Enhancing the Vascularization of Three-Dimensional Scaffolds: New Strategies in Tissue Regeneration and Tissue Engineering

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Summary

An essential element in tissue engineering is blood supply. The prevention of implant failure caused by hypoxia and following infection is still a challenge. There are different therapeutic strategies to enhance angiogenesis and wound healing in diseased or injured tissues: (i) implantation of modified bioactive materials, (ii) implantation of cells and (iii) implantation of biohybrids assembled of cells and scaffolds (1-3). From our point of view, cell-based tissue engineering provides a successful treatment in wound healing disorders.

Collagen, a natural, porous and degradable material was used as a scaffold for cell seeding. Cross-linking with 1-ethyl-3(3-dimethyl-aminopropyl) carbodiimide (EDC) and N-hydroxysuccinmide (NHS) was performed to improve its integrity and stability. Required immunological compatible, unrestricted available and easy to harvest cells were isolated from (i) human dermis and (ii) human femoral bone marrow. Fibroblasts and adult bone marrow mesenchymal stem cells (BMSC) are believed to play a key role in wound healing by releasing different growth factors (e.g. VEGF) and their own high mitogenic activity (4, 5).

These cell types were seeded on different scaffolds and cultured for 3 days. Fibroblasts and BMSC showed improved cell proliferation and invasion in vitro in modified matrices in relation to the controls. Further, they supported human umbilical vein endothelial cell (HUVECS) growth. Integrated angiogenic activity by growth factor releasing cells could become a successful tool in tissue engineering.

Key words: angiogenesis, adult bone marrow mesenchymal stem cells, fibroblasts, collagen

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Introduction

Therapeutic approaches that promote vascularized tissue growth represent an important field of tissue engineering. Attention has been directed to administration of vascular growth factor-producing cells that can induce physiological new blood vessel formation. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) represent most potent endothelial cell mitogens with increased expression in ischemic tissue and during wound healing (6, 7). Clinical applications of VEGF or bFGF showed improved regional blood supply in under-perfused regions of the heart or leg (8, 9). Functional blood vessel formation requires prolonged exposure to the angiogenic activity. In therapeutic angiogenesis, bolus injections or systemic delivery of high doses of VEGF or bFGF are rapidly cleared from the target site or cause severe vascular leakage and hypotension (8). The pharmacokinetics described above have forced the development of cell-seeded biomaterials that allow localized sustained release of growth factors (10-13).

As natural biopolymers, e.g. fibrin, collagen, and hyaluronic acid, they provide biologically specific signals for molecular interaction with the delivered cells and interact specifically with cells of the target tissue (13-16). We, as others, have tried cellular approaches that speed up endothelial proliferation (4, 17). The design of these biohybrids is motivated by their capacity of growth factor expression.

Since the beginning of application towards human wound healing and the improvement of patient-oriented strategies, most available biomatrices do not induce adequate sprouting of capillaries. Often, implant failure caused by delayed hypoxia can not be prevented. Principal therapeutic strategies to reduce impaired wound healing are: (i) the implantation of modified bioactive materials, (ii) the implantation of multipotential cells and (iii) the implantation of biohybrids assembled of cells and scaffolds.

Collagen, obtained by freeze-drying of collagen suspensions is often-used in tissue engineering as a natural, degradable and porous material. Inducing or speeding up angiogenesis could, e.g. be performed by manufacturing a growth factor-releasing scaffolds (11, 13). Due to the short half-life time (T½) of most growth factors (e.g. VEGF and bFGF have 3 min. T½) and the cost of these cytokines, this approach seems not to be the most successful one for any defect. Cell-based tissue engineering requires cells that are immunologicaly compatible, available or expandable *in vitro* and easy to harvest.

In the presented work, we evaluated adult bone marrow mesenchymal stem cells (BMSC) and dermal fibroblasts, which were attached to collagen scaffolds in order to accelerate the proliferation of human umbilical vein endothelial cells (HUVEC). Dermal fibroblasts regulate matrix deposition during wound healing by the release of collagen type I and IV, elastin and laminin. Expressing a large range of growth factors like insulin-like growth factor (IGF), keratinocyte growth factor (KGF), platelet derived growth factor A (PDGF-A), transforming growth factor (TGF) and vascular endothelial growth factor (VEGF), those cells can guide, e.g. endothelial and epidermal cell proliferation and differentiation (2, 3, 18).

