant radiation doses in radiotherapy, radio-oncology or in an accidental exposure context. The biological

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Received February 20, 2014; Accepted March 24, 2014; Published March 26, 2014

Citation: Riccobono D, François S, Valente M, Forcheron F, Drouet M (2014) Advances in Stem Cell Therapy: Specific Applications in the Treatment of Cutaneous Radiation Syndrome. J Stem Cell Res Ther 4: 186. doi:10.4172/2157-7633.1000186

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responses of the skin occur in a characteristic temporal pattern and mainly depend on radiation quality, dose rate, total dose, and cellular conditions. Immediately after irradiation, synthesis and action of cytokines by skin cells (epidermal keratinocytes, dermal fibroblasts, and resident immunocompetent cells including dermal dendritic cells, neutrophils, eosinophils, and lymphocytes) is initiated and continues as a cascade during all stages of the cutaneous radiation syndrome leading to progressive late symptoms. The predominant one is fibrosis. Interactions between these different cell types within skin tissue are mediated in large part by cytokines. The major cytokines involved in the response of skin cells to high doses of ionizing radiation include IL (interleukin)-1, IL-6, tumor necrosis factor (TNF-alpha), transforming growth factor (TGF-beta), and the chemokines IL-8 and eotaxin [8,9]. Moreover, skin and its appendages provide a protective barrier against the assaults of the environment. To perform this role, epidermis is in constant renewal from keratinocyte stem cells (KSC) residing in a special microenvironment called niche in basal epidermis, adult hair follicle, and sebaceous glands. The KSC control the balance between proliferation and differentiation/apoptosis called homeostasis [10]. Irradiation of KSC and niche induces DNA double strand breaks at the level of KSC (γ H2AX marker), decreases of KSC functionality, and colony-forming activity in culture as well as a delayed hair cycle *in vivo*. In fact niche damage results in the decrease of regeneration capacity with reduction of the production of differentiated keratinocytes over time and so, a limitation of the process of re-epithelialization. This is the rationale of skin autograft or transient flap surgery realized for important defect in wound repair.

Clinically, the classical evolution of CRS is characterized by the delayed onset of the manifestation stages and consists in transient early erythema, dry and then wet desquamation, derma ischemia or necrosis for doses above 20Gy [11]. It is associated with recurrent and uncontrolled inflammatory waves leading to irreversible necrotic processes and followed by a poorly spontaneous revascularization and incomplete healing [12,13]. Consequently, the evolution of skin

lesions often becomes uncontrolled and surgery is the final option, until amputation, leading to a major disability [14,15]. CRS is also characterized by paroxysmal and chronic pain resistant to opiates [16].

Even if these symptoms are very similar to thermal burn, the clinical evolution of CRS is very specific. There are two major differences. First, in opposition to thermal burn, the symptoms onset is delayed. This latency period is very variable: shorter for higher doses and depends on radio-sensitivity (few days to several weeks). It may induce diagnosis delay and even therapeutic delay. Secondly, radiation burn is a very extensive lesion, and first symptoms (erythema or blister) are not an indication of the ultimate extent of injury [16].

Therapeutic management of CRS is difficult due to unpredictable inflammatory waves which lead to extension and to delayed onset of clinical symptoms on the skin. Indeed, when cutaneous fibrosis or necrosis outcome (from 85 to 120 days post irradiation in minipig model according to intrinsic radiosensitivity), deep dermal and underlying muscle structures are already disorganized or necrotic (from 10+/-1 weeks post irradiation) (Figure 2). Until the late 90's, the treatment schedule consisted in excision of the highly exposed necrotic area (skin and subcutaneous tissues) followed by transient coverage of the wound bed and then by autologous skin grafts [17]. The efficacy was limited because limits between radio-injured and safe tissues are not well defined and because each operative act stimulated inflammatory and ischemic processes [14].

In this context, many *in vivo* models such as mouse and rabbit have been investigated to establish a therapeutic strategy [18]. The disadvantages of these models are mainly the different skin structure and the excessive doses of radiation needed to observe the development of CRS [19,20]. Pig seems to be the most relevant animal model to develop and study the effects of cell therapy on CRS and other skin diseases. The porcine species have similarities to human in organ morphology and function, and are easier to maintain and breed than monkey. Pig skin is also very close to human in terms of histology and vascular morphology of the dermis [21-23]. CRS kinetics is a little bit delayed but the symptomatic evolution is similar to human [24]. Moreover it is also a pertinent model to evaluate long-term effects of cell therapy.

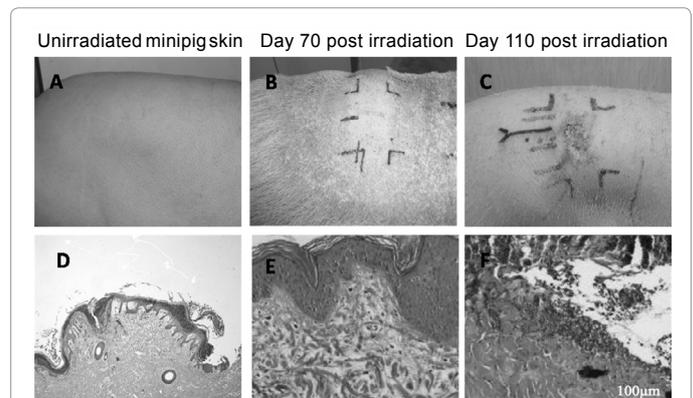


Figure 2: Contrast between speed lesion evolution of the skin and underlying tissues in minipig. A, B, C. Evolution of skin injury after irradiation. After irradiation a latency period is observed on cutaneous tissue and an edema appears on day 70 post irradiation (B). This injury leads to dry and then wet desquamation (C) before necrosis. D, E, F. Evolution of underlying tissues after irradiation: histological analysis with hematoxylin-eosin staining. A disorganization of underlying structures appears previously at the 10th week (E), leading to necrosis (F).

