

Adult Bone Marrow Mesenchymal Stem Cells as Feeder Cells for Human Keratinocytes: New Approaches in Bilayered Skin Replacements

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Summary

The authors have developed an autologous cultured skin substitute by culture of human adult bone marrow mesenchymal stem cells (hMSC) and human keratinocytes on cross-linked bovine collagen. The spongy collagen layer is the dermal substitute and essential for attachment and proliferation of hMSC. The keratinocytes are necessary for the coverage of the wound surface.

This study is designed to investigate the growth behaviour of hMSC and keratinocytes as a co-culture on the spongy matrix in contrast to a monoculture. The results suggest that a spongy collagen matrix populated with hMSC has the highest potency in regard to the proliferation of keratinocytes. This approach may be useful in composite tissue engineering for cutaneous wound repair.

Key words: mesenchymal stem cells, keratinocytes, cross-linked collagen, composite graft, skin substitute

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Introduction

The skin acts as a barrier to water and temperature loss, exogenous substances, pathogens, and trauma. The use of skin substitutes is a new approach for treating patients with extensive skin loss caused by burns, venous ulcers, diabetic ulcers, or injury. However, although much progress has been made towards the development of skin replacement products, nothing works as efficient as a patient's own skin. Because of the increasing survival rate of patients with extensive wounds, whose available donor sites for autografting are very limited, an off-the-shelf skin replacement product that can be applied to a wound and yield a permanent, dependable dermis and epidermal skin replacement still remains a vision (1). Tissue engineering products can cover wounds and provide a microenvironment that stimulates wound repair (1).

Graftskin® (APLIGRAF, Organogenesis Inc, Canton, MA, and Novartis Pharmaceuticals Corporation, East Hanover, New Jersey) is one of the allogenic bioengineered skin products. It is a bilayered skin construct consisting of dermis and epidermis.

Keratinocytes and fibroblasts are obtained from neonatal foreskin. Studies showed its effectiveness in healing venous ulcers, particularly those, that have proved hard to heal with conventional modalities. The disadvantage is related to the allogenic origin of used cells and, therefore, it can only be used as wound coverage and not as a composite graft for tissue replacement. Integra Artificial Skin®, an acellular collagen-glycosaminoglycan (C-GAG) dermal equivalent requires a two-stage grafting procedure. It is not beneficial in terms of replacing the need for traditional split-skin grafts. Pre-seeding Integra Artificial Skin® with cultured autologous fibroblasts and keratinocytes for *in vivo* application, as a single-stage grafting procedure needs further testing. New biotechnological developments try to engineer skin equivalents, the closely match native skin in terms of histological and functional properties. Strategies for developing new culture techniques, construction of kinetic models of cell growth, evaluation of cell

properties based on image-analyzing techniques, and design of bioreactor systems are the modern tools of tissue engineers (2).

Cell-cell interactions play an important role in tissue formation and regeneration, both in embryonic and adult stages. Mesenchymal cells (fibroblasts, embryonic stem cells and adult bone marrow stem cells) have been shown to improve performance of composite skin substitutes in the animal models (3-6). The process of wound healing requires coordinated cellular activities, including phagocytosis, chemotaxis, mitogenesis, differentiation, synthesis and reorganization of collagen and extracellular matrix (7). New studies report that mobilized bone marrow mesenchymal stem cells (hMSC) do actively participate in skin wound healing (5, 8, 9). Collagen deposition, epithelial and epidermal differentiation are believed to take place under conditions of mesenchymal-epithelial communication (6, 9-11). This makes multipotent hMSC attractive candidates for autologous cell transplantation (5, 12). The composition of the biomaterial that should guide a tissue-specific cell differentiation and proliferation is also important (13). The objective of this study was to produce composite autologous bilayered grafts as a skin and dermal substitutes for permanent wound closure. Furthermore, this article exploits the interdependency of epithelial keratinocytes and hMSC. It should be investigated, if the aid of a feeder layer of hMSC will accelerate the attachment and proliferation of the keratinocyte cells and thereby wound repair.

