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# Human Bone Marrow Mesenchymal Stem Cells Seeded on Modified Collagen Improved Dermal Regeneration In Vivo

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In the correction of functional and aesthetic impairments, loss of soft connective tissue creates the need for adequate implant material. The reconstruction of defects resulting from radical excisions, trauma, or hereditary diseases has seen the use of combined grafts and flaps. With the aim of minimizing donor site morbidity, new methods have been evaluated. Because of a low rate of vascularization, with artificial dermal templates the take has only been poor. As shown in previous studies, improved angiogenetic potency and epidermal formation has been obtained in modified, cell-seeded collagen matrices. We have now investigated the suitability of adult bone marrow mesenchymal stem cells (hMSC) for soft tissue engineering. In this study, hMSC were isolated and expanded. Cells ($10^6$) were seeded onto EDC cross-linked collagen sponges and implanted in 30 immunodeficient mice. Collagen sponges without cells were used as controls. The grafts were evaluated after 2 and 6 weeks. After explantation, macroscopic appearance, weights, and histology (scaffold degradation, cellularity, and invasion depth of the seeded cells) were all assessed. After 2 and 6 weeks in vivo, new vessels were found macroscopically on all cell-seeded collagen grafts. The control grafts appeared to be degraded with a lower rate of vessel ingrowth. In the experimental group, weight gain was significant after 2 and 6 weeks in vivo compared to the same grafts after 72 h in vitro, while weight increased only slightly in the control group. Histologically, populated scaffolds showed a high density of vascularization under a capsule. The control sponges showed single capillaries and a thicker capsule. Compared to the controls, cellularity (cells/field) was greater in cell-containing collagen grafts after 2 and 6 weeks. The results obtained demonstrate that in vitro cultured human mesenchymal stem cells seeded on modified collagen sponges may be able to act as a replacement for soft tissue.

Key words: Human bone marrow mesenchymal stem cells; Soft tissue engineering; Collagen matrices; Scaffolds; Animal model

## INTRODUCTION

Soft tissue plays both a crucial functional role (cushion for tendons, muscles, and bones) and an aesthetic (natural body shape) role. The reconstruction of acquired or hereditary soft tissue defects (e.g., due to burns, acute and chronic wounds, Poland's syndrome, tuberous breast deformity, etc.) places an enormous strain on healthcare resources. The total costs go beyond the cost of hospital services. Loss in income, pain and suffering, rehabilitation, and long-term costs of the injury must also be included. Transplantation of autologous tissue (flaps, composite grafts) is limited by donor sites and the patient's general condition. Artificially engineered tissues could revolutionize the field of reconstructive surgery. As far back as 20 years ago, Pruit and Levine (23) formulated the criteria that ideal skin replacement should meet. However, no adequate nonautologous tissue substitute has yet been established. Recent studies have shown that mesenchymal (bone marrow stem cells, fibroblasts, preadipocytes) cell seeding might be the answer to inadequate vascularization and incorporation. (4,8,9). By contributing to collagen deposition and by supporting the regeneration of cutaneous vascular, epithelial, and dermal structures, bone marrow-derived mesenchymal stem cells (hMSC) play an important role in wound healing (3,10,11). hMSC are characterized by their potential to differentiate into endodermal, mesoder-

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mal, and ectodermal cells in vitro and in vivo (12). For autologous cell transplantation, these multipotential and easy to harvest cells are seen as a promising source. Increasing the number of hMSC at the wound site may provide a useful therapeutic tool in wound healing.

Because collagen sponges are biodegradable, act like natural tissue, and provide a three-dimensional structure for the cells, they are a logical choice for engineering soft tissues. However, the drawback is the low angiogenetic capacity of collagen, which reduces incorporation and initiates apoptosis, resulting in implant failure (25,26).

This study thus focused on developing a modified collagen–cell-based assembly for the regeneration of soft tissue defects. Initially, collagen matrices were modified by cross-linking with EDC [1-ethyl-3(3-dimethylaminopropyl) carbodiimide]. Following this, hMSC-populated matrices were implanted subcutaneously into immunodeficient mice. Cultured soft tissue substitutes with hMSC showed enhanced vessel ingrowth, cell density, and weight. This was in contrast to cell-free constructs used in immunodeficient mice.

## MATERIALS AND METHODS

### *Collagen Sponges*

The collagenous sponge scaffolds were produced by Dr. Suwelack Skin and Health Care GmbH, Billerbeck, Germany. Collagen suspensions (mostly bovine collagen type I and 3% elastin) were frozen and vacuum dried. This results in a nondirected porous structure. The collagen fibers were cross-linked by admixing EDC. In these experiments, we used specimens measuring $7 \times 7 \times 5$ mm. Before being used in the animal model, the collagen sponges were disinfected for 24 h in 70% ethanol and then soaked twice for 24 h in sterile 0.9% NaCl solution.

