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The effect of adipose tissue derived MSCs delivered by a chemically defined carrier on full-thickness cutaneous wound healing

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ABSTRACT

Mesenchymal stem cells (MSCs) have properties which make them promising for the treatment of chronic non-healing wounds. A major so far unmet challenge is the efficient, safe and painless delivery of MSCs to skin wounds. Recently, a surface carrier of medical-grade silicone coated by plasma polymerisation with a thin layer of acrylic acid (ppAAc) was developed, and shown to successfully deliver MSCs to deepithelialised human dermis *in vitro*. Here we studied the potential of the ppAAc carrier to deliver human adipose tissue derived MSCs (AT-MSCs) to murine full-thickness excisional skin wounds *in vivo*. Further we investigate the mechanism of action of MSCs in accelerating wound healing in these wounds. AT-MSCs cultured on ppAAc carriers for 4 days or longer fully retained their cell surface marker expression profile, colony-forming-, differentiation- and immunosuppressive potential. Importantly, AT-MSCs delivered to murine wounds by ppAAc carriers significantly accelerated wound healing, similar to AT-MSCs delivered by intradermal injection. More than 80% of AT-MSCs were transferred from carriers to wounds in 3 days. AT-MSCs were detectable in wounds for at least 5 days after wounding. Carrier delivered AT-MSCs were demonstrated to have the capacity to down-modulate TNF-α-dependent inflammation, increase anti-inflammatory M2 macrophage numbers, and induce TGF-β_1-dependent angiogenesis, myofibroblast differentiation and granulation tissue formation, thereby enhancing overall tissue repair.

1. Introduction

Worldwide 200 million difficult-to-treat chronic wounds in elderly individuals suffering from diabetic foot ulcers [1], pressure ulcers [2], and chronic venous leg ulcers [3] among others present an increasing socioeconomic burden and a currently unmet challenge for societies – Demographic developments suggest this will increase. In contrast to acute wounds, chronic wounds fail to progress through the normal pattern of wound repair which involves inflammation, granulation tissue formation and remodelling [4], but instead remain in a chronic inflammatory state with little signs of healing [3,5,6]. Common effector molecules produced from highly activated M1 macrophages, such as enhanced concentrations of tumour necrosis factor-alpha (TNF-α), play a critical in chronic wounds [7]. Scarring is another problem with poor wound healing and skin scars can range from barely visible fine white lines to major scars and keloids and also cause physical morbidity and psychological suffering and affect the quality of life of these patients [8].

There is an increasing interest in exploiting the beneficial anti-inflammatory and trophic effects of multipotent mesenchymal stem cells (MSCs) [9] to repair and regenerate non-haematopoietic tissues [10–12]. Bone marrow derived MSCs were first discovered in the 1970s [13]. Since then, MSCs have been isolated from a variety of

Abbreviations: AT-MSCs, adipose tissue derived mesenchymal stem cells; ppAAc, plasma-polymerized acrylic acid; CFSE, carboxyfluorescein diacetate succinimidyl ester; CFU-F, colony forming unit – fibroblast; TGF-β_1, transforming growth factor beta 1; TNF-α, tumour necrosis factor-alpha; PFA, paraformaldehyde; β2M, beta-2 microglobulin; α-SMA, alpha smooth muscle actin; PECAM-1, platelet endothelial cell adhesion molecule-1; VEGF, vascular endothelial growth factor; PBS, phosphate buffered saline; RIPA, radio-immunoprecipitation assay; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

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other tissues (for a review see [14]) among them adipose tissue [15]. MSCs of different origins are endowed with the potential to differentiate into a wide variety of histogenetically distinct cell types and thus contribute to tissue repair [16]. MSCs in addition constitute the stromal niche which supports haematopoietic stem cells maintenance/differentiation and vascularisation [17,18].

