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The use of glandular-derived stem cells to improve vascularization in scaffold-mediated dermal regeneration

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ABSTRACT

Clinical success in tissue regeneration requires improvements in vascularization capacity of scaffolds. Several efforts have been made in this field including cellular and acellular technologies. In this work we combined the use of stem cells derived from pancreas or submandibular glands expressing green fluorescent protein (GFP^+) with a commercially available scaffold for dermal regeneration. Cells were isolated, characterized and seeded in a scaffold for dermal regeneration. Scaffolds containing cells were used to induce dermal regeneration in a full skin defect model. After 3 weeks of *in vivo* regeneration, tissues were harvested and vascularization was analyzed. Results showed that gland-derived stem cells displayed stem cell features and presented multipotential differentiation capacity because they were able to differentiate in cell types representing the 3 different germ layers. After seeding, cells were homogeneously distributed and formed focal adhesions with the scaffold. Metabolic assays showed that cells can be cultured for at least 3 weeks in the scaffold. *In vivo*, the presence of pancreatic or submandibular stem cells significantly enhanced the vascularization compared to empty scaffolds. Presence of gland-derived stem cells in the regenerating tissue was confirmed by the detection of GFP expression in the wound area. In order to explore the possible mechanisms behind the improvement in vascular regeneration, *in vitro* experiments were performed, showing that gland-derived stem cells could contribute by angiogenic and vasculogenic mechanisms to this process. Our results suggest that the combined use of stem cells derived from glands and scaffold for dermal regeneration could be a rational alternative to improve vascularization in scaffold-mediated dermal regeneration.

1. Introduction

Autologous tissue transplantation is the main surgical procedure for the treatment of full skin defects. Although its general clinical outcome is good, this technique has several limitations. Autologous split thickness skin grafting is limited by the availability and morbidity of the skin donor site and, allogenic donor transplantations are limited to very rare cases due to immunological problems and risk of disease transmission. Skin wound healing is a process where wound closure is accomplished by scar tissue formation which is mainly composed of fibroblasts and extracellular matrix, generating a non-functional tissue. Scar formation is a major problem of special importance in cases of massive wounds (e.g. burn injuries) or when wounds are located in specific areas that require high motility such as joints, hands or neck. In order to avoid tissue transplantation procedures or scar tissue formation, much research effort has been focused on induction of skin regeneration using scaffolds for dermal regeneration. These scaffolds are three-dimensional structures that can be placed over the wound bed serving as a backbone for cell infiltration triggering further tissue regeneration [1]. The clinical benefit of these scaffolds has been poorer than expected because its use is associated with high infection risk and low regeneration rates. The lack of vascularization is believed to be the main reason for these problems

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because it impairs the delivery of immunocompetent cells, oxygen and nutrients. Therefore, enhancement of vascular tissue regeneration in skin as well as in any other tissue-engineered structures is one of the main goals in tissue engineering and regenerative medicine [2]. We have recently described that cell-seeded scaffolds enhance neodermal vascularization [3]. The question of which is the best cell population to activate scaffolds in tissue engineering is still a matter of ongoing research. However, there is strong evidence showing that the combined use of scaffolds and adult stem cells is a promising approach in regenerative medicine. In this context, combined use of scaffolds and mesenchymal stem cells derived from bone marrow or fat tissue has been extensively described, showing impressive results in the regeneration of several tissues including bone, cartilage, nerves, heart and skin [4–6]. Although those results are promising, the therapeutic potential of stem cells derived from other sources must be analyzed in reliable preclinical studies. In this work, we investigate the regenerative potential of stem cells obtained from pancreas or submandibular glands using an *in vivo* model of scaffold-mediated dermal regeneration. Previously, we described gland-derived stem cells as adult pluripotent stem cells that can spontaneously differentiate into multiple cell lineages [7]. Moreover, we had recently shown that stem cells derived from rat pancreas can be used to improve both, epidermalization and vascularization in skin regeneration [8]. Here we extend these data to other glands, emphasizing in their possible mechanisms of contribution, showing for the first time the therapeutic potential of pancreatic stem cells (PSC) and submandibular stem cells (SSC), showing their potential to enhance vascular regeneration and elucidating the possible cellular and molecular mechanisms behind the therapeutic effects of these newly described stem cell populations.