### *In Vitro Tissue Culture*

*Isolation.* After acquiring informed consent, human bone marrow was aspirated from femoral bones during total hip replacements. Cells were isolated according to a successful protocol (19). Bone marrow was rinsed several times with stem cell medium (MSCBM, Cellsystems®, Germany). The suspension was centrifuged at $500 \times g$ for 10 min at room temperature. The cell pellet was resuspended in 10 ml of fresh medium and seeded in a T-75 culture flask (Cellstar® Greiner Bio-One GmbH, Germany). The further expansion was carried out at 37°C, in a 5% $CO_2$ and 20% $O_2$ humidified atmosphere. A medium change after 24 h removed nonadherent cells. Further medium changes were performed every 3–4 days. At confluency, the cells were trypsinized with stem cell trypsin (Cellsystems®) and seeded at a density of 5000 cells/cm$^2$.

*Cell Labeling.* To enable us to detect the hMSC after transplantation, cells were labeled using the fluorescent Cell Tracker™ CM-DiI (chloromethylbenzamido; Molecular Probes, Germany) diluted in dimethyl sulfoxide (DMSO). Twenty-four hours before being used in the seeding experiments, the hMSC were incubated with 1 μM CM-DiI/ml culture medium. This enabled us to label all lipophilic cell structures fluorescent red, which proved to be stable throughout fixation and paraffin embedding.

*Culture and Preparation of Cell-Containing Grafts.* At confluency, the hMSC of the second passage were trypsinized. The cell pellet was resuspended in 500 μl of stem cell medium and counted in a Neubauer chamber. A suspension of 50 μl, containing $1 \times 10^6 \pm 4 \times 10^4$ cells, was seeded on the upper surface by dropping it gently onto the medium-prewetted scaffolds. The sponges were incubated for 1.5 h at room temperature. Afterwards, 2 ml medium was added to the cell–matrix assemblies. Cell-free controls were only prewetted with medium. To enable cell attachment, controls and experimental matrices were kept for 24 h in the incubator.

### *In Vivo Experiments*

*Transplantation.* Seventy-two hours after seeding the cells in the scaffolds, 30 fabricated cell–sponge constructs were transplanted subcutaneously to the left paravertebral area of 30 immunodeficient, 6-week-old female mice (NOD. CB 17-Prkdcscid/J, Charles River), weighing 25–30 g on arrival. The base of the constructs was placed on the muscle fascia. In each of these animals a control matrix (cell free) was transplanted to the right paravertebral area through a separate incision.

All animal experiments were performed in compliance with the National Research Council's guidelines for human care and had official approval.

*Explantation.* Two groups were randomly formed. After 2 weeks (group A, $n = 13$) and 6 weeks (group B, $n = 13$), the mice were sacrificed and the grafts explanted.

*Weight.* The weight of each sponge was assessed 24 h after the cell seeding (before transplantation) and after explantation (2 or 6 weeks). The mean ± SE of the weights was calculated by measuring all mice at each examination time [before transplantation, 2 weeks (group A) and 6 weeks (group B) in vivo].

### *Histology*

The explanted material was fixed in 4% buffered formaldehyde, dehydrated in a series of alcohols, and embedded in paraffin. Vertical sections of 6 μm were then produced and stained with hematoxylin-eosin (H&E) and DAPI (4′,6-diamidino-2-phenylindole).

*Microscopy.* The samples were evaluated independently by three blinded investigators (M.M., E.K., S.L.) using light and fluorescence microscopy (Zeiss, Germany).

*Cellularity.* The overall cellularity was assessed by counting all DAPI-stained cells in the five fields at 200× magnification (Fig. 1Fig. 1).

*Cellularity of Seeded hMSC.* The cellularity was assessed by counting all CM-DiI-labeled cells in the five fields at 200× magnification (Fig. 1).

*Penetration of Seeded Cells.* In representative cross sections, the penetration depth of CM-DiI labeled cells was calculated at 200× magnification by using an intraocular micrometer (Zeiss, Germany).

### *Statistical Analysis*

The calculation of the mean value ± SD (weight of the grafts, overall cellularity, and penetration depth) was made by Sigma Plot and Sigma Stat (Jandel Scientific, USA). The significance of differences at 2 and 6 weeks was evaluated by Student's *t*-test and a value of $p < 0.05$ was considered significant.

## RESULTS

### *In Vitro*

hMSC adhered well to the plates. At first hMSC consisted of three different morphological cell types. At passage 3, the culture became more uniform. Confluence was achieved after approximately 10 days. During this time, an initiation of angiogenic differentiation could not be observed. Seventy-two hours after seeding on the collagen scaffolds, individual cells were located outside the sponges. The method of CM-DiI labeling proved to be reliable and detected the seeded cells. Inside the collagen matrices, individual cells could be identified filling the spaces of the sponge porous system. At the time of transplantation, hMSC presented elongated cell morphology but showed no signs of differentiation. Analogous to preliminary experiments, microscopic evaluation of the modified specimens demonstrated a higher overall cellularity after 72 h, in comparison to the unmodified scaffolds (19).