Materials & methods

Materials

Collagen scaffolds were manufactured by Dr. Suwelack, Skin and Health Care GmbH, Germany. According to an established method of Wissink *et al.* (14) and Steffens *et al.* (15), cross-linking was performed using 1 mg EDC/0.6 mg NHS (E) in 500 µl reaction solution. EDC and NHS were purchased from Sigma-Aldrich, Germany. Cubes of 10 x

10 x 2 mm (weight: 10–12 mg) with a pore size of 50–60 μm were used for further experiments. Scaffolds were disinfected by incubation in 70% ethanol for 24 hours. Afterwards, the ethanol was rinsed out with sterile 0.9% NaCl-solution for another 24 hours. Final sponge pre-wetting was performed in stem cell culture medium (Clonetics®, BioWhittaker, Germany) 24 hours before cell seeding.

Isolation of keratinocytes

After informed consent, keratinocytes were obtained from fresh human epidermis of adults who underwent elective split-thickness skin grafting at the Department of Plastic Surgery and Hand Surgery, Burn Centre. The sterile split-thickness skin was cut into small pieces and incubated in dispase (0.5 U/ml; Roche Mannheim, Germany) overnight at 4°C. Then, the skin was shaken for 3 min at 37°C. Enzyme reaction was stopped by immersing the epidermal stripes in cold phosphate buffered saline (PBS). Next, the epidermis was incubated in EDTA-trypsin (0.5 g/l trypsin and 0.2 g EDTA/l diluted 1:250 in 1x phosphate buffered saline; PAA Laboratories GmbH, Austria) and shaken for 15 min at 37°C. The solution was shortly vortexed and the EDTA-trypsin was inactivated by adding pure fetal bovine serum (FBS, Biochrom Berlin, Germany). Then, the suspension was filtered and centrifuged at 400g for 10 min. at room temperature. Finally, the cells were resuspended in complete medium seeded onto collagen-layered (Biochrom Berlin, Germany) T-75 culture flasks (Cellstar®, Greiner Bio-One GmbH, Germany) and cultured further at 37°C, in a 5% CO₂ and 20% O₂ humidified atmosphere.

The complete culture medium consisted of DMEM/F-12 medium (PAA Laboratories GmbH, Austria) supplemented with 10% FBS (Biochrom Berlin, Germany), 100 $\mu\text{g}/\text{ml}$ penicillin/streptomycin (PAA Laboratories GmbH, Austria), 0.4 $\mu\text{g}/\text{ml}$ hydrocortisone (Sigma-Aldrich, Germany), 10⁻¹⁰ M cholera toxin (Sigma-Aldrich, Germany), 2 x 10⁻⁹ M triiodothyronin (Sigma-Aldrich, Germany), 5 $\mu\text{g}/\text{ml}$ insulin (Roche Mannheim,

Germany), 5 µg/ml transferrin (Sigma-Aldrich, Germany) and 10 ng/ml epidermal growth factor (EGF; Sigma-Aldrich, Germany).

Isolation of hMSC

After informed consent from patients that underwent total hip replacements, bone marrow was obtained by aspiration from femoral heads. Cells were isolated as previously described (16). In brief, the bone marrow was rinsed several times with stem cell medium (MSCBM, Cellsystems®, Germany), then the suspension was centrifuged at 500g for 10 min. at room temperature. The cell pellet was resuspended in 10 ml of fresh medium (MSCBM, Cellsystems®, Germany) and seeded in a T-75 culture flask (Cellstar®, Greiner Bio-One GmbH, Germany). Cell culturing was carried out at 37°C, in a 5% CO₂ and 20% O₂ humidified atmosphere. After overnight incubation, non adherent cells were washed out with the medium. Afterwards medium changes continued every 3-4 days. After confluency, cells were dissociated by stem cells trypsin (Cellsystems®, Germany) and seeded at a density of 5000 cells/cm² to expand hMSC.

Model of composite skin graft

Human full-thickness skin equivalents were generated *in vitro* by using biodegradable EDC-collagen as dermal scaffolds. To analyze the effects of the mesenchymal cells on the epithelial regeneration, keratinocytes were seeded onto the hMSC-populated scaffolds and cultured at 37°C, in a 5% CO₂ and 20% O₂ humidified atmosphere. First, 1 x 10⁶ hMSC were seeded onto the three-dimensional collagen scaffolds and cultured for 3 days in the basal medium (MSCBM, Cellsystems®, Germany). Secondly, keratinocytes were overlaid on the hMSC-containing scaffolds and cultured for further 7 days in complete keratinocyte medium to construct an artificial bilayer skin. As control, keratinocytes were cultured on cell-free matrices.

