Artificial skin and tissue regeneration

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Chapter 4

Higher Numbers of Autologous Fibroblasts in an Artificial Dermal Substitute Improve Tissue Regeneration and Modulate Scar-tissue Formation

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Abstract

Cultured skin substitutes are increasingly important for the treatment of burns and chronic wounds. It is a general assumption that wound healing is accelerated when more fibroblasts are present in a living-skin equivalent. Therefore, we investigated the quality of dermal tissue regeneration in relation to the number of autologous fibroblasts seeded in dermal substitutes, transplanted instantaneously or precultured for 10 days in the substitute.

A full-thickness porcine wound model was used to compare acellular dermal substitutes (ADS) to dermal substitutes seeded with fibroblasts at two densities, i.e. 1x10^5 (0-DS10) and 5x10^5 cells/cm² (0-DS50), and to dermal substitutes seeded 10 days before operation at the same densities (10-DS10 and 10-DS50) (n=7 for each group, 5 pigs). After transplantation of the dermal substitutes, split-skin mesh grafts were applied on top. Additional dermal substitutes (n=5) were simultaneously prepared for each group, fixed on the day of operation, and analysed for the number of fibroblasts and myofibroblasts present in the matrix. Wound healing was evaluated blinded for six weeks, both macroscopically and microscopically (biopsies taken after 1, 3 and 6 weeks). Cosmetic appearance was scored for skin colour, smoothness of wound surface and tissue elasticity/suppleness. Wound contraction was measured by planimetry. The wound biopsies taken after 3 weeks were stained for myofibroblasts (α-smooth-muscle actin). The percentage of dermal area positive for myofibroblasts and the intensity of staining in this area were determined by image analysis. Six weeks post-wounding, scar tissue in wound cross-sections was identified by thin immature collagen bundles organized parallel with the epidermis, and by the absence of elastin staining. Collagen maturation was investigated with polarized light.

The overall wound cosmetic parameter scores and wound contraction showed an improvement in the following order: 10-DS50 > 10-DS10 = 0-DS50 > 0-DS10 > ADS. For wound cosmetic parameters, the 10-DS50 and 0-DS50 treatments scored significantly better than the ADS treatment, as the 10-DS50 treatment did for wound contraction (p<0.05, paired t-test). Three weeks after wounding, a similar trend was observed in the granulation tissue showing a reduction in cell density and early deposition of extracellular matrix (ECM). In addition, an area with myofibroblasts positive for α-smooth-muscle actin was observed in the granulation tissue, which was significantly smaller for 0-DS50, 0-DS10 and 10-DS50 than for the ADS treatment (p<0.04, paired t-test). The intensity of staining became less as the size of the area diminished. After 6 weeks, the wounds treated with 0-DS50, 0-DS10 and 10-DS50 had significantly less
scar tissue and significantly more mature collagen bundles in the regenerated dermis. This observed improvement of wound healing was correlated with the numbers of fibroblasts present in the dermal substitute at the moment of transplantation.

In conclusion, dermal regeneration of experimental full-skin defects significantly improved by treatment with dermal substitutes containing high numbers of (precultured) autologous fibroblasts.

Introduction

Patients with full-thickness burns suffer from a substantial loss of dermal tissue. In the past, these wounds were successfully grafted with autologous split-skin grafts and cultured keratinocytes. Nevertheless, the lack of a dermal component often negatively influences the outcome, because split-skin grafts alone often fail to correct contour defects, are prone to contractures, and can lead to a poorly developed dermal-epidermal junction. In addition, cultured epithelium grafts are also known to regenerate slowly the dermal-epidermal junction and to blister and ulcerate for several months after grafting. Several composite grafts, comprising both cultured fibroblasts and keratinocytes applied in an one-stage transplantation procedure, were tested clinically and were able to prevent this blister formation. It is now generally accepted that a dermal component is needed to improve the final outcome of cultured epithelial grafts. Most artificial skin substitutes include fibroblasts to promote dermal-epidermal regeneration and to stimulate epidermal growth and differentiation, but so far little attention was paid to the influence of the fibroblasts on dermal tissue regeneration. Nowadays, for practical reasons more effort is put into the use of allogeneic fibroblasts. The current view is that allogeneic cells provide and maintain graft integrity while they are being replaced by host cells. Whether or not this substitution causes inflammatory and immunological reactions and contributes to or prolongs the wound-scarring process remains to be elucidated. Pathological conditions of the skin associated with inflammatory responses in general result in tissue fibrosis and do not mediate true tissue regeneration.

