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Three-dimensional spheroids of adipose-derived mesenchymal stem cells are potent initiators of blood vessel formation in porous polyurethane scaffolds

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ABSTRACT

Adipose-derived mesenchymal stem cells (adMSCs) exhibit a high angiogenic activity. Accordingly, their incorporation into tissue constructs represents a promising vascularization strategy in tissue engineering. In the present study, we analyzed whether the efficacy of this approach can be improved by seeding adMSCs as three-dimensional spheroids onto porous scaffolds. Green fluorescent protein (GFP)-positive adMSCs expressing CD13, CD73, CD90 and CD117 were isolated from C57BL/6-TgN(ACTB-EGFP)1Osb/J mice for the generation of spheroids using the liquid overlay technique. Porous polyurethane scaffolds were seeded with these spheroids or a comparable number of individual adMSCs and implanted into the dorsal skinfold chamber of C57BL/6 wild-type mice. The vascularization of the implants was analyzed and compared to non-seeded scaffolds by means of intravital fluorescence microscopy and immunohistochemistry. The adMSC spheroids exhibited a homogeneous diameter of ~270 μm and could easily be incorporated into the scaffolds by dynamic seeding. After implantation, they induced a strong angiogenic host tissue response, resulting in an improved scaffold vascularization with a significantly higher functional microvessel density when compared to non-seeded scaffolds and scaffolds seeded with individual adMSCs. Immunohistochemical analyses revealed that a high fraction of ~40% of all microvessels within the center of spheroid-seeded scaffolds developed from GFP-positive adMSCs. These vessels inosculated with ingrowing GFP-negative vessels of the host. This indicates that adMSC spheroids serve as individual vascularization units, promoting the simultaneous development of new microvascular networks at different locations inside implanted tissue constructs. Thus, adMSC spheroids may be used to increase the efficacy of MSC-based vascularization strategies in future tissue engineering applications.

1. Introduction

The fate of scaffold-based tissue constructs is crucially dependent on a rapid vascularization after implantation, which determines their long-term survival and function at the defect site [1]. Accordingly, numerous vascularization strategies have been introduced in the field of tissue engineering during the last years, including the seeding of scaffolds with mesenchymal stem cells (MSCs) [2–4]. These pluripotent cells promote the ingrowth of new microvessels by hypoxia-induced paracrine secretion of angiogenic growth factors such as vascular endothelial growth factor (VEGF) or fibroblast growth factor (FGF)-2 [5,6]. In addition, MSCs possess a direct vessel-forming capacity, because they can differentiate into endothelial cells and perivascular smooth muscle cells [7–10].

Pluripotent MSCs can be found in the stromal fraction of many postnatal tissues. However, for clinical applications adipose tissue represents the most attractive source for the isolation of these cells, because it can easily be harvested in large amounts via liposuction with low donor site morbidity [11]. Moreover, the estimated fraction of MSCs in adipose tissue is markedly higher when compared to other common MSC sources such as bone marrow [12]. Under specific culture conditions, adipose-derived mesenchy-

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mal stem cells (adMSCs) can differentiate into osteogenic, adipogenic, chondrogenic or even hepatogenic and neurogenic lineages [13–16]. Thus, there is no doubt that adMSCs will play a major role in future regenerative medicine.

Like many other cell types, adMSCs exhibit the ability to aggregate into multicellular spheroids when cultured in suspension or on non-adhesive surfaces [11,17,18]. Thereby, the three-dimensional (3-D) arrangement of the cells with increased cell-to-cell interactions mimics much better the in vivo environment of a real tissue when compared to conventional monolayer cultures [19]. Accordingly, adMSC spheroids have been shown to exhibit an improved differentiation potential [18]. Moreover, they are more resistant against hypoxia and apoptotic cell death [6]. In addition, they secrete higher amounts of VEGF and FGF-2, which may markedly improve their angiogenic efficacy under in vivo conditions [6].

Based on these findings, we analyzed in the present study whether adMSC spheroids are more suitable than individual adMSCs to promote the vascularization of porous polyurethane scaffolds. For this purpose, we seeded the scaffolds with adMSC spheroids or a comparable number of individual adMSCs and assessed their vascularization in comparison to non-seeded scaffolds using intravital fluorescence microscopy and immunohistochemistry [20].

2. Materials and methods

2.1. Animals

In the present study we used 12- to 16-week-old transgenic C57BL/6-TgN(ACTB-EGFP)1Osb/J mice (The Jackson Laboratory, Maine, USA) and corresponding C57BL/6 wild-type animals (Charles River, Sulzfeld, Germany) with a body weight of 22–26 g. The mice were housed one per cage and had free access to tap water and standard pellet food (Altromin, Lage, Germany). All experiments were approved by the local governmental animal care committee and were conducted in accordance with the German legislation on protection of animals and the NIH Guidelines for the Care and Use of Laboratory Animals (NIH Publication #85-23 Rev. 1985).