Structure and morphometric analysis

At day 7, the culture assembly was fixed with 4% formalin and embedded in paraffin. Longitudinally sectioned tissue slices of 6 μm were prepared and stained with hematoxylin-eosin (H&E). In these stained samples the structure of the epidermal layer was assessed. The thickness of the epidermis was measured by an objective micrometer in 12 randomly chosen fields (magnification 200x) to evaluate the impact of the hMSC on the proliferation of the keratinocytes.

Statistics

The significance of the differences between the mean value \pm standard deviation (of thickness of the epidermis) was evaluated by the Student's *t*-test. When $p < 0.05$, differences were considered significant.

Results

Tissue engineering of skin, based on collagen using a designed co-culture system is reported. Collagen has been proven to be a biocompatible material to support both adult human epidermal keratinocytes and hMSCs (17, 18). Sponges of collagen were prepared using a cross-linking method of lyophilized collagen (14, 15). The obtained artificial bilayer skin was flexible and had good mechanical properties. Histology revealed good cell attachment (only single keratinocytes were found around the matrix) and proliferation (build a multilayered epidermis) of human keratinocytes. HMSCs promoted the stratification of keratinocytes, forming a fully differentiated stratified epidermis after one week in culture. Basal, prickle cell, granular and cornified layers could easily be detected (Fig. 1A). In addition, hMSCs induced the keratinocytes to organize in a rete ridge-like structure (Fig. 1A).

In contrast, keratinocytes seeded on cell-free matrix migrated into the porous scaffold and formed only a significantly ($p < 0.05$) thinner epidermal layer (Fig. 1B). The significance of the differences between the mean value \pm standard deviation (of thickness of the epidermis) was evaluated by the Student's *t*-test.

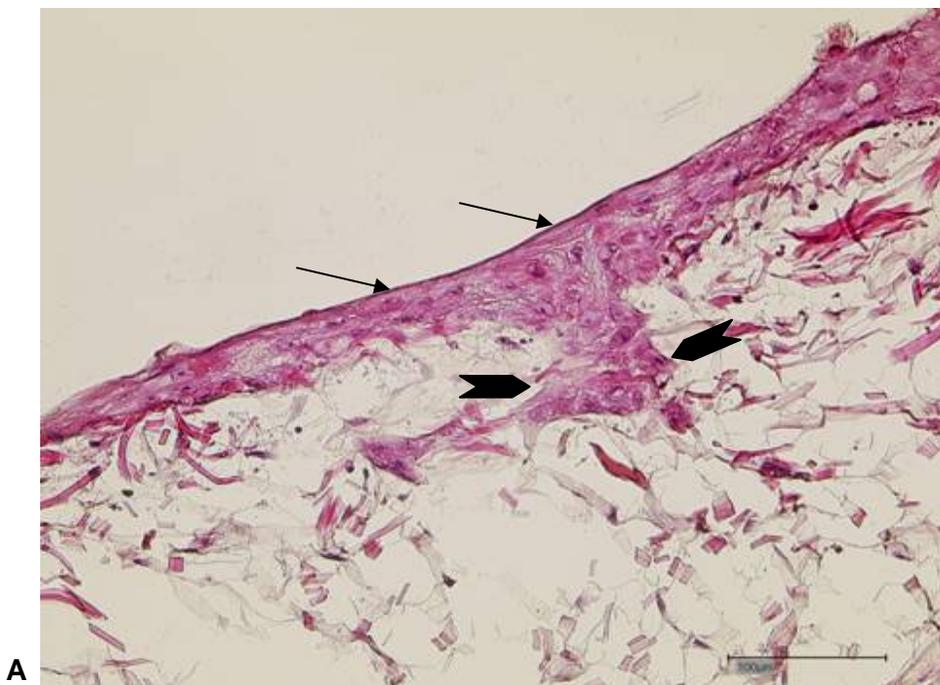


Fig. 1A: Structure of regenerative epidermal-dermal equivalents based on EDC-collagen after one week (original magnification 200x, H&E staining). hMSCs promote stratification (\rightarrow) and proliferation of keratinocytes and result in a fully differentiated multilayered epidermis with organisation of rete ridge-like structures (\blacktriangleright).