Currently, standardized therapeutic protocols and evidence-based approaches for the management of local injuries do not exist. In the past 10 years, several strategies have been yet needed in order to enhance the beneficial effect of stem cell therapy and to counteract the deleterious effect of an irradiated tissue environment [25]. Based on advances in understanding of cell biology, stem cell therapies such as bone marrow mesenchymal/ stromal stem cells (BM-MSC) have emerged as promising approaches to prevent and cure CRS.

Pre-clinical and clinical applications of MSC

BM-MSC trials: Multipotent stromal cells also named mesenchymal stromal cells or mesenchymal stem cells (MSC) are capable of dividing and their progeny is capable of further differentiating *in vitro* into one of several mesenchymal phenotypes such as osteoblasts, chondrocytes, myocytes, marrow stromal cells, tendon-ligament fibroblasts, and adipocytes [26]. MSC have been isolated predominantly from hematopoietic tissues, such as bone marrow (BM-MSC), peripheral blood and umbilical cord blood but also from parenchymal non-hematopoietic tissues such as muscle, fat or liver. MSC isolated from adult tissues are not ethically restricted, but the isolation efficiency of MSC differs according to the source tissue [27,28]. To uniform characterization of MSC and facilitate the exchange of data among investigators, the guidelines of the International Society for Cellular Therapy (ISCT) proposes a minimal set of standard criteria to define these multipotent precursors : (1) MSC must be plastic-adherent when maintained in standard culture conditions; (2) MSC must express CD105, CD73 and CD90, and lack the expression of CD45, CD34, CD14 or Cd11b, CD79a or CD19 and HLA-DR surface molecules; (3) MSC must differentiate to osteoblasts, adipocytes and chondrocytes *in vitro* [29].

In just ten years, since the description of the multilineage potential of MSC by Pittenger et al. [30] until now, there have been more than 90 clinical trials worldwide using BM-MSC, 20 of which are being made in East Asia, 19 in Europe and 18 in north America. The potential of these cells for cell-based therapies relies on several key properties: (1) their capacity to differentiate into several cell lineages [31]; (2) their lack of immunogenicity and their immune-modulatory properties [32]; (3) their *ex vivo* expansion potential [33]; (4) their ability to secrete soluble factors which regulate crucial biological functions such as proliferation and differentiation over a broad spectrum of target cells [34,35]; and (5) their ability to home to damaged tissues and tumor sites [36]. Based on these properties MSC are being exploited worldwide for a wide range of potential clinical applications including cell replacement strategies, treatment of graft-versus-host disease, autoimmune diseases and rejection after solid organ transplantation as well as their use as vehicles to deliver anti-cancer therapies. Importantly, the low inherent immunogenicity of MSC means that they could be used not only for autologous but also for allogeneic cell therapies [37].

Different routes of administration are currently discussed concerning stem cell therapy. The main ones are direct injection in wound bed, systemic injection with a homing of MSC in injury location, conditioned media injection and biomaterials. This strategies of stem cells delivery have been elaborated based on the stem cells properties i.e. their capacity to produce and secrete cytokines/trophic factors and to transdifferentiate. Recent studies highlighted synergic and specific effects of these two biological pathways (Figure 3). Cytokines mitigate cell proliferation [28], immune-modulation [38], and pro/neoangiogenesis [39]. Otherwise, direct differentiation of MSC has been reported in fibroblasts, keratinocytes and endothelial cells [38].

Recent works have highlighted the transdifferentiation of MSC (BM-MSC and adipose tissue derived stem cell) in keratinocytes *in vivo* in mouse model [40-42] under β -catenin, bone-morphogenic protein 4 and epithelial growth factor stimulation [42,43] (Figure 3) and have suggested that cells residing in the bone marrow can migrate to the different tissues, transdifferentiate into various epithelial cell types and integrate long-term in these tissues [40]. This transdifferentiation process remains rare but could be underestimated and crucial in skin rejuvenation. In wound repair, closure of epithelial gap and restoration of the epithelium as barrier function is achieved by a combination of keratinocytes migration and differentiation. Moreover, according to the concept of Ito et al. [44], wound healing mimics embryonic development by activating the hair follicle stem cell niche which raises the possibility that stem cells can restore loss of stem cell niche following injection. Finally, as described in Figure 3, MSC can also differentiate in endothelial cells with an impact on vessels integrity and neovascularization which are key processes in wound healing.