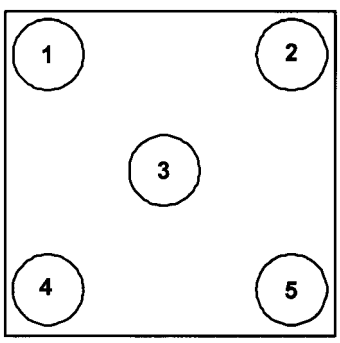


**Figure 1.** Assessment of cellularity. Microscopic fields from the cross section of the constructs were evaluated.

### *In Vivo*

Four animals died during the operative procedure. In the observation period, no infections occurred and no wound dehiscences were observed. Thus, 26 hMSC-populated collagen sponges and 26 cell-free scaffolds were evaluated.

*Macroscopic Examination of Transplants.* After 2 weeks, each graft was easily identifiable. Macroscopically, the square shape of the scaffolds was unchanged. The hMSC-populated constructs showed new vessels on the surface (Fig. 2A), in contrast to the white and more degraded control grafts (Fig. 2B).Grafts populated with hMSC were found inside a thin fibrovascular capsule that showed the ingrowth of vessels and connected tissue into the sponge. In case of the controls, the capsule consisted of avascular fibers.

After 6 weeks, identification of the scaffolds was still easy. The shape of the grafts looked irregularly round. Cell-seeded specimens (Fig. 3A) presented capillary tissue on the top that adhered tightly to the scaffold and that could not be removed. Connections to the mouse tissue and fine ligaments surrounded the hMSC-populated constructs. The controls without cells did not lead to such adhesions and showed a higher rate of degradation and still no formation of vessels (Fig. 3B).

*Macroscopic Examination of Weights.* To determine the effects of hMSC on graft weight, we measured the scaffolds after 72 h in vitro and 2 and 6 weeks after transplantation. Figure 4 shows only a slight significant weight increase at 2 and 6 weeks compared to the weights after 72 h in vitro in the cell-free constructs. The hMSC populated grafts revealed significantly higher weights at 2 and 6 weeks ($p < 0.05$).

Soft tissue substitutes with hMSC started gaining twice as much weight after 2 weeks as controls. This process continued in the populated grafts and they reached the doubled weight after 6 weeks in vivo.

*Histological Assessment.* After 2 weeks, all grafts presented a thin capsule layer of fibrovascular tissue. No neutrophils were found. The scaffold appeared almost unchanged, with minimal signs of degradation (curling) at the edges. Microscopic examination of the hMSC-containing grafts revealed a high quantity of vessels and bundles of new collagen fibrils, in contrast to cell-free specimens. Due to the cell tracking, human and mouse endothelial cells could be separated. Both cells contributed to neovascularization inside the scaffold, whereas the vessels on top of the sponges seemed to be of mouse