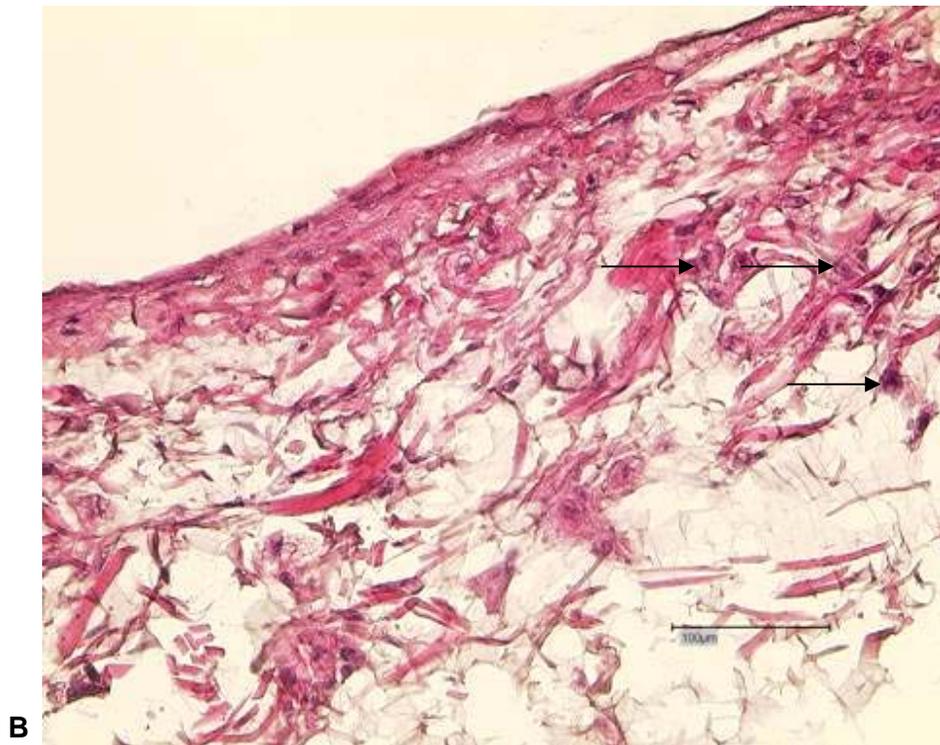


Fig. 1B: Structure of regenerative epidermal-dermal equivalents based on EDC-collagen after one week (original magnification 200x, H&E staining). Keratinocytes seeded alone on the collagen matrix invaded into the spongy structure (→) and formed only a thin, irregular epidermal layer.

Discussion

The data of this study suggest that the used technique is suitable for preparing a bilayered skin *in vitro*. Our composite skin substitute is compound of cultured epidermal keratinocytes, hMSC and a cross-linked collagen sponge. We have shown that hMSC promote epidermal regeneration and led to a fully differentiated, thick and multilayered epidermis. Keratinocytes without hMSC-support formed only an irregular and thin epidermal layer. This suggests that bone marrow-derived mesenchymal stem cells and thereby mesenchymal intercellular communications are crucial for proliferation and stratification of human keratinocytes. The formation of rete ridge-like structures under conditions of hMSC-keratinocyte co-cultures implicates a vital role of the cellular interactions during human wound healing.

Efforts for closure of full-thickness skin defects with autologous keratinocytes has often ended in blistering and prolonged secondary wound healing. The missing of dermal components was blamed for these suboptimal results (19, 20). Although hMSC are not the major cell type in the normal dermis newer studies pointed out to the participation of these cells in skin wound healing, e.g. by the mechanism of homing (5, 21). Aoki *et al.* showed that bone marrow stromal cells accelerated epidermal regeneration better than preadipocytes and dermal fibroblasts (11). Experiments are needed to clarify the mechanisms and cytokines involved in the epidermal-dermal regeneration by hMSC to make them safely applicable in cellular therapies. Our results illustrate that these technique could be exploited as a suitable therapy for repair and regeneration of skin defects.

Conclusion

The approach to skin modelling reported here showed that non-skin-localized hMSC can promote skin regeneration. The work suggests that direct intercellular contact is required for a skin-specific morphology. Co-cultures of hMSCs and keratinocytes may improve the performance of composite skin grafts in clinical applications.

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