The potential therapeutic effect of BM-MSC infusion for the correction of tissue injury (skin [19,24], liver [45], intestine [46]) induced by acute high-dose irradiation has been demonstrated in animal models including (1) xenotransplantation of BM-MSC in NOD/SCID mice 24 hours after exposure [19] and (2) Autologous BM-MSC injected locally (intradermal in multiple points) at various times after irradiation [47]. A beneficial effect was observed for both types of transplants. In grafted animals, BM-MSC led to local accumulation of lymphocytes at the dermis border and improved vascularization, with an increase of reepithelialisation. The development of lesions was delayed and limited to moist desquamation. In these cases of acute irradiations, the BM-MSC have been shown to be implicated in tissue homeostasis by maintaining vascular system integrity via the activation of anti-apoptotic and anti-oxidative processes [31], and stimulation of endogenous host progenitor cells to improve the regenerative process to reduce tissue damage [48].

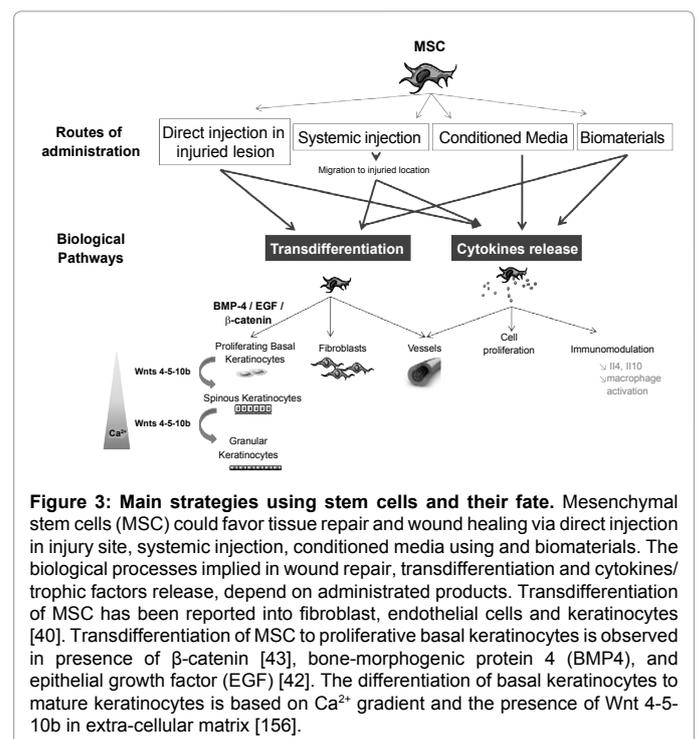


Figure 3: Main strategies using stem cells and their fate. Mesenchymal stem cells (MSC) could favor tissue repair and wound healing via direct injection in injury site, systemic injection, conditioned media using and biomaterials. The biological processes implied in wound repair, transdifferentiation and cytokines/trophic factors release, depend on administrated products. Transdifferentiation of MSC has been reported into fibroblast, endothelial cells and keratinocytes [40]. Transdifferentiation of MSC to proliferative basal keratinocytes is observed in presence of β -catenin [43], bone-morphogenic protein 4 (BMP4), and epithelial growth factor (EGF) [42]. The differentiation of basal keratinocytes to mature keratinocytes is based on Ca²⁺ gradient and the presence of Wnt 4-5-10b in extra-cellular matrix [156].

Due to their beneficial effects, BM-MSC was used for the first time to treat CRS in humans in 2006. Four patients reported with cutaneous radiation syndrome (up to 70 Gy of local exposure) associated with a heterogeneous irradiation (hematopoietic syndrome associated) have received a compassionate protocol using autologous BM-MSC. This regenerative cellular therapy has become the reference treatment used for radiation skin injury according to the International Atomic Energy Agency [49]. It consists first on a physical dosimetric reconstruction of doses absorbed by tissues. This dosimetry-guided surgery performs the most accurate excision in order to prevent iterative surgery and vicious circle induced by the successive inflammatory waves. In this compassionate therapy, autologous bone marrow total cells are harvested from unexposed areas and expanded in culture media supplemented with human platelet lysate. At the confluence step, MSC are harvested. Four quality controls are archived on MSC: a phenotype characterization (CD45⁻; CD105⁺; CD73⁺; CD90⁺), a clonogenic efficiency is calculated, telomerase activity is performed and a contamination control for bacteria, mycoplasma and fungi before administration is realized [50]. Different schedules of MSC injected have been realized: 200 (± 50). 10⁶ stem cells were injected twice at day 90 and 99 post irradiation or five times from day 191 to 268 post-irradiation [50,51] according to clinical evolution and delay between exposition and therapeutic management (Figure 4). This cell therapy is combined with surgery: skin autologous graft +/- transient skin flap. Unlike classical evolution, the size of the wound progressively decreased and almost complete healing was achieved by 1 month after cell therapy. Additionally, there were no recurrent inflammation and a better control of pain was observed. Many mechanisms are involved in radiation burn repair and it has been reported that MSC were implicated in all stages of tissue repair including inflammation [52], epithelialization [53], angiogenesis [39,53,54], and proliferation of fibroblasts [53]. So this cell therapy protocol is efficient but given its complexity and dependence on the production and quality of the stem cells, only a very low number of patients can be treated at the same time.

