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ORIGINAL PAPER

Epithelization of skin lesions in animal model treated with mesenchymal stem cells and derivatives

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Abstract

Human mesenchymal stem cells (hMSCs) are used in regenerative therapies for various conditions, involving defects of one of the mesodermal lineages. We attempted to investigate the differentiation of human MSCs towards epithelial lineage and assayed the involvement of these cells in epithelization of epidermal lesions in mouse animal model. Bone marrow-derived hMSCs were grown in specific medium supplemented with EGF, KGF, FGF and IGF-2 in different concentrations for 3 weeks, expressing positive staining of early and late differentiation markers of epithelial-like cells. We used 20 B6D2F1 male mice as animal model, supplementary immunosuppressed with i.p. Dexamethasone for 3 weeks, when the skin lesion was induced. Human MSCs and epithelial-like derived cells were superficially injected at the site of the lesion in mouse model, and we followed the repair process. After 10 and 14 days, according to the cell type, animals were sacrificed and the presence of human cells (MSCs and epithelial-like cells) at the site of the injury was assessed. In mice not treated with cells the healing occurred in 15 days. Lesions treated with hMSCs stained positive for Vimentin and beta1-Integrin, while treated with epithelial-like cells stained positive for E-cadherin. We may conclude that epithelial-like differentiated cells induced a faster healing of skin lesions, while undifferentiated MSCs had a minor immediate contribution to epithelization process, when injected at the site of the injury. On the long term, MSCs could have an important contribution to renewal of stem cell population within the epidermis.

Key words: epithelial-like cells, differentiation, engraftment

Introduction

The epidermis is the outer covering of the skin, consisting of stratified squamous epithelium called interfollicular epidermis (IFE), and associated hair follicles, sebaceous glands, and sweat glands [1]. The epidermis is renewed throughout adult life, starting from proliferation of basal layer and moving to the suprabasal layer and tissue surface, as the cells undergo terminal differentiation. It has been appreciated for many years that the epidermis is one of the tissues in adult mammals with a stem cell compartment [2], based on the permanent shedding from the skin of differentiated cells from IFE and hair follicles. But these fully differentiated cells lack nucleus and are unable to proliferate, so that the stem cells must be less highly differentiated than their progeny and must lie in regions of the epidermis (e.g. IFE basal layer and hair follicles) where the proliferation takes place. It seems more likely that there are discrete populations of stem cells in the IFE, so that microenvironmental cues direct the progeny to differentiate towards the appropriate lineage.

Human IFE can be grown in culture on a feeder layer, generating confluent sheets of epidermis that could be used as autologous grafts for extensively burned victims, as demonstrated by Green and co-workers [3]. But the success of the autografts depends on the nature of the wound bed, which will not always allow appropriate engraftment of the novel epidermis. Other approaches would be infusion of the stem cells at the injury site, so that, under the microenvironmental conditions, these cells could proliferate and differentiate for reconstitution of epidermal defects, giving limitless possibilities for transplant procedures. Bone marrow-derived mesenchymal stem cells (MSCs) are adherent to culture plastic surfaces, with fibroblast-like morphology, long-term proliferation *in vitro* and self-renewal capacity, and with the ability to differentiate into mineralizing cells (bone), cartilage and fat [4]. MSCs could be the election choice for epithelization purposes considering that in response to appropriate stimuli, MSCs multipotent cell population is capable of differentiation towards cellular lineages beyond their developmental mesodermal layer, giving rise to ectodermal-derived cells (neurons, glial cells, and epithelial cells) and endodermal-derived progenitors (hepatocytes, pancreatic islet cells) [5].

We investigated the ability of human MSCs to differentiate towards the epithelial lineage under the *in vitro* conditions, using chemical induction of a combination of different growth factors, and we evaluated the ability of both undifferentiated MSCs and epithelial-like derivatives to induce reconstitution of epidermal layers when injected in superficial skin lesions in immunosuppressed mouse animal model.