In recent studies on rodents, MSCs have been well documented to enhance cutaneous wound healing with reduced scar formation, enhanced wound closure and restoration of the skin tensile-strength [16,19–22]. In a proof of principle study on 3 patients by Falanga and coworkers [23] bone marrow aspirates were topically applied to chronic wounds which had not healed for years and resulted in complete healing. While their anti-inflammatory and regenerating properties make MSCs a highly attractive cell source for the treatment of chronic human wounds, there is a need to develop an efficient, safe and painless delivery method for applying these cells to the wound site *in vivo* suitable for routine clinical use. Most studies have used systemic intravenous or local subcutaneous or intraperitoneal injections, procedures which are invasive, painful and simply not convenient for any routine clinical service. Falanga and colleagues delivered bone marrow MSCs in a fibrin polymer spray, which requires a double-barrelled syringe containing fibrinogen and thrombin to deliver cells in a polymerized gel [24]. Unfortunately, fibrin spray contains blood products, which potentially induce allergic reactions in recipient patients, requires complex preparation and has a short working life [24].

The problem of delivering cultured cells from the laboratory to the clinic was solved for keratinocytes [25,26] using a chemically defined carrier consisting of medical-grade silicone coated by plasma polymerisation with a very thin layer acrylic acid (ppAAc). This was used to deliver autologous keratinocytes to patients suffering from burns injuries [27] and chronic ulcers [28,29]. More recently this synthetic carrier approach was extended to co-cultures of melanocytes and keratinocytes [30]. Using an *in vitro* human wound bed model of de-epidermized acellular demis (DED), almost all cells grown on the carrier were shown to transfer to DEDs within 48 h [31]. Very recently the *in vitro* delivery of bone marrow derived MSCs from ppAAc carriers to DED was reported [32]. In addition, the issue of cell transport on these carriers from culture laboratories to patients who may be at geographically distant locations was addressed as the functional properties of keratinocyte-melanocyte co-cultures were fully maintained for at least 72 h on these carriers [33]. These data indicate that the ppAAc carrier may be particularly suited for a non-invasive, painless and efficient delivery of MSCs to wounds.

Accordingly the aims of this study were to evaluate the use of a ppAAc carrier for the delivery of MSCs for wound healing *in vivo* and to investigate the mechanism of MSCs induced wound healing as important steps in developing MSC-based therapies for difficult-to-treat wounds. Thus we set out to evaluate the efficiency and efficacy of the ppAAc carrier to deliver human adipose tissue derived MSCs (AT-MSCs) to acute cutaneous wounds *in vivo* employing a full-thickness excisional murine wound model.

2. Materials and methods

2.1. Plasma-polymerized acrylic acid (ppAAc) carriers

A thin layer of acrylic acid was deposited on the sheets of medical-grade silicone by plasma polymerization and the carrier surfaces were analysed by X-ray photoelectron spectroscopy and sterilised with gamma irradiation as detailed previously [32].

2.2. Adipose tissue derived mesenchymal stem cells (AT-MSCs)

Human AT-MSCs at passage 2 were purchased from PromoCell (Heidelberg, Germany). AT-MSCs were seeded at a density of 3000 cells/cm^2 in complete MSC growth medium with SupplementMix (PromoCell), and cultured at 37 °C under 5% CO_2. AT-MSCs were harvested with Accutase (PAA Laboratories, Pasching, Austria) at 70–80% confluence. AT-MSCs at passage 4 to 6 were used in subsequent experiments.

2.3. Murine full-thickness excisional wound model

One day before wounding, ppAAc carriers were cut into 8 mm round pieces (surface area 0.50 cm^2) with sterile biopsy round knives (Stiefel Laboratories, Ireland), placed into 48-well plate (surface area 0.95 cm^2) and equilibrated with MSC growth medium for 1 h at 37 °C. 2×10^5 AT-MSCs were seeded into each well and incubated at 37 °C for 24 h to allow them to attach to the carriers. Based on the surface area of the carrier, approximately 1×10^5 AT-MSCs were seeded on each carrier membrane. The homogeneity of AT-MSCs seeded on carriers was confirmed by phase contrast microscopy. The carrier membranes without AT-MSCs were used as negative controls. The carriers with or without AT-MSCs were washed gently with 37 °C PBS for three times to remove the serum proteins in the culture medium prior to use.