Adult bone marrow mesenchymal stem cells are multipotent and high proliferating cells that can release different growth factors (e.g. VEGF) (4, 19). Furthermore, recent findings indicate that these cells can differentiate into mature, non-haematopoietic cells of various tissues including cells of skin, lung, liver, kidney , gastrointestinal tract and muscle fibres. Recent research work showed the contribution of BMSC to collagen deposition and epithelialization (3, 19, 20).

Seeded on an optimized scaffold [in this study: 1-ethyl-3(3-dimethyl-aminopropyl) carbodiimide (EDC) and N-hydroxysuccinamide (NHS)-cross linked collagen] they could guide angiogenesis and wound healing (13, 21). The accelerated proliferation of HUVEC was used as an indicator for the mitogenic capacity of the biohybrids. Cell-based biohybrids might become a successful tool in the treatment of extended acute defects, chronic ulcers and wound healing disorders.

Materials & methods

Materials

Collagen scaffolds were manufactured by Dr. Suwelack, Skin and Health Care GmbH, Germany. Mainly, bovine collagen type I sponges showed a pore size between 15 - 25 µm. Cubes of 10 x 10 x 2 mm (weight: 10 – 12 mg) were used for the experiments. Cross-linking was performed according to the procedure of Wissink *et al.* (22) and Steffens *et al.* (13). We tested E0 and E1 specimens, where E1 referred to 1 mg EDC/0.6 mg NHS (E) in 500 µl reaction solution. E0 underwent the same chemical procedures but without the addition of EDC/NHS. EDC and NHS were purchased from Sigma-Aldrich, Germany. Scaffolds were sterilized by washing with 70% ethanol for 24 hours followed by washing in sterile 0.9% NaCl-solution for other 24 hours. The final sponge wetting was carried out in stem cell culture medium (Clonetics®, BioWhittaker, Germany) for 24 hours before cell seeding.

Isolation and culture of human dermal fibroblasts

After appropriate informed consent, dermal fibroblasts were isolated from human cutaneous tissue of adults undergoing elective surgery at the Department of Plastic Surgery and Hand Surgery, Burn Centre. The dermis was treated with collagenase I/dispase solution (collagenase I 0.1 U/ml in PBS; dispase II 0.8 U/ml in PBS, Boehringer Mannheim, Germany) for 1 h at 37°C with cells further cultured in DMEM/F12 medium (Cellsystems®, Germany) with additional fetal bovine serum (10%) and penicillin-streptomycin (100 U/ml, Sigma-Aldrich, Germany).

Isolation and culture of human adult bone marrow mesenchymal stem cells

BMSC were isolated from bone marrow obtained from femoral heads of adults undergoing total hip replacements at the Department of Orthopaedic Surgery (23). Aspirated bone marrow was washed thoroughly with stem cell medium (MSCBM,

Cellsystems®, Germany). After centrifugation of the cell suspension (500 x g for 10 minutes at room temperature) the resultant pellet was resuspended in 10 ml of fresh medium and cultured in T-75 culture flask (Cellstar®, Greiner Bio-One GmbH, Germany). Expansion was carried out at 37°C, in a 5% CO_2 and 20% O_2 humidified atmosphere. Medium was changed on the following day to remove non-adherent cells. Culture medium was changed every 3-4 days. At confluence, cells were trypsinized with stem cells trypsin (Cellsystems®, Germany) and seeded at a density of 5 x 10^3 cells/cm².

Isolation and culture of human umbilical vein endothelial cells (HUVEC)

HUVEC were isolated according to van Wachem *et al*. (21). Cells were cultured in tissue culture flasks (Cellstar®, Greiner Bio-One GmbH, Germany) with endothelial culture basal medium-2 (ECBM-2, Clonetics®, BioWhittaker, Germany) at 37°C, in a 5% CO_2 and 20% O_2 humidified atmosphere. The second passage was detached from flasks by incubation with trypsin (Trypsin-EDTA, PAA Laboratories GmbH, Austria). Further culturing was performed in 6-well plates (Cellstar®, Greiner Bio-One GmbH, Germany) with 3 x 10^4 cells per well.