Materials and Methods

Human cells isolation and culture

Normal human mesenchymal stem cells (MSCs) were obtained from bone marrow of 8 healthy Orthopedics patients undergoing hip replacement surgery. Approximately 10 ml of bone marrow were placed in culture plates, and the fibroblastic-like, plastic adherent fraction, was isolated following multiple passages and used in our experiments. The MSCs were further cultured and expanded in *alpha*-minimum essential medium (MEM; Gibco BRL, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal calf serum (FCS; PromoCell, Heidelberg, Germany) and 2% Penicillin/Streptomycin mixture (Pen/Strep, 10,000 IU/ml; PromoCell), by incubation at 37^{o}C in 5% CO_2 atmosphere. Medium replacement was performed every third day and when reaching 80-90% confluence, the cells were passed using 0.25% Trypsin-EDTA solution (Sigma-Aldrich Company, Ayrshire, UK) followed by centrifugation (10 minutes, 300g) and replated in T75 culture flasks at a density of 10,000 cells/cm^2 to ensure optimal proliferation. Starting with passage two, part of the cells were used for further phonotypical analyses and differentiation assays, while MSCs expanded to passages 2-5 were used in the subsequent experiments.

All tissue samples were obtained after signing the informed consent elaborated under an approved protocol, according to the World Medical Association Declaration of Helsinki.

Epithelial differentiation of human MSCs

Human MSCs at passage three, obtained as described above, were plated on adherent uncoated culture flasks, and the differentiation process was induced using a protocol previously described [6]. Shortly, the induction medium was constituted of basal DMEM low glucose (1 g/l, Gibco BRL, Invitrogen, Carlsbad, CA, USA) with 10% FCS (PromoCell, Heidelberg, Germany), supplemented gradually with 10 ng/ml keratinocytes growth factor (KGF, Peprotech Inc., Rocky Hill, NJ), 20 ng/ml epidermal growth factor (EGF, Peprotech Inc., Rocky Hill, NJ), 10 ng/ml hepatocyte growth factor (HGF, Peprotech Inc., Rocky Hill,

NJ) and 60 ng/ml insulin-like growth factor-2 (IGF-2; Human Recombinant, Peprotech Inc., Rocky Hill, NJ). The differentiation process was evaluated after 14 days of culture, when the cells acquired a rounded or polygonal shape and expressed epithelial markers (data not shown) [7].

Murine whole blood harvesting and analysis

Experimental model employed 20 partially immunosuppressed male adult mice, B6D2F1 model (Charles Rivers Laboratories, Research Models and Services, Germany GmbH), 75 ± 5 g weight, divided in two groups: control group (n = 10) and experimental group (n = 10). Experimental group was supplementary injected intraperitoneally with 100 µl of Dexamethasone solution 4 mg/ml (Sigma-Aldrich Company, Ayrshire, UK) for 3 weeks. Following the immunosuppression period, both mice groups were submitted to procedures for harvesting approximately 100 µl whole blood from the caudal vein.

Flowcytometric analysis of mononuclear cells required a first step of red blood cells lysis using 200 µl BD FACS™ Lysing Solution (BD Biosciences, San Jose, CA, USA) followed by 20 minutes incubation at room temperature and centrifugation for 10 minutes at 300 g. Mouse cells were incubated for 30 minutes at room temperature in the dark with fluorochrome-conjugated anti-mouse antibody CD8 (APC, BD Biosciences), CD4 (FITC, BD Biosciences), CD3 (PE, BD Biosciences), and CD45 (PerCP, BD Biosciences) at a dilution specified by manufacturer's protocol. Centrifugation for 10 minutes at 300g and resuspension in 500 µl BD FACS™ Lysing Solution were performed prior to acquisition and data analyses, which were accomplished using CellQuestPro software (Becton-Dickinson) on a four color capable FACSCalibur (Becton-Dickinson) flow-cytometer.