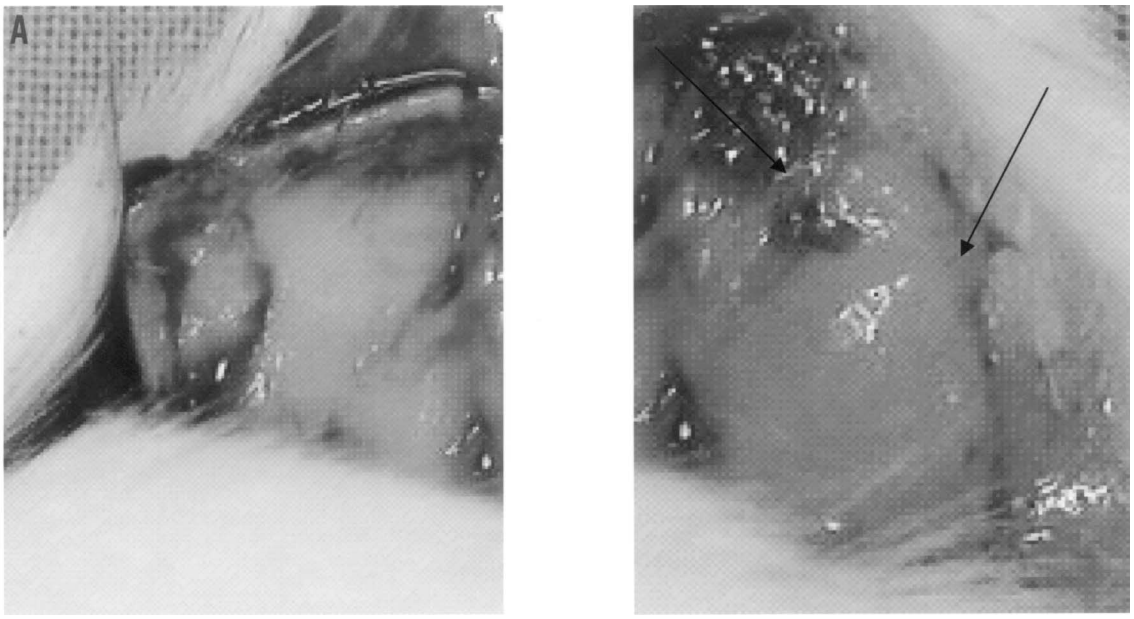


**Figure 2.** After 2 weeks, new vessel formation was observed macroscopically on hMSC grafts (A). The control sponge from the same animal revealed no angiogenetic capacity (B; arrows show the silhouette of the graft).

origin (no CM-DiI labeling). These grafts also showed a higher cell number compared to the cell-free grafts. The cell density was $164 \pm 52$ (cells/field) compared to $115 \pm 45$ (cells/field) in the control grafts without hMSC. The cells in the hMSC-populated grafts were evenly distributed and reached the center of the sponge. In contrast, in the controls cells remained on the edges of the scaffold (Fig. 5).

After 6 weeks, layers of fibrovascular tissue that was thicker in the cell-free constructs encapsulated all grafts. The number of macrophages and giant cells slightly increased. The structure of the scaffolds had changed to a

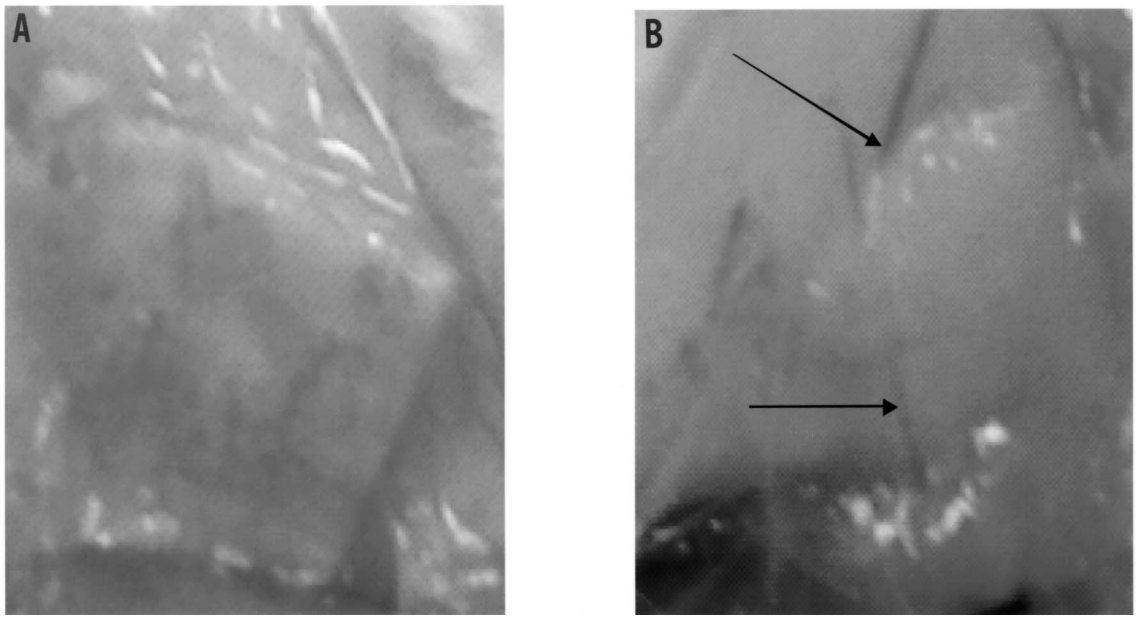


**Figure 3.** After 6 weeks, all hMSC-seeded scaffolds were covered by vascular tissue (A), in contrast to the contralateral control from the same animal that had a white and more degraded appearance (B, arrows show the silhouette of the graft).