2. Material and methods

2.1. Cell isolation

Glandular derived stem cells were isolated as previously described [7]. Biopsies obtained from pancreas or submandibular glands derived from a GFP^+ mouse (strain C57BL/6-Tg(ACTB-EGFP)1Osb/J, Charles River, Germany) were cut into small pieces and then digested in medium containing HEPES-Eagle-medium (pH 7.4), 0.1 mM HEPES-buffer (pH 7.6), 70% (v/v) modified Eagle-medium, 0.5% (v/v) Trasylol, 1% bovine serum albumin, 2.4 mM $CaCl_2$ and collagenase NB 8 (Serva, Germany). The digestion was accompanied by mechanical chopping with a scissor and gassing with carbogen. After digestion, the tissue was dissociated by up-and-down suction through different glass pipettes with restrictive orifices and filtered through a nylon mesh. Cells were then suspended in Dulbecco's modified Eagles' medium (DMEM) supplemented with 20% fetal bovine serum and were centrifuged (800 rpm, 4–5 min). Cell-pellet was resuspended in DMEM and cultured at 37 °C in a 5% CO_2 humidified atmosphere. After 3 days of culture, adherent growing cells were observed. Cells were washed with phosphate buffered saline (PBS) and further cultured with DMEM supplemented with 10% fetal calf serum, 1 U/ml penicillin and 10 mg/ml streptomycin. Media was frequently changed until cells could be subcultured using 0.1% trypsin. All studies were run with cells derived from passages 11–14.

2.2. Immunocytochemistry

Adherent cells derived from mouse pancreas or salivary glands were cultured on chamber slides, fixed for 5 min in methanol: acetone (7:3) containing 1 g/ml DAPI at 20 °C and washed three times in PBS. After incubation with 1.65% normal goat serum (Vector, USA) at room temperature for 20 min, the specimens were incubated with primary antibodies in a humid chamber for 60 min at 37 °C. Primary antisera were directed against: nanog (rabbit polyclonal, 1 μg/ml, Chemicon, USA); nestin (mouse monoclonal, 1:500, Chemicon, USA); Cytokeratin 18 (mouse monoclonal, 1:200, Santa Cruz, USA) and α-smooth muscle actin (α-SMA, mouse monoclonal, 1:100, DAKO, Denmark), neurofilaments (NF-Pan-Cocktail, rabbit polyclonal, 1:500, Serotec, Germany); albumin (chicken polyclonal, 1:100, USBiological, USA), collagen II (mouse monoclonal, 1:20, DSHB, USA) and glial fibrillary acidic protein (mouse monoclonal, 1:100, DAKO, Denmark). After rinsing three times with PBS, slides were incubated for 60 min at 37 °C with either Cy3-labelled anti-mouse IgG, FITC-labeled anti-chicken IgG or FITC-labeled anti-rabbit IgG (Dianova, Germany) diluted 1:400 and 1:200, respectively. Slides were washed again, covered in Vectashield mounting medium (Vector, USA) and analysed with a fluorescence microscope (Axioskop Zeiss, Germany). Negative controls were carried out using only the secondary antibodies.

2.3. Transcription factor stem cell chip

Nuclear extracts from cells were analyzed for the presence of stem cell-related transcription factors (TFs). Nuclear extracts were prepared with a nuclear extract kit (Active Motif, Rixensart, Belgium) according to the manufacturer's instruction. Two million PSC or SSC grown to 80% confluence were harvested and nuclear fractions were stored at −80 °C. The protein content was determined using Bradford's assay. The transcription factor analysis was performed using the TF stem cell chip kit (Eppendorf, Germany), following manufacturer's instruction. The hybridization procedure was carried out overnight using 30 μg nuclear proteins. The colorimetric detection of the slides was performed by Silverquant detection kit (Eppendorf, Germany). For further TF chip processing the slides were scanned with the Silverquant scanner and data acquisition was accomplished with the TF Chip data analysis tool. Expression of active transcription factors was calculated as arbitrary units (AU).