Skin lesion and cells injection

Epidermal lesions of 0.5 cm^2 were induced in both mice groups (control and experimental), after anesthetic procedure consisting of 8% Sevofluran (DB01236) induction and 3.5% Sevoflurane/Oxygen maintenance. Human MSCs and epithelial-like cells, differentiated as described above, were injected at the injury site, for each mouse in an amount of 100,000 suspension cells in 100 µl of physiological solution (Aqua ad injectabilia Braun, B. Braun Melsungen AG). Skin lesions were performed following the same procedures in a group of 5 supplementary B6D2F1 mice, which were not injected with cells, being used for control of the healing process *per se*.

Immunohistochemical analysis

When the healing process finished, evaluated by formation of uniform cornous layer, the animals were sacrificed and the newly formed epidermal layer was harvested. Initial histological investigation included hematoxylin-eosin staining of 3 µm thick sections, fixed with 4% Paraformaldehyde on Poly-L-Lysine slides (Sigma-Aldrich Company), for comparative evaluation of the healing process. Tissue fragments were further processed and investigated for expression of the human proteins of interest, using for labeling the following antibodies: monoclonal mouse anti-swine Vimentin (clone V9, Dako, Glostrup, Denmark), monoclonal mouse anti-human E-cadherin (clone NCH-38, Dako), and monoclonal mouse anti-human Integrin beta 1 (P4G11, Abcam, Cambridge, UK). All antibodies were tested for human specificity and cross-reactivity. Staining protocol continued with secondary biotinylated antibody binding, substrate addition (DAB), and hematoxylin (Hematoxylin, Mayer's Lillie's Modification, Dako) counterstaining of the nuclei (LSAB2 System-HRP, Dako) following the manufacturer procedures. Microscopy analysis was performed on a Nikon Eclipse E800 microscope.

All animal experiments described herein comply with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Directive 86/609, Strasbourg, 1986) and the experimental protocol was reviewed and

approved by the University of Medicine and Pharmacy Timisoara Board for Animal Experiments.

Results and discussions

Flowcytometric aspects of the immunosuppressed murine blood

Flowcytometric analysis of murine blood revealed the following distribution of white blood cells populations within the control group: 52.9% Neutrophils (Neu), 5% Eosinophils (Eo), 10% Lymphocytes (Ly), and 10.9% Monocytes (Mo). CD3+ cells represented 33.84% of total Ly, while CD4+ T lymphocytes were 28.71% and CD8+ 51.6%. CD3- cells (B and NK cells) represented 66.16% of total Ly (Figure 1A).

When compared with the control group, the experimental starting condition, after i.p injection with Dexamethasone, showed markedly decrease of neutrophils, and significant increase in lymphocyte population: 10.7% Neu, 9.5% Eo, 64.1% Ly, and 9.3% Mo. CD3+ cells were 30.37%, and CD4- approximately 70%. CD4+ cells increase, so that the value after additional immunosuppression is almost double compared with the control (47.5%); CD8+ cell population present significant lower values (39.8%) (Figure 1B).

These results show that our initial immunosuppression in experimental murine model was augmented by i.p. Dexamethasone, thus providing appropriate environment for injection of human cells and study of epithelization process.