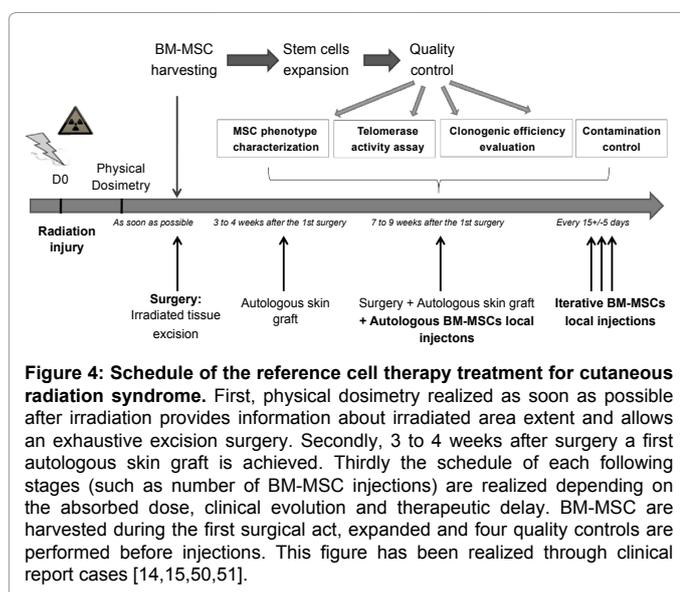
In retrospect, the data from various pre-clinical and clinical trials using BM-MSC, show their strengths and potential weaknesses that limit their use (Table 1). It is essential to optimize the quantity

and the quality of injected stem cells, as only a small amount can be harvested and their engraftment is transient [47]. Some important points to consider in the optimization of this cell therapy will be culture standardization, the choice of source tissue and donor fat status and age. To address the problem of cell quantity an abundant source tissue and cell banking could be a solution. The adipose tissue is such a promising abundant source. Stem cells derived from this tissue (ADSC) exhibit the same properties as BM-MSC which are required to favor wound repair [27]. Furthermore MSC secretome would eliminate the delay of cell culture and extend their use to mass casualties and no non-irradiated area cases.

MSC from another connective tissue ADSC: The clinical use of BM-MSCs has presented problems, including poor engraftment, low cell number upon harvest (they only represent about 0,001%-0,01% of the total bone marrow nucleated cells [55]), migration to tumor tissue potentially favoring the growth of breast and prostate cancers [56] and promoting drug resistance in tumors cells [57]. This has led many researchers to investigate alternate sources for MSCs. Adipose tissue, like bone marrow, is derived from the mesenchyme and contains a supportive stroma that is easily isolated. Based on this, adipose tissue may represent a source of stem cells that could have far-reaching effects on several fields. In recent years, interest in the developmental plasticity and therapeutic potential of stromal cells isolated from adipose tissue (which can be isolated from lipoaspirates) has rapidly grown [58]. Their amount is approximately 500-fold greater [59,60] than BM-MSC so adipose tissue represents an abundant, practical, and appealing source of donor tissue for autologous cell replacement.

There is a confusing inconsistency in the literature when using terms describing multipotent precursor cells from adipose tissue stroma, such as processed lipoaspirate (PLA) cells, adipose tissue-derived stromal cells (ADSC), preadipocytes, adipose stroma vascular cell fraction, and others. The stromal-vascular cell fraction is free of adipocytes discarded after centrifugation and is described as cells obtained immediately after collagenase digestion. Accordingly, the term ADSC will be used throughout this review. Considerable effort has been made to characterize the cellular and molecular properties of ADSC. A large number of research teams have demonstrated that mesenchymal stem cells within the stromal-vascular fraction of subcutaneous adipose tissue can be maintained *in vitro* for extended periods with stable population doubling and low levels of senescence [61]. ADSC are more genetically and morphologically stable in long term culture [28]. They also have a higher proliferative capacity and a lower senescence ratio than BM-MSC which allow an easier cell expansion [62]. ADSC differentiate into various cell types including adipogenic, chondrogenic, myogenic, and osteogenic cells in the presence of lineage-specific induction factors [63]. They also have the capacity to transdifferentiate into keratinocyte-like cells (KLC) and furthermore are able to engineer a stratified epidermis. Chavez-Munoz et al. [64] have reported that ADSC co-cultured with human keratinocytes or with keratinocyte conditioned media developed a polygonal cobblestone shape characteristic of human keratinocytes after a 14-day incubation period. This study suggests that adipose tissue is potentially a readily available and accessible source of keratinocytes, particularly for severe wounds encompassing large surface areas of the body and requiring prompt epithelialization.

Otherwise, ADSC have similarities to BM-MSC in terms of cell surface phenotype (Table 2) and they present multilineage differentiation capabilities regardless of the adipose tissue they were



collected from [65]. This information supports the use of ADSC in cell-based therapies such as autologous fat grafting. Currently, 77 clinical studies using ADSC are underway, 27 are made in Europe. Seven of these clinical studies are performed on severe skin disorders, particularly for the reconstruction of the breast and in patient lipodystrophy [30].