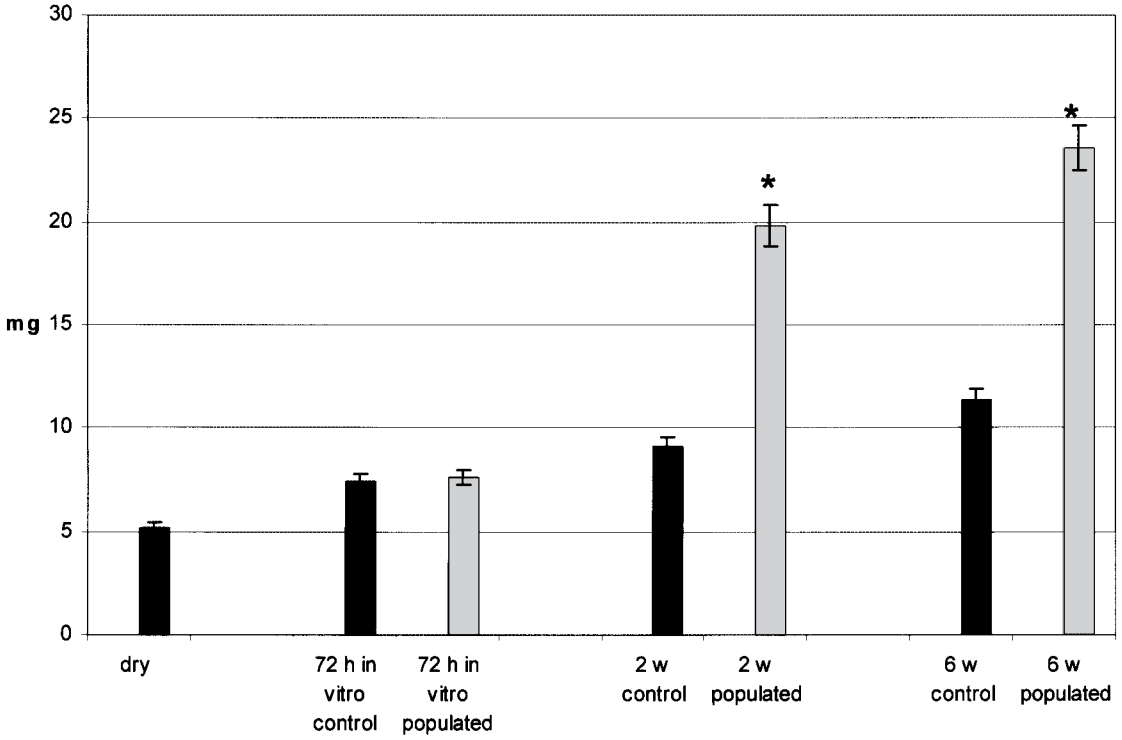


**Figure 4.** Assessment of weight of hMSC collagen and control grafts. A significant increase after the wetting and seeding of the collagen scaffolds was observed. The difference between hMSC-seeded grafts and control grafts was also significant ($p < 0.05$).

more irregular one (shrinkage and curling). Some pores appeared to have collapsed. Newly formed extracellular matrix mainly composed of collagen fibers was found inside the scaffolds. Vessels were distributed through the complete hMSC-containing grafts (Fig. 6A), in contrast to cell-free specimens (Fig. 6B). Again, hMSC in combination with mouse cells formed the vessels inside the specimens and mouse cells surrounded the sponges with fibrovascular tissue.

The cellularity in the hMSC grafts was $237 \pm 44$ (cells/field) while the cell-free grafts showed $170 \pm 49$ cells/field. The specific cellularity slightly increased between 2 weeks ($45 \pm 16$ cells/field) and 6 weeks ($76 \pm 19$ cells/field). In addition, the penetration depth of the stem cells significantly increased from $945 \pm 265$ µm at 2 weeks to $1560 \pm 437$ µm at 6 weeks posttransplantation (Figs. 7 and 8). HMSC survived the procedure and had enough oxygen and substrates to migrate into deeper regions of the scaffolds.

## DISCUSSION

Traditionally, full-thickness defects are covered by local or free flaps. Cases where skin loss is extensive, donor sites limited, or general state of the patient is not good make this technique difficult or impossible to perform. Split-thickness skin grafts adhere well to the wound bed and shrink as they heal, making it impossible to prevent less than optimum mechanical and cosmetic outcomes. The effect of contraction-inhibition is adjusted for by dermal components (9,16). In cases of this kind, xenografts (e.g., collagen prosthesis) have become widespread for soft tissue reconstruction. Lack of angiogenetic activity and poor performance in terms of healing time are the drawbacks in this context.

Recent studies have revealed improved performance of grafts and enhanced extracellular matrix formation in combination with mesenchymal cells (2,9,15). Anderson et al. identified biomaterials that support appropriate cellular attachment, proliferation, and gene expression for tissue engineering and cell therapy with human embryonic stem cells (2). Erdag et al. demonstrated that fibroblast-seeded skin substitutes formed a thicker epidermis with only 2% contraction. In addition, fibroblasts containing grafts revealed many larger vessels than cell-free constructs (9). Further, von Heimburg et al. achieved successful results in soft tissue reconstruction by using human adipose precursor cells seeded on hyaluronic acid grafts in a nude mice model. Differentiation into adipose tissue in combination with accelerated vascularization was the major goal observed in cell-containing carriers (30).