In our opinion, the use of autologous fibroblasts in artificial skin is a feasible concept. In the practice of Dutch burn centres, if the patient is stable and fluid loss is under control, further surgical treatment is postponed for two to three weeks in order to observe healing of partial-thickness wounds. In addition, usually a temporary wound coverage such as cadaver skin conserves a good wound environment for future grafting. Normally, if skin biopsies or split skin are used for the isolation of
fibroblasts, culture periods longer than 2 weeks are required to obtain sufficient cells to treat large wound areas. However, cell yields could be improved by the use of subcutaneous fat as a source for fibroblasts\textsuperscript{28}, which is easily obtained by liposuction or lipectomy\textsuperscript{29}.

With respect to skin substitution with autologous cells and efforts to obtain sufficient numbers of fibroblasts, we felt it was important to investigate in which quantity and in which fashion fibroblasts contribute to dermal tissue regeneration. Therefore, we studied dermal tissue regeneration in relation to the number of autologous fibroblasts in dermal substitutes. Fibroblasts were seeded at different densities and transplanted instantaneously or after a precultured period of 10 days in the dermal substitute. This relatively short culture period was chosen because of the limited time available for cell amplification in a clinical setting. For allografted substitutes, long pre-culture periods are employed to increase the numbers of fibroblasts, the deposition of ECM, and the amount of growth stimulatory proteins in the substitute.\textsuperscript{16,22} The general hypothesis is that wound healing is accelerated by the presence of more fibroblasts and ECM in the dermal substitute. However, in earlier experiments we found that high numbers of fibroblasts in the early granulation tissue were associated with the presence of higher numbers of myofibroblasts, which finally resulted in wound healing with more wound contraction.\textsuperscript{28,30}

In this study, we used the porcine full-thickness wound model and an elastin/collagen dermal matrix as dermal substitute.\textsuperscript{31} One week and 3 weeks post-wounding, the cellularity of the granulation tissue and the presence of ECM were scored, and the percentage of dermal area containing myofibroblasts was determined. Six weeks post-wounding, we investigated ECM remodelling, maturation of collagen, and elastin regeneration, and then correlated the histological data with macroscopic appearance and contraction results.

**Materials and methods**

**Preparation of dermal substitute grafts**

After 1 week of acclimatization and 4 to 6 weeks prior to operation, dermal fibroblasts were isolated from 5-10 punch biopsies (4 mm) from each pig. The epidermis was cut off, dermal tissue was minced and digested for 1-2 h at 37°C in a phosphate buffered saline solution (PBS) containing 0.25% (w/w) collagenase A and dispase (Boehringer Mannheim, Mannheim, Germany) (1ml/biopsy). The digest was sieved
over a 70 µm cell strainer (Falcon, Becton Dickinson, Mountain View, CA), washed in culture medium and subsequently cultured in DMEM supplemented with 10% (v/v) FCS, 1 mM L-glutamine and antibiotics (penicillin (100 IU/ml), streptomycin (100 mg/ml)) (all from Life Technologies, Breda, The Netherlands). The dermal substitute was a non-cross-linked native bovine collagen matrix (type I collagen from bovine skin) coated with a 3% (w/w) α-elastin hydrolysate (80% MW 60 kDa and 20% MW 200 kDa from bovine ligamentum nuchae) and was kindly provided by Dr. P. Tewes-Schwarzer (Dr Otto Suwelack Nachf. GmbH & Co., Billerbeck, Germany). Dermal substitutes were seeded with cultured porcine fibroblasts by inoculating the upper side of the pre-moistened matrices with culture medium containing the fibroblasts (1 ml/10 cm² of substitute). The seeding volume was absorbed by the dermal substitute. Two groups were treated with dermal substitutes seeded 10 days before the operation with 1x10⁵ and 5x10⁵ fibroblasts/cm² (10-DS10 and 10-DS50) respectively. The dermal substitutes were cultured in petri-dishes (non-TC-coated; d=20 cm) and 4 h after seeding 20 ml of medium supplemented with 25 mg/ml ascorbic acid (Sigma, St.Louis, MO) was added. Ascorbic acid was added to stimulate the deposition of collagens and glycosaminoglycans. The media were changed every day and supplemented with freshly prepared ascorbic acid. The other two groups were treated with dermal substitutes seeded with 1x10⁵ and 5x10⁵ fibroblasts/cm² (0-DS10 and 0-DS50) 4-16 h prior to grafting. As controls, the dermal substitutes without cells (ADS) were also incubated in culture media for 4-16 h. For each group, extra dermal substitutes were prepared which were fixed on the day of operation with 4% paraformaldehyde PBS solution for 16 h at RT.