Female C57BL/6 mice between 8 and 12 weeks of age were used in this study. Mice were specific pathogen free, and kept with free access to food and water in the animal care facility at the University of Ulm in compliance with the German Law for Welfare of Laboratory Animals. The animal experiments were approved by the Institutional Review Board and the Ethical Committee at the University of Ulm.

C57BL/6 mice were randomly divided into 4 groups. Two full-thickness excisional wounds were produced with 6 mm sterile biopsy round knives (Stiefel Laboratories) under anaesthesia on both sides of the shaved backs of mice as described previously [34,35]. The back and the abdomen were completely shaved for carrier fixation and dressing. Immediately after wounding, the wounds were covered by ppAAc carriers with AT-MSCs placed downside onto the wound bed and fixed with Hansaplast® (BSN medical, Hamburg, Germany) and further dressed with cotton bandages (Beiersdorf, Hamburg, Germany) to prevent damage by self-grooming. In the second experimental group serving as negative controls, wounds were covered with empty carriers followed by identical fixation and dressing. In addition, AT-MSCs cultured on conventional tissue culture plates were harvested and washed with PBS and then applied by intradermal injections around the wounds. These served as positive controls. In this group, each wound received three injections of 50 μl of AT-MSCs suspension at a density of 6.67×10^5 AT-MSCs/ml, with 1×10^5 AT-MSCs per wound, or injections were performed with 50 μl PBS serving as a sham control. All animals were shaved and dressings were applied as mentioned above.

Dressings and carriers were removed on day 3 post-wounding and carriers were collected for further analysis. At days 0, 3, 5, 7, and 9 post-wounding, wounds were photographed and wound areas were quantified as previously described [7,34,35] using Adobe Photoshop 7.0.1 software (Adobe Systems). In addition, wound tissue was harvested after sacrifice of mice at the indicated time points.

2.4. Assessment of viability, proliferation and cell death

AT-MSCs were seeded into 6-well plates with or without ppAAc carriers and cultured for 4 days. Following staining with 0.4% trypan blue (Sigma–Aldrich), numbers of viable cell were assessed based on trypan blue exclusion. AT-MSCs were labelled with 10 μM CFSE (Invitrogen, NY, USA) for proliferation analysis. Unlabelled AT-MSCs served as controls. CFSE labelled and unlabelled AT-MSCs were cultured on carriers or culture plastic plates for a period of 4 days. Thereafter AT-MSCs were harvested and subjected to flow cytometric analysis for CFSE. To determine cell death, detached AT-MSCs were stained with 1 μg/ml propidium iodide (PI, Sigma–Aldrich) for 10 min at room temperature and subsequently assessed for PI$^+$ cells by flow cytometry.

The cytotoxicity of ppAAc carriers to wound host cells was assessed by the TUNEL (TdT-mediated dUTP-X nick end labelling) assay using the *in situ* cell death detection kit (Roche, Switzerland) according to the manufacturer's instructions. Briefly, 5 μm cryosections of wound tissues which had been treated with or without the carrier for 3 days were fixed with 4% PFA and permeabilized with 0.1% TritonX-100 in PBS. Thereafter sections were incubated with the mixture of fluorescein labelled nucleotide and terminal deoxynucleotidyl transferase (TdT) at 37 °C for 1 h. Cell nuclei were counterstained with DAPI (Fluka, MO, USA). Coverslips were mounted with fluorescent mounting medium (DAKO, Denmark). Sections treated with 100 U/ml DNase I (Qiagen, Germany) for 10 min at room temperature prior to TUNEL reaction were used as positive controls, while sections incubated with fluorescein labelled nucleotide without TdT enzyme served as negative controls.

2.5. Flow cytometry

Fluorochrome (FITC or PE or APC) conjugated anti-human antibodies CD3, CD14, CD29, CD31, CD34, CD44, CD59, CD71, CD73, CD90, CD105, HLA-ABC, HLA-DR were purchased from eBioscience (CA, USA). Subsequent to culturing either on ppAAc carriers or on tissue culture plates for 4 days, AT-MSCs were harvested, washed with PBS, and incubated with antigen-specific antibodies for 30 min at room temperature. Non-specific staining was controlled by isotype-matched antibodies. Flow

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