Cell labelling

The fluorescent cell tracker CM-DiI (Chloromethylbenzamido, Cell Tracker™ CM-DiI, Molecular Probes, Germany; working concentration of 1µM with DMSO = dimethylsulfoxide) was used 24 hours before cells (fibroblasts and BMSC) were seeded on the matrices.

Culturing on collagen scaffolds

Cells of the second passage were trypsinized after confluence. The cells were resuspended in 0.5 ml of cell medium (fibroblasts in DMEM/F12 medium; BMSC in

MSCBM, Cellsystems®, Germany) and counted in a Neubauer chamber. A suspension of 50 µl, containing $5 \times 10^5 \pm 4 \times 10^4$ cells, was seeded on the upper surface by gently dropping on the medium-wetted scaffolds. The sponges were left at room temperature for 2 hours to allow the cells to attach. Afterwards, 2 ml medium was added and further cell culturing was carried out in 6-well plates (Cellstar®, Greiner Bio-One GmbH, Germany) for 3 days at 37°C, in a 5% CO_2 and 20% O_2 humidified atmosphere.

Proliferation of HUVEC

Biohybrids (fibroblasts and BMSC) were cultured for 24 hours in cell specific medium. The medium was removed from the endothelial culture and the biohybrid was placed on the monolayer. Further expansion was performed with ECBM-2 in 6-well plates (Cellstar®, Greiner Bio-One GmbH, Germany) at 37°C, in a 5% CO_2 and 20% O_2 humidified atmosphere for further 3 days. Cell proliferation was quantified by microscopic counting in a Neubauer chamber. Non-seeded scaffolds served as controls. Since there was no ingrowth of cells from the monolayer, no further cell removal from the sponges was performed (24).

Statistical analysis

Data were statistically compared using the Student's *t*-test for paired samples. Values represented the mean ± standard deviation. When $p<0.05$, differences ere considered significant.

Microscopy

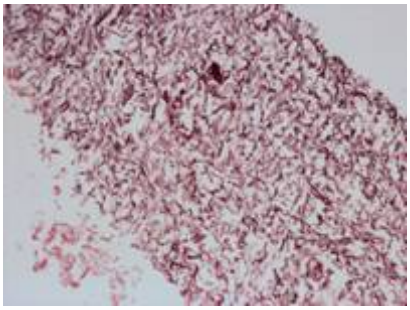
Formalin fixed biohybrids were dehydrated in series of increasing concentrations of alcohol, embedded in paraffin and cut in 6-µm sections. For histological analysis sections were stained with haematoxylin-eosin (HE) or DAPI (4',6-Diamidino-2-phenylindole)

and tissue composition was assessed using a light and fluorescence microscope (Zeiss, Germany).

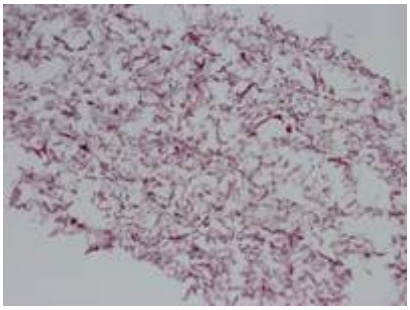
Results

Isolated dermal fibroblasts and mesenchymal stem cells adhered well to the plates. On morphological characterization, cultures of dermal fibroblasts presented elongated cell morphology, whereas BMSC seemed to consist out of three different cell types or cell developmental stages. In both cell cultures, confluence was obtained after approximately 10 days in fibroblasts and BMSC cultures. CM-DiI labelling proved to be reliable in detecting seeded fibroblasts and BMSC, which could be useful in further animal transplantation studies (25, 26).