Anti-apoptotic and pro-angiogenic effects of ADSC have been clearly described through paracrine factors secretion *in vitro* [66,67] and *in vivo*, in mice models [68,69] as well as trans differentiation processes into endothelial cells also *in vitro* and *in vivo* [70]. Kim et al. [71] have demonstrated in a NOD/SCID model that ADSC improve angiogenesis through VEGF, bFGF and MMP3 and 9 more efficiently than BM-MSC in a comparative study using the same amounts of transplanted cells and Koh et al. [72] have related the formation of vascular networks 2 weeks after injections of ADSC in Matrigels™ (mouse model). It was also found with an *in vitro* experiment that ADSC were better at inhibiting the differentiation of dendritic cells, at decreasing their expression of co-stimulation factors and at stimulating their secretion of immunosuppressive cytokine IL10 [73]. Moreover the same group highlighted a significant inhibition of Ig production in incubated peripheral blood mononuclear cells to a much greater extent than BM-MSC [74]. In addition, it has been reported that ADSC decrease inflammatory reaction via PGE 2 [52,75] and improve wound healing [76,77] in various models (mice, rats, dogs) and pathologies as cardiac ischemia [78] brain lesion [79], musculoskeletal regeneration [80] and spinal cord injury [81]. Furthermore, the onset of senescence for ADSC after freezing has been demonstrated to occur later than for BM-MSC [82] which provides another advantage if stem cell banking is planned for cutaneous radiation syndrome treatment. Due to their

properties described above, the ADSC appear as excellent candidates to counteract the development of CRS, thus pre-clinical and clinical studies were investigated in wound healing.

In the French Armed Forces Biomedical Research Institute (IRBA), autologous ADSC have been evaluated in a specific minipig model of acute radiation syndrome; the experimental design is illustrated in Figure 5. Minipigs were locally exposed to 50 Gy as described in Riccobono et al. [83] (local gamma irradiation) and they received 4 injections of 50.10⁶ ADSC in the irradiated area. These stem cells had been harvested one month before exposure to avoid systemic radio-induced consequences. In contrast to controls that developed a typical evolution of CRS with final necrosis, injected animals ultimately exhibited wound healing. Histological analysis highlighted a progressive and transient lymphocyte infiltration, an increase of vascularization and a sustained epidermis recovery of the skin with a multilayer appearance similar to healthy skin whereas controls skin exhibited a progressive disorganization of epidermal layers [84]. Moreover in opposition to controls analgesic treatment was unnecessary for ADSC treated individuals from 6 weeks post-irradiation.

Autologous ADSC have also been evaluated on severe human cases of chronic radiation injury of the skin in Japan [85,86]. Unlike previous work, ADSC were non cultured cells sampled from liposapirate. This protocol started with an excision of the defective portion of the irradiated skin. During this surgical step, ADSC were harvested by liposuction and grafted in association with a temporary artificial dermis impregnated with bFGF. A regenerative tissue matured in 1.5 years.

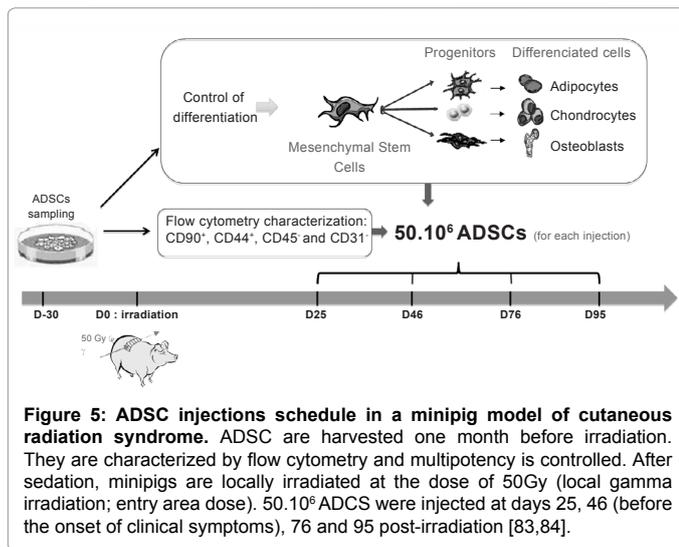
Finally, in case of mass casualties or important heterogeneous

Strengths	Weaknesses
Lack of immunogenicity	Heavy surgery :puncture of the bone marrow
Regulation of crucial biological functions: <ul style="list-style-type: none"> • Proliferation • Differentiation • Inflammation 	Low amount of MSC in bone-marrow (1/10000 nucleated cells)
Expansion <i>in vitro</i> obtained in not complex culture medium: <ul style="list-style-type: none"> • Basic fibroblast growth factor-enriched media. • Serum-enriched/serum-free media • Cytokine-free media 	With increasing passage number: <ul style="list-style-type: none"> • Decrease of proliferation rate • Loss of their typical morphology
Good expansion yield in culture with retain of: <ul style="list-style-type: none"> • Growth • Multilineage potential. Stable morphology (fibroblast) up to 20-25 passages No chromosomal abnormalities for MSC expanded <i>in vitro</i> Isolation efficiency of BM-MSC : 100%	The discrepancy between pro or anti-tumoral effect of MSC related to: <ul style="list-style-type: none"> • Different tissue sources • Culture conditions • Individual donor variability • Injection timing of MSC Decrease of differentiation potential with age of donor

Table 1: The strengths and weaknesses of BM-MSC for their clinical use. This table has been realized through the results and reflections brought by the pre-clinical and clinical investigations using BM-MSC as reparative therapy [28,29,33,157-159].