The optimum matrix for hMSC transplantation is not

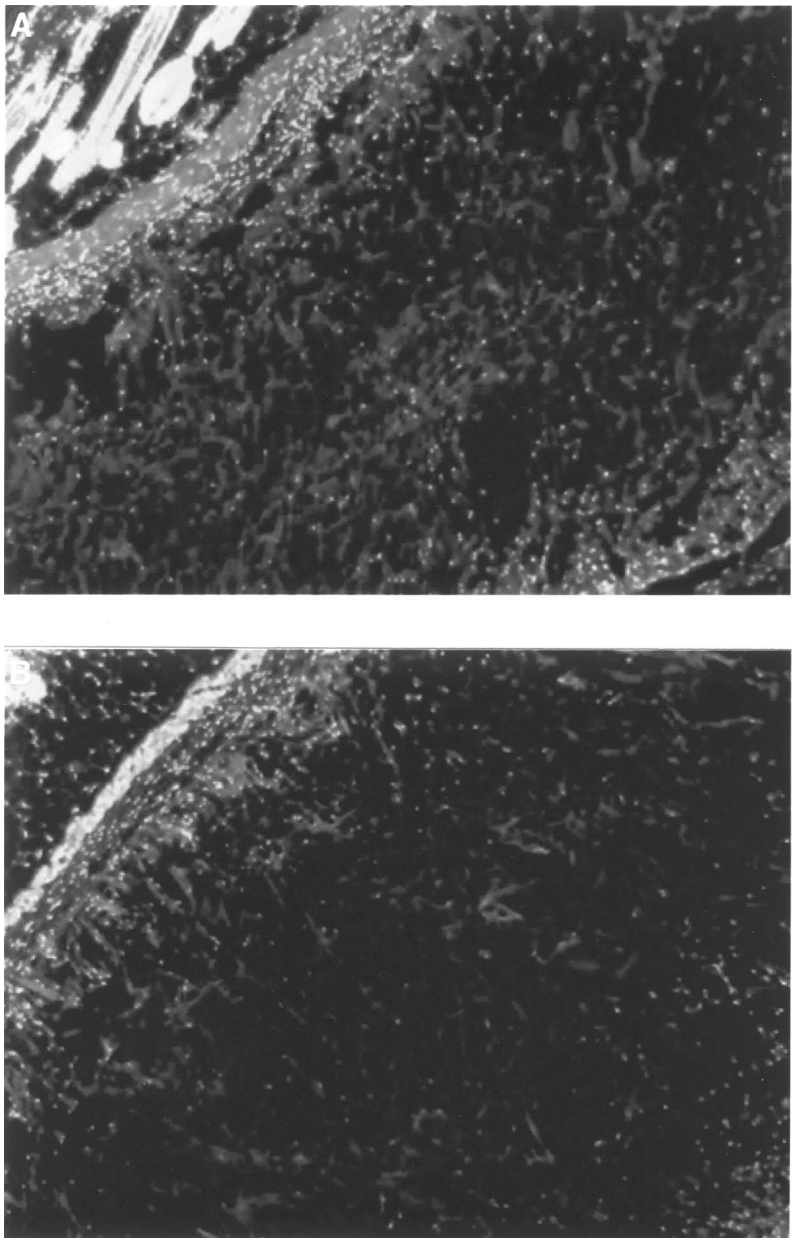


**Figure 5.** Paraffin sections of dermal substitutes after 2 weeks with (A) or without hMSC (B) (magnification 200×: DAPI stained).

yet known. Although Chunmeng et al. demonstrated the positive impact of dermal multipotent cells (7) and Satoh et al. of mesenchymal stem cells (24) in wound healing in rats, a biocompatible material is needed for an adequate volume in soft tissue replacement. Porous collagen is a widely used biomaterial in tissue regeneration that supports cellular ingrowth and new matrix formation (18). Mauney et al. outlined the potential role of MSC–extracellular matrix interactions in mediating the retention of MSC proliferative and differentiation capacity after ex vivo expansion on collagenous biomaterials (22).

In preliminary work, we have been able to demonstrate that native collagen scaffolds do not promote endothelial and hMSC proliferation in an appropriate manner. The mitogenic and angiogenetic potential of these collagen scaffolds produced by Dr. Suwelack Skin and Health Care GmbH could be accelerated by cross-linking with EDC (19,26). Toxicologically, EDC proved to be absolutely safe when implanted in vivo (29). We saw

no differences in growth of a HUVECs' culture in collagen extracts and EDC–collagen extracts (20). The lack of local and systemic toxicity is explained by the fact that, after activating the amino and carboxylic groups during the cross-linking procedure, the molecules are completely washed out.

This study presents an efficient method of cell-based soft tissue replacement in the immunodeficient mouse. The hMSC survived the mouse transplantation procedure and promoted vascularization at the recipient site, penetrating scaffold in vivo to a depth of more than 1900 μm, while some infiltrated the whole scaffold.