Microscopical examination of the modified specimens (EDC/NHS cross-linked) demonstrated a higher overall cell density after 3 days in culture, compared to the control scaffolds. Unmodified scaffolds were degraded to a larger extent and occurred with an inhomogeneous structure (shrinkage and curling) with pores that appeared collapsed (Figure 1A, 1B). Mean penetration depth of cells inside the cross-linked sponges was higher (up to 400 µm) in comparison to the untreated ones (250 µm) (Figure 2A, 2B). Cell-seeded EDC-matrix enhanced HUVEC proliferation two-fold in contrast to cell-free-matrix specimens (Table 1). BMSC and dermal fibroblasts seemed to promote endothelial proliferation. There were no significant differences in mitogenic capacities between the two mesenchymal cell types among the used conditions.

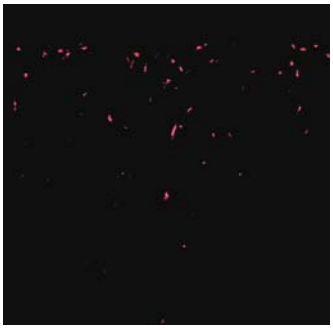


1 A:

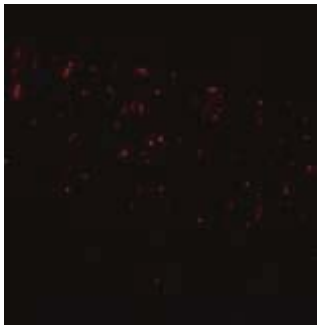


1 B:

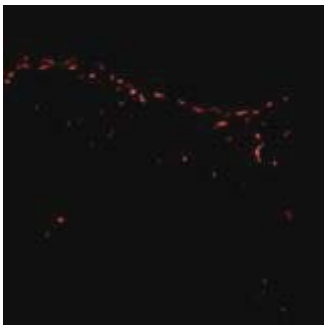
Fig. 1: Fibroblasts seeded on native and modified collagen [HE staining, magnification 50x].
1A: After 3 days, E1 scaffold maintained its structural integrity.
1B: E0 collagen matrices showed a high grade of degradation.



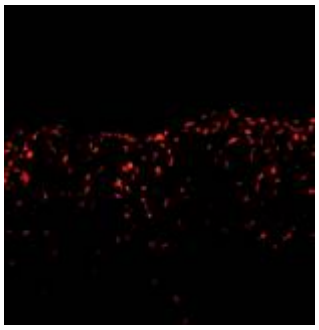
2 A:



2 B:



2 C:



2 D:

Fig. 2: Mesenchymal cells seeded on collagen scaffolds [DiI labelled, magnification 200x].
2A: Fibroblasts in E0 collagen proliferated most on the upper side of the sponge.
2B: Fibroblasts in E1 showed a high overall cellularity as compared to unmodified matrices after 3 days.
2C: The growth of BMSC in E0 was located on the upper side of the matrix.
2D: BMSC in E1 revealed a high cell density with penetration depth up to 400 µm.