	POSITIVE surface Antigen	NEGATIVE surface Antigen
Adhesion molecules	CD9; CD29 (β ₁ -integrin); CD49d (α ₄ -integrin); CD54 (ICAM-1); CD105 (endoglin); CD166 (ALCAM)	CD11b (α ₅ -integrin); CD18 (β ₂ -integrin) CD49f (α6-integrin); CD50 (ICAM-3); CD56 (NCAM); CD61, CD62e (E-selectine); CD104 (α ₄ -integrin); CD106 (VCAM); CD133
Receptor molecules	CD44 (hyaluronate); CD71 (transferrin)	CD16 (Fc receptor)
Enzymes	CD10 ; CD13 (aminopeptidase) CD73; aldehyde deshydrogenase	
Extra cellular matrix molecules	CD90; CD146; Collagen I and III; osteopontin; osteonectin	
Complement cascade proteins	CD55; CD59 (protectin)	
Histocompatibility Antigen	HLA ABC	HLA DR
Stem Cell markers	CD34; ABCG2	
Stromal cell markers	CD29; CD44; CD73; CD90; CD166	
Hematopoietic cells markers		CD14; CD31; CD45

Table 2: Phenotypical characteristics of ADSC. This table has been realized based on data from research articles and reviews on ADSC phenotype [28,61,160].



irradiation associated to CRS, a stem cell bank use may be the only valuable strategy. Technical challenges have to be investigated in this context. The major ones are cryo-protector agents required to allow frozen process without cell damage. They are known to be toxic and currently several safe protocols are proposed using sericin [87] or methyl cellulose [88] to decrease dimethyl sulfoxide concentration. This is a major issue that many groups are working on [89-91]. Otherwise the use of cell bank implied donor-recipient compatibility. Because of MSC low immunogenicity, allogeneic ADSC injections, without HLA typing have been evaluated for treatment of cutaneous radiation syndrome with the same minipig model described earlier [83,84]. In opposition to results achieved previously, allogeneic ADSC injections delayed the onset of symptoms but did not improve wound healing in comparison to controls [83], in spite of low immunogenicity of ADSC and their lack of expression of HLAI [92,93]. Wound repair enhancement was described in pre-clinical studies of allogeneic ADSC grafts on murine models [80,94,95] which suggest that allogeneic grafts may be harder to develop in high mammals closer to human than expected. The effect of stem cell donor and donor-recipient compatibility on wound repair efficiency requires further studies.

In this context, to avoid donor-recipient compatibility problems, MSC conditioned media may represent a valuable therapeutic alternative.

Stem cell therapy strategies under assessment

Conditioned media: The value of MSC secretome: Current data suggest that, following injection, the contribution of MSC differentiation to wound healing improvement is limited as poor engraftment and survival are observed at the site of injury. Indeed studies in mouse models have demonstrated that the percentage of MSC in the injected site was reduced to half 4 days after transplantation [19]. It has also been shown that the release of soluble trophic factors and cytokines of transplanted MSC promote endogenous repair of damaged areas. MSC engrafted mechanisms involved in wound repair are enhanced by paracrine signaling. MSC secretome also called MSC-conditioned medium (CM) is defined as the collection of secreted proteins processed via the endoplasmic reticulum and Golgi apparatus through the classical secretion pathway as well as encompassed proteins shed from the cell surface and intracellular proteins released

through non-classical secretion pathway or extracellular vesicles. These secreted proteins include numerous enzymes, growth factors, cytokines and hormones or other soluble mediators. They constitute the most biologically significant role of MSC under injury conditions [96]. Thus the concept of MSCs-conditioned media (MSC-CM) from BM-MSCs and ADSC may be an attractive method for amplifying the beneficial effect of engrafted stem cells in injury area. CM from BM-MSC but also from ADSC have been described to have a major effect on wound repair through an accelerated epithelialization [35]. Extra-cellular matrix proteins (fibronectin, type 1 collagen) and several cytokines were identified in MSC-CM such as IL-6, IL-8, TGF-beta1, MCP-1, EGF, RANTES, SPARC, IGFBP-7, MCP-1 and CXCL1. These biofactors play an important role in stimulating wound healing and migration [97-99] ADSC-CM have been reported to promote human dermal fibroblast proliferation and enhance type I collagen secretion [100]. Other investigations report that the protein Cyr61 (Cysteine-rich angiogenic inducer 61) implicated in cell adhesion, migration, proliferation, differentiation, through interaction with cell surface integrin receptors is present in MSC-CM [54,101]. It was established that this protein promotes angiogenesis *in vitro* and neoangiogenesis *in vivo* in the NOD/SCID mouse model. Otherwise, a better angiogenesis potential was observed with ADSC-CM in comparison with BM-MSC-CM [71].

Thus using stem cell-conditioned medium might be a viable alternative to stem cell graft, which is often hampered by low grafting efficiency. It may overcome the stem cell isolation and culture period and overcome the compatibility between donor and recipient in the case of allogeneic grafts. However, current stem cells culture media contain components that are not intended for human use, such as 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and bovine serum, necessitating development of an alternative medium that is safe for human clinical use. Bhang et al. [102] provided a new culture process: 3D spheroid culture with a clinically relevant medium composed of vitamins, glucose, amino-acids and human serum. This process showed a cellular proliferation 4 times higher than the one achieved with monolayer culture in conventional media for ADSC. Another promising strategy for conditioned media is the use of extracellular vesicles (EV) which include exosomes and micro vesicles [103]. Extracellular vesicles are membrane vesicles secreted by various cell types including MSC. These EV contain paracrine factors secreted by MSC. Furthermore, in a porcine model, it was shown that the fraction of the CM containing products greater than 1000 kDa provided an improvement of myocardial injury repair [104]. These products were identified as EV. This result suggests that most of the secreted factors in CM are contained in EV [105]. Given that EV are easily isolated by ultra-centrifugation of culture media [103], they represent a pertinent strategy for CRS managing.