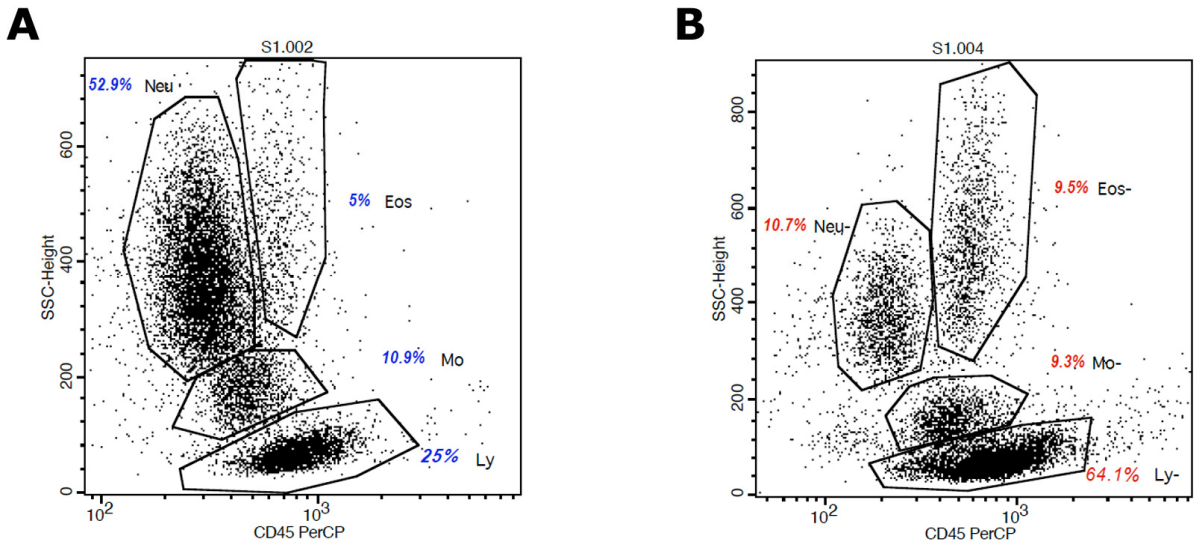

Fig.1. Analysis of the immune status of partially immunosuppressed B6D2F1 mice, which were additionally injected i.p. with Dexamethasone for 21 days (**B**) or left untreated (**A**). Following Dexamethasone treatment, neutrophil relative counts were markedly decreased, possibly impairing innate immune response and lowering inflammation associated with the infusion of human mesenchymal stem cells.

Human cells engraftment in murine experimental model

Infusion of human mesenchymal stem cells in both control and experimental model induced a slower healing process of 14 days, compared with the B6D2F1 mice left untreated in which the epidermal injury healed in 15 days *per se*. The architecture of the epidermal outer layer was seriously deranged in both cases, showing that epithelization process following the injury is disturbing normal structure of epidermal components (Figure 2B). Injection of epithelial-like differentiated mesenchymal stem cells induced a faster healing of

the injury in experimental model of 10 days, the epidermal layer eliciting a continuous and smooth aspect, findings revealed by hematoxylin-eosin staining (Figure 2A). Control animals infused with human MSCs and epithelial differentiated cells behaved similarly to the untreated mice, thus showing that human cells were not engrafted within the murine IFE, possibly due to the competent immune system which removed them and did not allow appropriate integration. Overall, Figure 2 presents the epidermis architecture restored after healing: interfollicular epidermis, hair follicles, and sebaceous glands.