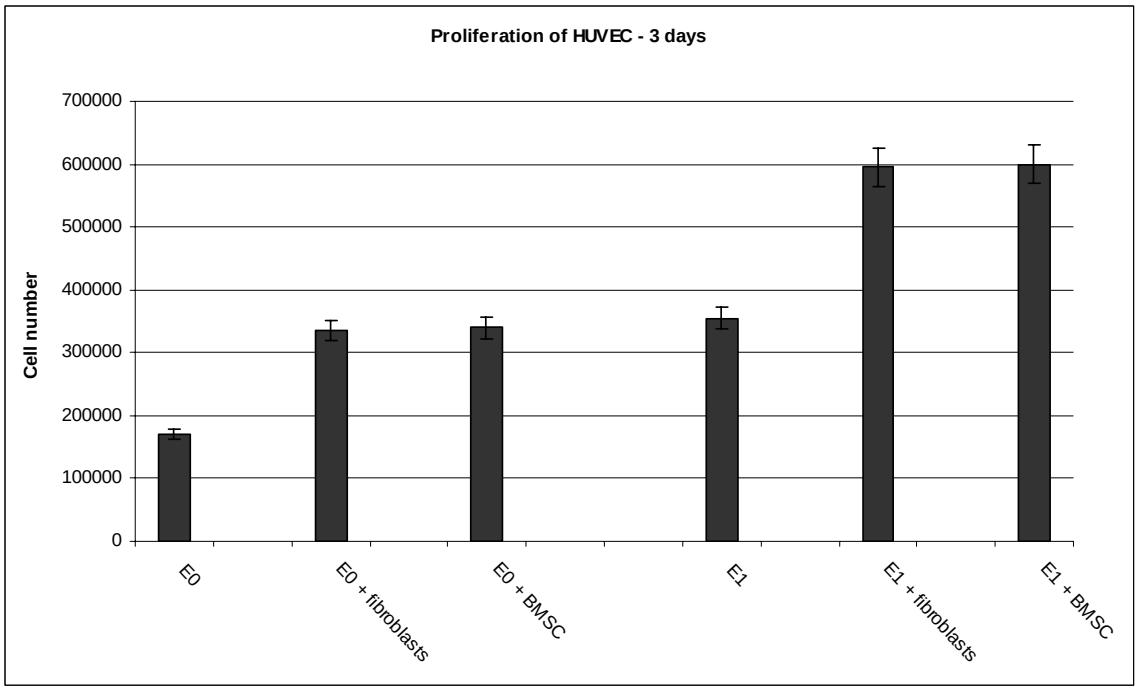


Table 1. Proliferation of HUVEC after 3 days in contact with different scaffolds.
Acceleration of proliferation of HUVEC by exposure to collagen matrices which were modified or non-modified. Further, matrices were either seeded with dermal fibroblasts or BMSC. Columns show the mean values. Error bars represent the corresponding standard deviations (*n*=6).

Discussion

This study presents a method of autologous cell culture that might be beneficial for application in wound healing and regeneration for accelerating the proliferation of endothelial cells. Human mesenchymal cells were isolated, cultured and seeded onto unmodified and EDC/NHS cross-linked collagen type I sponges. Fibroblasts and BMSC survived, proliferated and migrated inside the (collagen) matrix. Cell-matrix-biohybrids promoted greater endothelial cell proliferation compared to non-seeded scaffolds. In this context, the cell-free EDC/NHS modified matrices were superior to cell growth enhancement compared to the native collagen scaffolds.

The ideal matrix for human cell transplantation and regeneration is not yet known. It is known that collagen, a natural biodegradable material, can support cellular ingrowth and matrix synthesis (27). Commercially available unmodified collagen scaffolds showed a reduced mitogenic activity and structural integrity by degradation. Cross linking proved to reduce the degradation of the collagen and enhance its mitogenic activity. Our *in vitro* findings encourage further investigations of cross-linked collagen sponges as potential cell-delivery matrices in soft tissue engineering. Isolated mesenchymal cells or attracted hMSC (dermis and bone marrow) by a scaffold might provide a successful approach for the treatment of full-thickness defects. The release of cytokines (e.g. VEGF, bFGF) could have a positive effect on wound healing.

Darland *et al.* observed that differentiated pericytes in co-culture with endothelial cells and multipotent mesenchymal stem cells produce VEGF that support the survival and/or stabilization of endothelial cells in microvessels (28). Furthermore, the studies of Hudon *et al.* confirmed our findings that a co-culture of dermal fibroblasts and HUVEC in a collagen matrix promotes the three-dimensional structure formation of a capillary-like network. (29)

In the future, research has to continue to evaluate other matrices and cell types to decide which combinations are optimal for speeding up wound healing processes. In addition, effects of longer culture have to be tested. To contribute to tissue repair, cell survival and proliferation need to be prolonged. Up to these data, it remains unclear for how long.

Conclusions

The present study demonstrates that isolated human dermal fibroblasts and bone marrow-derived stem cells seeded on modified freeze-dried collagen scaffolds showed improved proliferation and scaffold penetration *in vitro*. These mesenchymal cell-matrix-biohybrids supported endothelial cell proliferation that was twice (Table 1) as high as in acellular constructs. Such biohybrids could be useful in clinical applications of cultured skin and soft tissue substitutes or cellular therapy of wound healing disorders.

Acknowledgements

This work was supported by a grant from the Medical Faculty of Aachen University of Technology RWTH IZKF "BIOMAT" (START-01/2003).

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