To our knowledge, no studies have been published on the use of CM to treat thermal burn or cutaneous radiation syndrome, neither *in vivo* nor *in vitro*. Nevertheless this therapeutic process remains very promising in overcoming stem cell culture requirements and it might present a real benefit for radio-injured patients that need a treatment quickly.

Gene therapy to optimize stem cell treatment: Gene therapy could be considered as an option to enhance and target the processes responsible for the beneficial effects of MSC in tissue repair. The genetically-manipulated stem cells can be used both as the therapeutic agents and the vehicle for gene delivery for wound treatment. This

method serves to provide regenerative cells and bioactive genes within an optimal environment of regulatory molecular expression for wound sites. This method has emerged as a promising strategy for wound regenerative therapy [106]. In gene therapy, it is important to consider the duration of transgene expression, as a crucial point for the choice of transfection technique. Currently two methods are used to target genes transfer [107] (1) integrative techniques with stable expression (retrovirus, lentivirus or specific sites recombinase), (2) non integrative techniques with transient gene expression (plasmid DNA transfection via adenovirus, electroporation or nucleofection and chemical transfection). For wound repair and especially cutaneous radiation syndrome, the most interesting optimizations concern anti-inflammatory properties, angiogenesis and cell proliferation. The pro-angiogenic effect and the stimulation of cell proliferation are processes involved in tumor growth. In this context, permanent gene therapy with integrative methods represents a risk of tumorigenesis and is not valuable. In opposition, non-integrative gene therapy may allow the transient specific secretion of selected proteins. A few studies have been realized on transient gene therapy with adenoviral vectors: adenoviruses are rarely integrated into the host genome but instead stay in the cytoplasm, which explains the transient nature of the gene expression [108,109]. Wound repair enhancement was observed in a rat spinal fusion model with BM-MSC or ADSC transduced with adenovirus and no difference was observed between these two types of stem cells [110]. However, the major inconvenience of virus vectors is their immunogenicity [111] and a non-zero risk of insertional mutagenesis [112]. Feasibility of transient gene therapy for cutaneous radiation syndrome has been evaluated *in vivo* in the minipig model of radiation burn previously described [83,113]. ADSC were nucleofected with a plasmid coding for a protein involved in angiogenesis and cell proliferation: Sonic Hedgehog (Shh). In this study, the choice of nucleofection method of Shh gene was based on two arguments: (1) this technique allows the genetic material to be delivered directly into the nucleus [114,115], resulting in higher transfection efficiency than electroporation [116], (2) this technique does not alter the phenotype and potential multilineage of ADSC [117]. In this *in vivo* study, we checked that nucleofection technique allowed the production of sufficient amounts of transfected ADSC to be grafted to a large animal model [113]. No signs of inflammation, pain or loss of appetite were observed in grafted animals showing a good tolerance to Shh-transfected ADSC. Other non-viral techniques are currently evaluated [118,119] and this genetic technology provides new possibilities for modulation and optimization of MSC to achieve beneficial clinical effects in the future.

Future development areas

Currently, several other therapeutic strategies are being considered at an earlier stage and represent promising strategies for the future, especially induced Pluripotent Stem Cells (iPSC), biomaterials and preconditioning of stem cells.

The emergence of iPSC is a therapeutic hope. These cells were presented in 2006 after a groundbreaking study by Yamanaka's group. They demonstrated that viral transduction of murine fibroblasts with only four transcription factors, Oct-4, Sox-2, Klf4 and c-Myc (OSKM), could reprogram these cells back to an undifferentiated embryonic state. It has opened the possibility of reprogramming adult cells of the skin. These completely reprogrammed cells share almost all the features of murine embryonic stem cells (ESCs) [120]. iPSCs have now been generated from multiple terminally differentiated somatic cells

and adult stem cells from mouse [120-124] as well as from human [125-127]. Fibroblasts derived from human iPSC have been reported to enhance angiogenesis *in vitro*. An elevated secretion of pro-angiogenic soluble mediators, including VEGF, HGF, IL-8, PDGF-AA, and Ang-1, that stimulated endothelial cell sprouting in a 3D model of angiogenesis was observed *in vitro* [128]. Moreover, nuclear transfer of isolated epidermal cells demonstrated that multipotent bulge stem cells could be more easily reprogrammed to pluripotent state than more committed epidermal cells, suggesting that these cells might be a better source of cells for nuclear reprogramming to pluripotent state [129]. Presently, many limitations still affect the possibility to apply this personalized medicine. The main limitations are related to ethical and technical issues, including the development of safe and efficient methods for iPSC generation (the use of retroviral vector to obtain these cells limits their clinical feasibility) as well as the choice of the most appropriate cell type for reprogramming [130]. Nevertheless, iPSC cell biology has admittedly become a new field within stem cell research that covers various important and attractive scientific areas such as wound repair and tissue regeneration.