**Operation and grafting procedures**

The protocol was approved by the University of Amsterdam Committee of Animal Welfare and five female Yorkshire pigs (15 kg at arrival) were included in the study. Before each surgical procedure, wound evaluation and bandage changing, the pigs were sedated by intramuscular injection with azaperon 4 mg/kg (Stressnill®, Janssen-Cilag, Gent, Belgium). Complete anesthesia was induced with a face mask with 5% isoflurane and a 50:50% mixture of nitrous oxygen and oxygen. Anesthesia was maintained with 1%-2% isoflurane and the same air mixture. If necessary post-operative pain was treated with a subcutaneous injection containing flunixin (50 mg/50 kg/day ; Finadyne®, Schering-Plough, Ségé, France).

For the operation in which the full-thickness wounds were created, 0.01 mg/kg atropine was included as additional sedative and to loosen muscle tension. After
complete anesthesia was induced, animals were intubated and artificial respiration was applied. During operation, all vital functions were monitored and fluid loss was compensated by an intravenous infusion of Ringers solution. Anesthesia was antagonized with 0.005 mg/kg sufentanil (Sufenta®, Janssen-Cilag, Gent, Belgium) and postoperative analgesia was provided with 0.05 mg/kg buprenorphine (Temgesic®, Reckitt & Colman Products Ltd, Hull, UK), both administered intravenously.

One week before the operation, the hair was clipped from both flanks and the skin was disinfected with hibiscrub, 70% alcohol solution containing 0.6% chlorohexidin, and with 2% iodine solution. A grid was tattooed by cutting the skin with a scalpel till subepidermal depth and applying tattoo paste. On the day of the operation, the skin was disinfected as described above and full-thickness wounds (3.0 x 3.0 cm) were created on the back of the pigs using a dermatome. First split-skin mesh grafts (0.2 mm thick) were harvested from the wound sites, moistened in PBS, and expanded at a ratio of 1:3. The wounds were re-excised to a depth of 2.5 mm up to the subcutaneous fat layer. In total, 14 wounds per animal were created, which were grafted with the dermal substitutes and covered with the split-skin mesh grafts. The wounds were protected against dehydration and bacteria with a polyether urethane top layer (Exkin®, X-Flow, Twente, The Netherlands). Protection against mechanical trauma was achieved by wound coverage with one layer of hydrophilic gauzes (5x5 cm) fixed with adhesive tape, two layers of hydrophilic gauzes (20x20 cm) fixed with elastic adhesive tape from the back to the midriff/abdomen, and elastic stockings (Tubigrip). The pigs were housed individually and fed twice a day. At the moment of the operation the pigs weighed 40-45 kg and 6 weeks later 75-85 kg.

**Evaluation of wound healing**

The wounds were evaluated weekly. Wound contraction was followed by tracing the wound edges and the tattooed grid on transparent film. Wound contraction was measured by planimetry and expressed as percentage of reduction of original wound area and was corrected for the local growth of the animal. The final wound evaluation included a blinded score for three wound cosmetic parameters by two independent experienced observers. They scored on a 1-5 scale wound colour (pink - purple/red), smoothness and skin level of the epidermis, and wound suppleness or stiffness. After 1 and 3 weeks, 4 mm punch biopsies were taken from similar locations in each wound. After 6 weeks, a cross biopsy was taken from the middle of the wounds (0.5 x 4.0 cm). The biopsies were fixed in 4% formaldehyde PBS solution for at least 16 h at RT, and subsequently embedded in paraffin according to standard procedures.
**Immunohistochemistry, image analysis, quantification of dermal regeneration**

For immunohistochemistry, the 4% formaldehyde fixed wound biopsies were embedded in paraffin and sectioned at 5 μm. The sections were deparaffinized, hydrated through a graded series of ethanol solutions, and incubated for 30 min in 0.02% \( \text{H}_2\text{O}_2 \)/methanol solution. Sections were subsequently incubated at RT with the following four solutions: (1) PBS solution/10% AB serum for 15 min; (2) anti-\( \alpha \)-smooth-muscle actin monoclonal antibody (clone 1A4, Dako) diluted in PBS (1:100) or anti-elastin monoclonal antibody (1:1000, Sigma) for 60 min; (3) with biotinylated rabbit anti-mouse IgGs antibodies (1:400, Dako) diluted in PBS 10% AB serum for 30 min; (4) and with streptavidin-AB-complex/horseradish peroxidase (1:100, Dako) diluted in PBS for 30 min. Between these incubations, the sections were washed twice with PBS. Colour reaction was performed for 7 min in 50 mM Tris-HCl buffer (pH 7.8) containing 0.05% diaminobenzidine and 0.03% \( \text{H}_2\text{O}_2 \). Finally, the slides were washed in water, counter-stained with haematoxylin, mounted in glycergel (Dako) and examined with bright-field microscopy. Sections of normal porcine skin served as positive controls. As negative controls, adjacent sections of the wound biopsies were stained with nonimmune IgG from the same species in the same dilution as the primary antibody. No specific signal was noticed in the negative controls.