Various studies on mesenchymal stem cells for tissue regeneration have shown improved proliferation of endothelial cells (5,13,21). Increased expression of VEGF (vascular endothelial growth factor) by hMSC has been demonstrated, indicating enhancement of vascularization (27). VEGF presents the most potent growth factor for angiogenesis (14). At 2 weeks, and to an even greater extent 6 weeks after transplantation, cell-containing grafts have shown greater densities of vessels. It is possible that the presence of hMSC affected endothelial proliferation through expression of VEGF. Besides supporting capillary formation, hMSC play a vital role in dermal regeneration (10,13). Wound contraction and graft degradation (weight reduction) could be considered

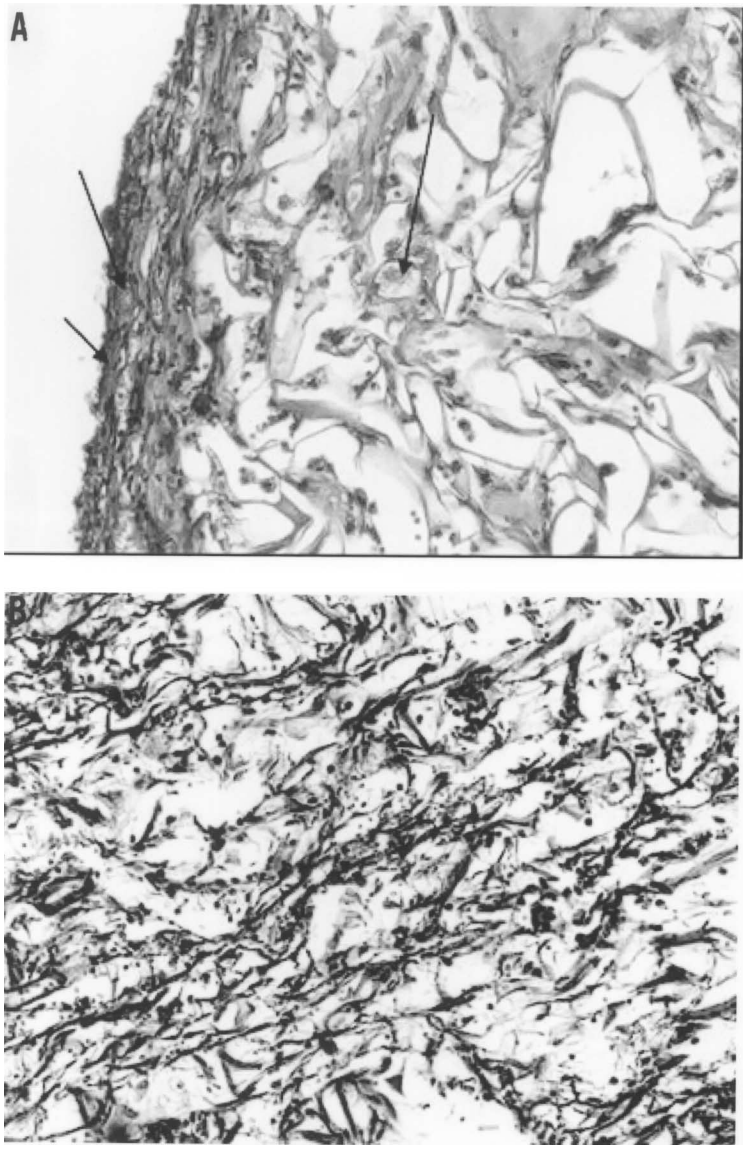


**Figure 6.** Cultured dermal substitutes with (A, arrows show capillaries filled with erythrocytes) or without hMSC (B) after 6 weeks (magnification 400×: H&E stained).

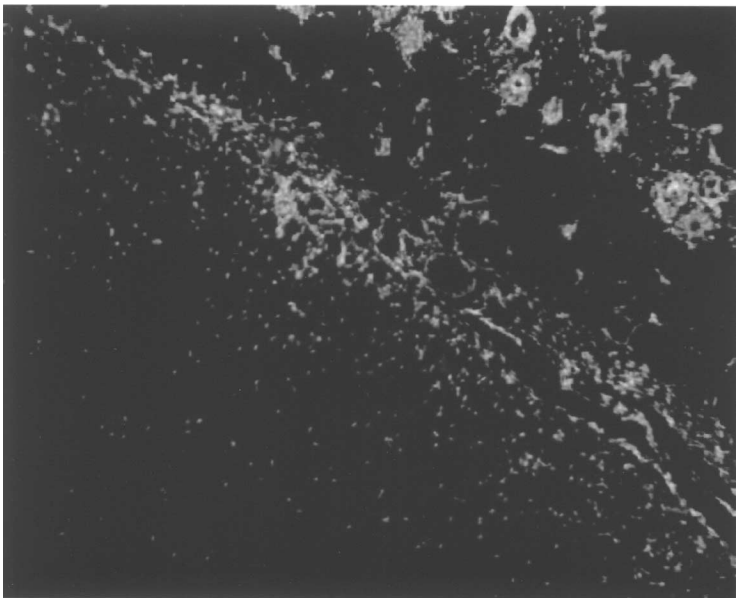

**Figure 7.** Homogenous distribution of invaded hMSC after 2 weeks in vivo (magnification 200×: CM-DiI-labeled cells and DAPI staining).