Otherwise, different biomaterials such as dermal/matrix scaffolds [131], nanofibrous structures [132] or dermal substitutes [133] associated with MSC are evaluated to cure wound damage. Tissue-engineered skin substitute biomaterials need to reproduce all skin physiologic, protective and immune functions. The majority of them was developed during the 1990's and is designed to mimic the basic properties of the extracellular matrix. These de-cellularized ECM represents a mature end-point structure both in term of architecture and molecular structure. Thus, it was hypothesized that biomaterials associated with MSC may improve cutaneous repair through synergic effects of biomaterials which provide a micro-environment and wound healing properties of stem cells. Preliminary results demonstrated that the combination of biomaterials and stem cells increase cellular organization through integration of the stem cells into the biomaterials (scaffold, dermal substitutes such as nanofibrous) [132] and exhibit synergistic angiogenesis promoting effects [131-133] which enhances the transport of essential nutrients for skin repair [134]. Thus they pave the way of new wound repair management.

Furthermore, an arising method to enhance MSC potential is the preconditioning of the stem cells. The aim of this strategy is to improve a well-known property of MSC such as cell migration, secretion of angiogenic factors etc, according to the intended effect, by cultivating the MSC under specific culture conditions, different from classic ones. The most studied preconditioning is hypoxia which has been reported to enhance stem cells (BM-MSC and ADSC) survival *in vitro* and *in vivo* [135,136] and to decrease apoptosis [137]. Hypoxia also favors immune-modulation through an inhibition of COX1/2 [138] and improves MSC migration [137,139]. Pro-angiogenic effects have also been reported with hypoxia preconditioning via an enhancement of transdifferentiation into endothelial precursors cells (EPC) and angiogenic factors secretion [140,141]. Many other preconditioning have also been evaluated such as co-culture with growth factors (TGF α , IGF-1, FGF-2), Tumor Necrosing Factor α , or erythropoietin which respectively enhance pro-angiogenic factors secretion [142], immunomodulation [138] and transdifferentiation in endothelial precursor cells [143]. Several works have been realized *in vivo* on murine models to evaluate preconditioning, mostly on cardiac lesions and have highlighted the benefit of preconditioning on myocardial repair. Concerning wound healing, *in vitro* a very specific preconditioning has already been evaluated by co-culture of ADSC with keratinocytes or

culture in keratinocytes CM. and the study has demonstrated a trans differentiation of these stem cells into keratinocytes [64]. *In vivo*, culture of ADSC under hypoxia has also been reported to enhance wound repair in a NOD/SCID model [144]. Thus, this preconditioning strategy is as promising as previous ones and offers a new development area for the future.

Conclusions

For the past 15 years, the treatment of cutaneous radiation syndrome has evolved due to the emergence of cellular therapy. Therapeutic strategies beyond surgery now include autologous BM-MSC injections and skin grafts in humans and many studies are underway to optimize this process and extend it to the cases of mass casualty and heterogeneous irradiation in pre-clinical experiment. Thus from numerous studies, ADSC may be more appropriate for CRS treatment and allogeneic ADSC injections may represent a very beneficial technique provided that compatibility donor-recipient is checked and donor variability is evaluated. To overcome these challenges conditioned media could be used if confirming their potential and autologous or allogeneic cell therapy could be optimized by gene therapy based on the transient expression of selected proteins involved in wound repair. Many other strategies arise such as iPSC, preconditioning or biomaterials which have not been evaluated presently for the treatment of CRS. They provide interesting biological processes which may supplement stem cells therapies currently studied.

For the treatment of CRS using MSC/ADSC, general inherent risks in cell therapy are still relevant today. In particular the possibility of immunological incompatibility in the case of allogeneic stem cells graft, the risk of tumor development given the anti-apoptotic and pro-angiogenic properties of stem cells and finally intrinsic risk of every injected treatment such as infection, which is favored here by the culture time. Many safety controls may be proposed or already exist to minimize these risks such as phenotype analyze, or telomerase activity evaluation and contamination control previously described [50]. Moreover tumor induction by MSC is presently a source of discussions with different results depending on the type of cancer. Indeed, supportive effect on tumor growth has been reported for cancers of colon [145], lymphoma [146], melanomas [147] through immunomodulation [148] or proliferation of MSC in tumor [149] whereas suppressive roles on tumor development were observed after injection of stem cells in liver cancer [150], leukemia [151], and pancreatic cancer [152] mostly via a modification of Akt signaling [148]. The only consensus in this field is the preferential migration of stem cells to tumor sites [153] which could be problematic for MSC systemic injections in case of unknown subclinical cancer. Nevertheless, these risks does not exclude cell therapy using for CRS but it has to be more investigated and the balance benefit/risk would have to be assessed for each patient with his informed choice.

To conclude, considering CRS, evaluated MSC/ADSC strategies are all currently based on a secretory theory. The arising of graft optimization such as biomaterials, and preconditioning may change the situation in the years to come. Of course, several challenges still need to be overcome before these strategies can be effectively used on human skin radio-injury. Nevertheless, pre-clinical and clinical trials show that stem cell therapy to mitigate CRS is a promising working hypothesis. All these strategies represent a hope and a huge benefit for local irradiated patients.

Acknowledgements

This work was supported by a grant from Direction Générale de l'Armement (Paris, France).

Conflict of Interest Statement

The authors declare no conflicts of interest, financial, or otherwise.

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