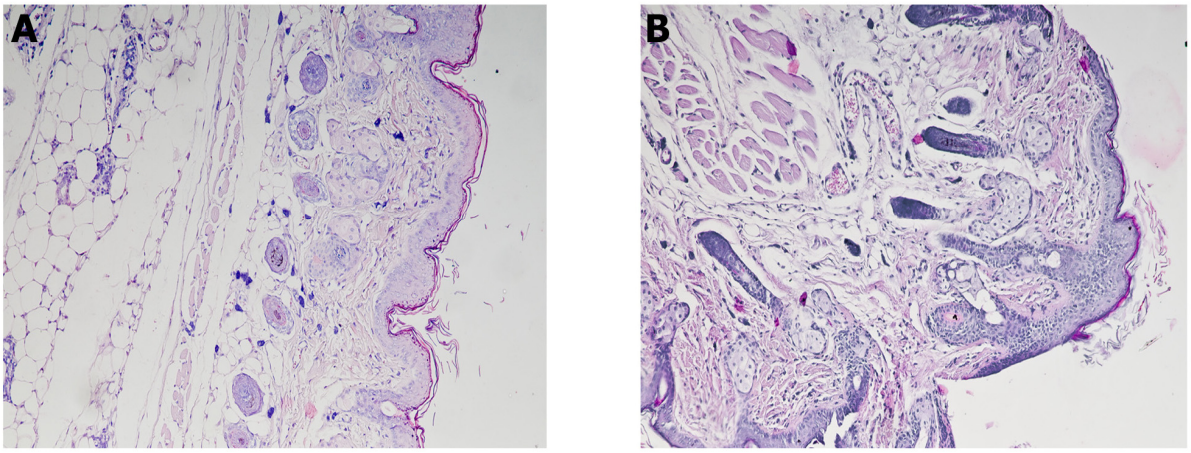

Fig.2. Comparison of epidermal regeneration of murine tissue infused with epithelial-differentiated human mesenchymal stem cells (**A**) and undifferentiated human mesenchymal stem cells (**B**). The first showed a better engraftment of foreign cells than the latter, reconstituting the normal architecture of the epidermal layer. Hematoxylin-eosin staining, magnification 100x.

E-cadherin expression was revealed at the level of constituted IFE, in human cells located in direct contact with cells of the tissue surface. Intense membrane staining is shown in Figure 3A, in experimental group of animals injected with epithelial differentiated mesenchymal stem cells. In control group, we could not find expression of E-cadherin, indicative of more competent immune system destroying human cells.

E-cadherin is a calcium-dependent adhesion molecule, which initiates intercellular adhesion by binding the homologous extracellular domain of E-cadherin on neighboring cells, being stabilized when associated with catenins. E-cadherin is required for epithelial histogenesis, tissue stability and differentiation functions [8]. Decreased expression of E-cadherin during organism development induces epithelial-mesenchymal transition, required for cellular migration and tissue rearrangements during embryogenesis. Thus, E-cadherin acts as key regulator of epithelial phenotype [9]. In our experiments, presence of this human epithelial marker is suggestive for engraftment of human epithelial cells and for the possibility of proliferation and regeneration of destroyed tissue.