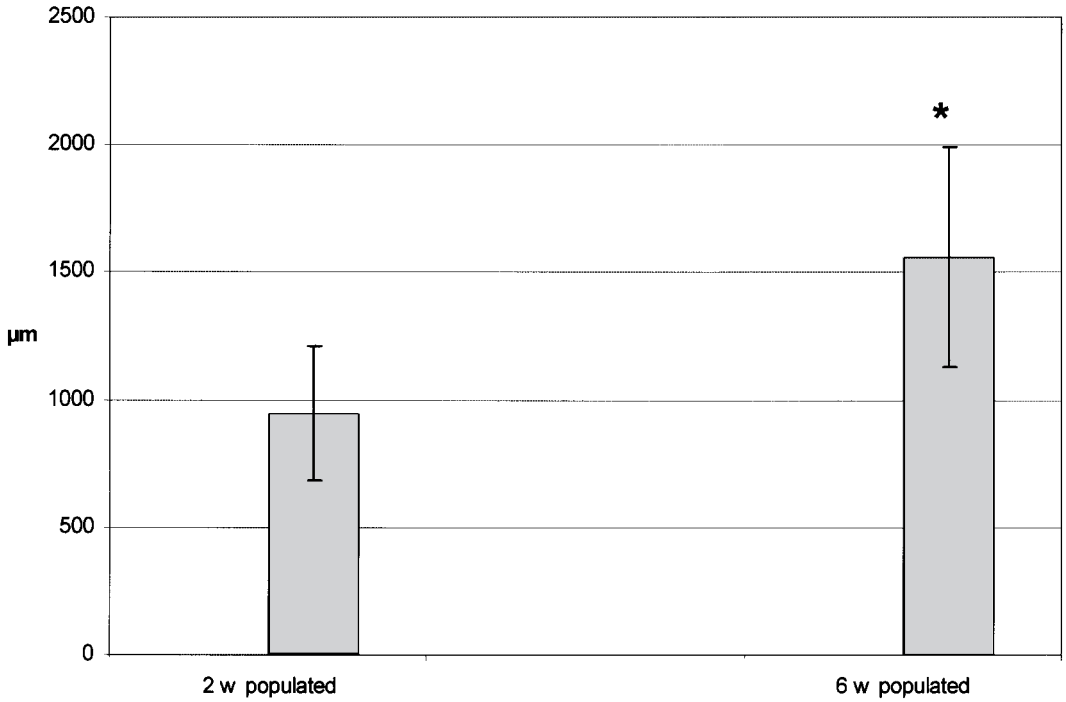


**Figure 8.** Assessment of mean maximum penetration depth of hMSC in collagen constructs of CM-DiI-labeled cells on cross sections in vivo (2 and 6 weeks after transplantation). A significant increase in penetration depth between 2 and 6 weeks was observed in vivo. In some scaffolds, hMSC migrated through the whole scaffold after 6 weeks (2.0 mm depth).

as two aspects of dermal healing. We have observed a significant increase in weights in hMSC-populated constructs compared to cell-free dermal templates. In our study, the participation of hMSC in collagen deposition may play an important role in dermal regeneration with artificial materials as dermal substitutes (10).

Currently, the comparison of our results to other studies is difficult because to our knowledge only a few investigations deal with soft tissue engineering using mesenchymal stem cells. Ueda et al. obtained positive results in soft tissue management using cultured epithelial grafting (28). Most works tried to engineer soft tissue using adipogenic differentiation (1,30). Choi et al. described an efficient model of adipose tissue engineering using mesenchymal stem cells and injectable PLGA spheres. Addition of hMSC promoted cellular invasion, vascularization, and produced larger grafts (6). These phenomena were also observed in our work.

We consider that adipose tissue might be useful for the reconstruction of volume defects but it is not helpful when full-thickness wounds have to be treated. Adipose tissue cannot be covered with split-thickness skin grafts, in contrast to collagen templates.

Our results support the use of modified collagen sponges as the mesenchymal stem cell delivery matrix in soft tissue engineering. Lewus et al. demonstrated the compatibility of bone marrow stem cells with the collagen gels (17).

To define the optimum material composition, other matrices and various modifications need to be tested.

## CONCLUSION

The ideal material for soft tissue replacement has not yet been found. Widely available and not leading to immune reactions, autologous hMSC are an ideal tool as donor sites for transplantation. This initial study confirms that isolated hMSC seeded on freeze-dried collagen scaffolds develop viable soft tissue-like structures following implantation into the immunodeficient mouse.

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