The dermal substitutes that were fixed on the day of operation were sectioned at 10 μm, deparaffinized, hydrated and stained for \( \alpha \)-smooth-muscle actin as described above, except that for the second and third steps were replaced with an incubation with goat anti-mouse IgG2a antibodies conjugated with Texas Red (Southern Biotechnology Associates, Birmingham, AL). To identify nuclei, adjacent sections were stained with a 5 μg/ml propidium iodide PBS solution for 15 min. Slides were washed in PBS and mounted in vecta-shield.

Image analysis of sections stained for \( \alpha \)-smooth-muscle actin was performed using white light, an infrared-blocking filter, and a low-magnification objective (2x). The sections of the dermal substitute, which were stained for \( \alpha \)-smooth-muscle actin and cell nuclei were analysed with a 10x objective. Images were recorded with a colour CCD camera (1024x1280@24 bits) attached to an Olympus AHTB3 microscope (Tokyo, Japan) and saved as a TIF file. The images were analyzed blinded with the Leica Qwin-colour analysis software package (Leica Imaging Systems Ltd., Cambridge, UK). For the \( \alpha \)-smooth-muscle actin staining, the total dermal area and the area with myofibroblasts were measured. Subsequently, the staining intensity in the area with myofibroblast was measured as the percentage of stained area relative to the total
positive dermal area. Colour selection was performed by exclusion of the highest colour intensities, resulting in the selective loss of most of the vascular staining. For an estimate of the numbers of cells in the dermal substitutes, images of fluorescent nuclei of one dermal substitute were merged together, giving a complete overview of the cross-sectioned dermal substitute. This image was analysed for the total number of fluorescent areas corresponding to the size of a nucleus.

Six weeks post-wounding, the maturation of collagen fibres was analysed with polarized light in H&E stained wound sections. The thickness of the regenerated tissue was measured at 4 different places at similar intermediate distances with a microscope with built-in calliper. The area with mature collagen fibres was expressed as the percentage of the total regenerated dermal tissue. The elastin stainings at six weeks were measured and analysed as described above using bright-light microscopy.

**Statistical analysis**

The different treatments were all applied at one side of each animal in a randomized fashion. The treatments were tested for significant differences with a paired Student's t-test, except for the evaluation of cosmetic parameters, for which the non-parametric Wilcoxon signed rank test was used. Correlation and correlation significance were tested with the Spearman Rank correlation test. $P<0.05$ was considered to be statistically significant.

**RESULTS**

![Figure 1](image.png)

Figure 1. Appearance of wounds treated with ADS (acellular dermal substitute) (A) and with 10-DS50 (dermal substitute seeded with $5 \times 10^5$ fibroblasts/cm$^2$, precultured for 10 days) (B), six weeks post-wounding. The ADS-treated wound had contracted more, showed a rougher skin surface, and was not uniform in wound colour (red areas). The colour of the 10-DS50-treated wound was almost comparable to the surrounding skin.
Macrosopic evaluation of wound healing

For all wounds the epithelium out-growth from the split-skin mesh grafts was similar and the wounds closed within two weeks. The wound colour changed from initially purple/red to pink, a colour comparable to the surrounding skin. The speed of wound-colour transition was different for the treatment groups. In general, the wounds treated with the cultured dermal substitutes (10-DS10, 10-DS50) and dermal substitutes seeded with the highest concentration of fibroblasts (0-DS50) became sooner pink. In addition, the wound tissue was more supple, and the epidermal upper layer was smoother compared to treatment with the acellular dermal substitute (ADS; controls). Representative examples for the ADS (A) and 10-DS50 (B) treatments are shown in figure 1.