2.2. Isolation and characterization of adMSCs

For the isolation of adMSCs epididymal fat pads were harvested from transgenic male C57BL/6-TgN(ACTB-EGFP)1Osb/J donor mice. In these mice, all of the tissues except erythrocytes and hair exhibit a green fluorescence under blue-light excitation [21]. The mice were anesthetized by intraperitoneal injection of ketamine (75 mg/kg body weight; Pharmacia GmbH, Erlangen, Germany) and xylazine (25 mg/kg body weight; Rompun, Bayer, Leverkusen, Germany). After midline laparotomy, the epididymal fat pads were carefully excised and transferred in Dulbecco's modified Eagle medium (DMEM; 10% fetal calf serum (FCS), 100 U ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin; PAA, Cölbe, Germany). After washing the fat pads three times in phosphate buffered saline (PBS), they were finely minced with a scissors and digested in collagenase NB4G (0.5 U ml^{-1}; Serva, Heidelberg, Germany) for 5–7 min with vigorous stirring at 37 °C in a humidified atmosphere with 5% CO_2. After neutralizing the collagenase with PBS containing 20% FCS, the cell suspension was incubated three to five times for 5 min at 37 °C for fractionated collection of fat supernatants. The remaining cell suspension was centrifuged gently for 10 min at 40*g* for the removal of pelleted microvascular fragments. The obtained supernatants were centrifuged for 5 min at 200 g, resulting in an adMSC-containing pellet. The cells were then cultured in endothelial cell growth medium MV (PromoCell, Heidelberg, Germany) for 11 days with medium change every 2 days until generation of adMSC spheroids.

For characterization of the freshly isolated adMSC-containing pellet, the expression of different stem cell markers was analyzed by flow cytometry ($n = 4$). For this purpose, the cells were incubated with the following antibodies: CD117-fluorescein isothiocyanate (FITC) (BD Pharmingen, Heidelberg, Germany), CD90-phycoerythrin (PE) (BD Pharmingen), unlabeled goat-anti-mouse CD13 (Santa Cruz, Heidelberg, Germany) with a rabbit-anti-goat IgG-Cy3 (dianova GmbH, Hamburg, Germany) as secondary antibody, unlabeled rat-anti-mouse CD73 (BD Pharmingen) with a goat-anti-rat IgG-Cy3 as secondary antibody (dianova GmbH), CD34-FITC (BD Pharmingen), Sca-1-FITC (BD Pharmingen) and VEGFR-2-PE (BD Pharmingen). FITC- and PE-labeled isotype-identical rat IgG2a$_\kappa$ (BD Pharmingen), goat-anti-rat IgG-Cy3 (dianova GmbH) and rabbit-anti-goat IgG-Cy3 (dianova GmbH) served as controls. Flow cytometric analyses were performed by means of a FACScan (BD Biosciences, Heidelberg, Germany). Data were evaluated by the software package CellQuest Pro (BD Biosciences).

2.3. Generation of adMSC spheroids

The liquid overlay technique was used for the generation of adMSC spheroids [22]. For this purpose, 30 µl of 1% microwave-melted agarose gel (Sigma-Aldrich, Taufkirchen, Germany) was added on the bottom of each well of a 96-well plate. After polymerization of the gel for 2 h at room temperature, 5000 adMSCs were seeded on the gel and cultured in a humidified atmosphere containing 5% CO_2. By this, the seeded cells aggregated spontaneously to 3-D spheroids, which were harvested after 3 days (Fig. 1A and B).

2.4. Seeding of polyurethane scaffolds

In the present study, we used polyurethane scaffolds with a size of ~3 × 3 × 1 mm and interconnected macropores with diameters ranging between ~200 and 600 µm. These biocompatible scaffolds were fabricated by a salt leaching-phase inverse process and exhibited nanosize hydroxyapatite particles on their surface to increase their osteoconductive properties for bone tissue engineering [23].

For seeding of the scaffolds with adMSC spheroids, each scaffold was fixed in the lumen of a modified 1 ml syringe (BD Plastipak; BD Biosciences) by means of a rubber ring (Fig. 1C). The tip of the syringe was filled with 100 µl PBS containing 40 spheroids. Subsequently, negative and positive pressure was alternately induced three to five times in the syringe. By this, the spheroids could pass the scaffold from both sides and were finally trapped in the scaffold pores (Fig. 1D and E). After the seeding procedure, the scaffold was carefully taken out of the syringe and embedded for histological analyses or directly transferred into dorsal skinfold chambers for further in vivo experiments.

In a second group, the scaffolds were seeded with individual adMSCs, which had also been cultured for 14 days comparably to the adMSCs of the spheroids. For seeding of the scaffolds with individual adMSCs, each scaffold was positioned in 3 µl PBS containing 200,000 cells and temporarily compressed with forceps. After removal of the forceps, the flexible scaffold restored its original shape, during which time the cells were sucked into the scaffold pores.

To clarify whether individual adMSCs or spheroids were lost during the seeding of the polyurethane scaffolds, we checked microscopically the remaining PBS which was not entrapped in the scaffolds after the seeding. We found that the PBS did not contain relevant numbers of individual cells or spheroids, indicating

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