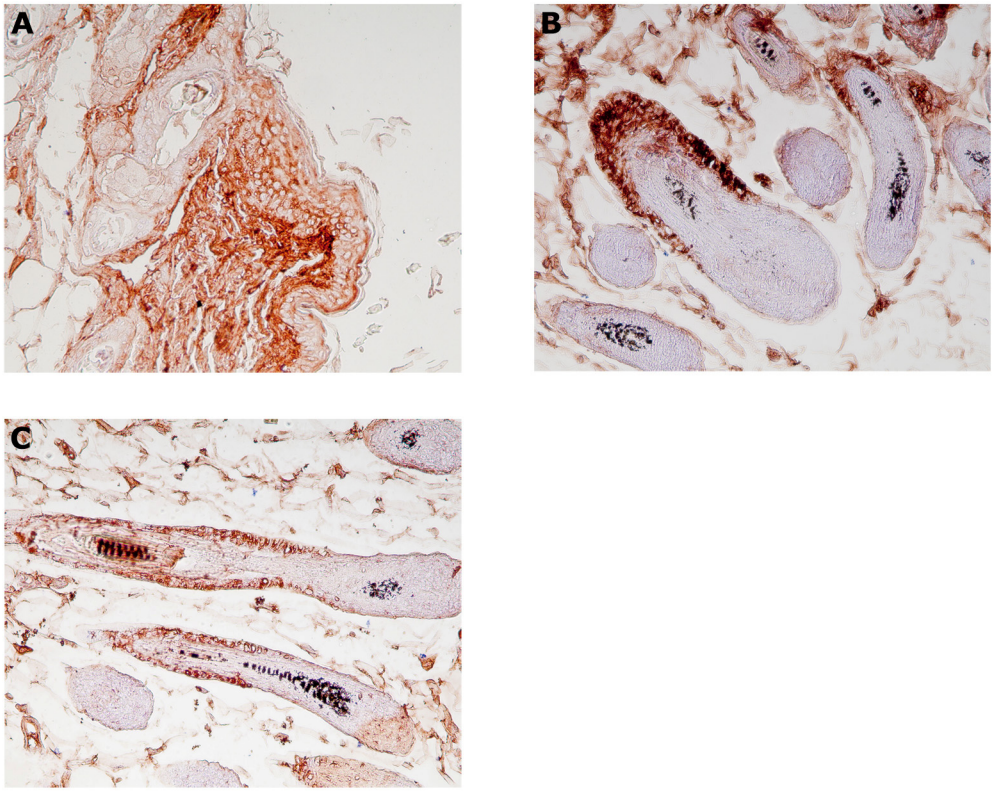

Fig.3. Immunohistological analysis of engrafted human mesenchymal-derived epithelial cells (A-C). Presence of human structural protein was revealed by staining with human specific primary antibody, which showed no cross-reactivity to mouse proteins. **A** The presence of E-cadherin positive cells (red) in the basal epidermal layer can be correlated to regeneration of the affected tissue. **B** Vimentin positive human cells (brown) migrated and located in the pilous bulb. **C** Mesenchymal derived human cells expressing beta1-integrin (red) likewise localized near the pilous bulbs, possibly contributing to stress signaling during epithelial regeneration. Magnification 200x.

Human Vimentin stained positive within the cytoplasm of MSCs injected into the experimental group. Cells were located at the level of murine pilous bulbs, demonstrating migration of stem cells towards their own niche (Figure 3B). One possible explanation could stress release of different cytokines and chemokines, as chemoattractant factors for human MSCs. Vimentin staining is also a quality control of the entire process, from harvest to tissue processing, Vimentin presenting a Formalin-sensitive epitope.

One of the attempts to find markers to distinguish stem from transit amplifying cells in the basal layer was based on fractionating basal cells according to the level of integrin. The reason for this was that epidermal cells undergo terminal differentiation, which can be inhibited by integrin ligation with extracellular matrix proteins or antibodies. When experimental group was injected with human MSCs, we could identify engraftment of human cells based on presence of beta1-integrin in cells located at the level of pilous bulbs (Figure 3C). In control group we were not able to find any beta1-integrin positive cells. Migration of human MSCs could be the result of ligand-receptor formation and chemotactic ability of these cells. Even though epitelization *per se* is not necessarily initially influenced by beta1-integrin positive cells, localization of human MSCs within this niche is concordant with distribution of murine stem cells. They lie within the undifferentiated, proliferative population, concentrated in the epidermal basal layer, but they undergo a program of terminal differentiation and move

through the suprabasal layers. Human MSCs could renew the local stem and also find appropriate signaling surrounded by this microenvironment and differentiate into more specific epithelial cells.

Several *in vitro* studies provided evidence regarding the pluripotency of mesenchymal stem cells, which could be used for regeneration and restoration of damaged tissues. Even though there are strong controversies regarding the ability of MSCs to regenerate tissues, MSCs could induce tissue repair in physiological and pathological condition, in different non-hematopoietic origin tissue, including different epithelial types [6]. *In vivo* studies revealed MSCs homing and differentiation in retina pigmentary epithelial cells [10,11], epidermis [12], ductal epithelial cells [13], and renal tubules epithelial cells [14,15].

Recent embryological studies showed that epithelium precedes mesenchyme, both evolutionary, as well as ontogenetically, so that mesenchyme derives from epithelium [16]. The primitive mesenchyme forms epiblastic cells during the epithelial-mesenchymal transition. Immediate process is reorganizing of primitive mesenchymal cells during mesenchymal-epithelial transition, to form more secondary epithelial structures [17,18]. Continuous epithelial-mesenchymal transition triggers development of different organs, such as kidneys and the heart [19,20]. We may conclude that both epithelial cells, as well as mesenchymal stem cells could recapitulate this transition *in vivo* under favorable conditions, having the transition molecular program imprinted from the embryonic development.

Conclusion

The present study provides strong evidence that epithelial-differentiated mesenchymal stem cells induce a faster healing of the epidermal lesions, engrafting at the level of differentiated cells layer in immunosuppressed murine models. Nevertheless, human MSCs could be useful therapeutic agents for skin regeneration on long-term process, engrafting within the basal layer of the epidermis, thus providing renewal and differentiation possibilities to the local stem cells pool.

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