2.4. Real-time PCR expression analysis

RNA was obtained from trypsinated and centrifuged glandular cells or from excised mouse tissue transplants after three weeks of regeneration. Tissue was collected in RNA later (Qiagen, Germany), shock frozen in liquid nitrogen and pulverized with a mortar and pestle. Total RNA was isolated using the RNA Mini kit (Qiagen, Germany) according to manufacturer's protocols. RNA concentration was determined by measuring the absorbance at 260 nm in a spectrophotometer. cDNA was synthesized of 1 μg RNA using QuantiTect reverse transcription kit (Qiagen, Germany) including a gDNA digestion step. Real-time PCR was carried out with 1 μl cDNA in a 25 μl reaction volume using QuantiFast SYBR Green PCR kit and QuantiTect Primers (both Qiagen, Germany) to amplify specific mRNA regions of β-actin (Mm_Actb_1_SG QuantiTect Primer Assay), Nestin (Mm_Nes_2_SG QuantiTect Primer Assay), Nanog (Mm_Nanog_3_SG QuantiTect Primer Assay), Amylase 1 (Mm_Amy1_1_SG QuantiTect Primer Assay), GFAP (Mm_Gfap_1_SG QuantiTect Primer Assay), PGP 9.5: Mm_Uchl1_1_SG QuantiTect Primer Assay, Neurofilament (Mm_Nefm_1_SG QuantiTect Primer Assay), α-SMA (Mm_Acta2_1_SG QuantiTect Primer Assay), PPARγ (Mm_Pparg_1_SG QuantiTect Primer Assay), amylase 1 (Mm_Amy1_1_SG QuantiTect Primer Assay) and von Willebrandt factor (Mm_Vwf_1_SG QuantiTect Primer Assay). The specificity of the amplified product was confirmed by melting point analysis. Automated gel electrophoresis was carried out with the QIAxcel capillary gel electrophoresis system.

2.5. Scaffold for dermal regeneration and cell seeding

Integra matrix (IM; Integra LifeScience Corporation, NJ, USA) is a scaffold based on bovine collagen fibers cross linked with glycosaminoglycans which forms a porous biodegradable structure. On top, the collagen structure is covered with a removable silicon layer, which acts as a temporal epidermis. Pieces of IM (15 mm diameter) were dried with sterile gauze, placed in a 24 well plate and 300 μl of medium containing 1×10^6 cells was dropped over the scaffold being quickly absorbed for it. After 30 min of incubation, 1 ml of DMEM 10% FBS was added into each well. Cell seeding efficiency was evaluated by removing the scaffold from the well and counting cells adhered to culture dish.

2.6. Cell visualization in the scaffold

Scaffolds containing cells were fixed in 3.7% paraformaldehyde (Sigma-Aldrich, Turkirchen, Germany) for 1 h and embedded in paraffin. Sections (10 μm) were deparafinized, stained for 1 h with TRITC-conjugated phalloidin (Sigma-Aldrich), used according manufacturer instructions. Finally, samples were mounted in prolong antifade containing DAPI (Invitrogen, Oregon, USA) and analyzed by a Axioskop 2 fluorescence microscope (Zeiss, Germany).

2.7. Quantification of metabolic activity in the scaffold

At different time points after cell seeding, scaffolds were incubated for 3 h in fresh medium containing 5 ng/ml of (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) (MTT) (Sigma-Aldrich, Turkirchen, Germany). Next, medium was removed and replaced by 300 μl DMSO. In order to quantify metabolic activity, absorbance at 560 nm was measured in the DMSO containing soluble formazan blue. Scaffolds without cells were used as negative control.

2.8. Scaffold based dermal regeneration model

24 scaffolds obtained from 12 athymic nude mice (6–8 weeks old; Takomi, Copenhagen, Denmark) were analyzed. Animals were distributed in 3 groups. 6 controls (12 empty scaffolds) and 6 animals containing either PSC or SSC (6 scaffolds per group). Before transplantation animals were anesthetized with ketamine