Six weeks post-wounding, these different parameters (wound colour, skin elasticity, and surface smoothness) were scored blinded on a scale of 1-5 (good - worse) by two experienced observers. The scores were totalled for each wound, and averaged per treatment group. The best score for a wound was 3, whereas the wounds which healed the worst could have a maximal score of 15. The latter, however, did not occur, and the maximum score given for a wound was 12. The average scores for the different treatment groups are shown in figure 2A. The 10-DS50 and 0-DS50 treatments showed a significant improvement in cosmetic appearance compared to the ADS treatment (p<0.03, unpaired Wilcoxon rank test). The cosmetic parameters of wounds treated with the 0-DS10 were comparable to those of wounds treated with ADS.

Figure 2. The average cosmetic scores and average wound contraction per treatment after 6 weeks of healing. A. The 0-DS50 and 10-DS50 wounds scored significantly better for cosmetic appearance compared to those treated with ADS (*p<0.04). B. The 10-DS50 treatment significantly diminished wound contraction compared to the ADS treatment (*p<0.05). SD is indicated by bars.
The percentage of contraction was determined as reduction in original wound area and was corrected for the local growth of the pigs. Wound contraction started after 5 to 7 days, was most pronounced for another two weeks, and continued slowly or was arrested after 3 weeks. After 6 weeks, the wound contraction for the 10-DS50 treatment was 24.8 (SD±13.4%) and was significantly reduced compared to the ADS treatment with an average contraction of 36.4 (SD±11.4%) (Fig. 2B).

**Histological observations after one and three weeks of healing**

After 1 and 3 weeks, 4 mm biopsies were taken from the wounds. One week post-wounding, most wounds showed a completely infiltrated dermal substitute in the H&E stained wound sections. Although the cell densities varied, this could not be attributed to one specific treatment. After 3 weeks, a clear difference in cell density was observed between treatments. For the cultured dermal substitutes 10-DS10, 10-DS50 and 0-DS50, the granulation tissue had not only a lesser cell density but it also contained more ECM in between the cells when compared to the ADS and 0-DS10 treatments. In figure 3, this is illustrated for the ADS (A and C) and the 10-DS50 treatments (B and D). In addition, the thickness of the granulation tissue was especially thick in wounds which showed the most contraction. Furthermore, in the middle of the granulation tissue of the ADS and 0-DS10 treatments and to a lesser extent for the 0-DS50 and 10-DS10 treatments an area in the section was observed with cells orientated parallel with the epidermis. This area was absent in the wounds treated with 10-DS50.

Staining of the sections for myofibroblasts with the marker α-smooth-muscle actin showed that this typical area contained high numbers of myofibroblasts. Comparing the acellular treatment ADS (Fig. 4A) to the cultured dermal substitute treatment 10-DS50 (Fig. 4B), a remarkable difference was observed: the 10-DS50 treatment showed strong vascular staining but was negative for myofibroblasts. Six out of seven 10-DS50 wounds did not stain for α-smooth-muscle actin containing myofibroblasts at all 3 weeks post-wounding. Using image analysis, the positive area with exclusion of most of the vascular staining was measured and expressed as the percentage of total dermal area (Fig. 4C). In this area, the intensity of the staining was also determined and shown in figure 4C as open bars. The area with myofibroblasts positive for α-smooth-muscle actin was significantly reduced for the cultured dermal substitute treatments (10-DS10 and 10-DS50, p<0.001) and the 0-DS50 treatment (p<0.04) compared to the acellular treatment (ADS). In addition, as the size of the positive area diminished, the intensity of the staining in the area also decreased. The
percentage dermal area containing myofibroblasts correlated significantly with the percentage of wound contraction after 6 weeks of healing (correlation coefficient of 0.72, p<0.001, Spearman Rank correlation test).

**Figure 3.** Granulation tissue of wounds treated with ADS (A, C) and 10-DS50 (B, D) substitutes, 3 weeks post-wounding. In the ADS-treated wounds more granulation tissue was formed with higher cell densities and less deposition of ECM than in the 10-DS50-treated wounds. Underlying subcutaneous fat is indicated with arrowheads and is not visible in A due to the thickness of the granulation tissue. A and B are shown at identical magnifications (x 55), C and D show a 2.5-times higher magnification of the granulation tissue in figure A and B, respectively.

**Figure 4.** Alpha-smooth muscle actin staining for the detection of myofibroblasts in the granulation tissue, 3 weeks after wounding. In the granulation tissue of ADS-treated wounds (A) a clear area with myofibroblasts was detected, whereas in the 10-DS50 wounds only smooth muscle cells in vascular structures stained positive (B). (C) Percentage of dermal area with myofibroblasts determined with image analysis. Open bars indicate the intensity of the staining in the areas positive for myofibroblasts. The wounds treated with 0-DS50, 10-DS10, and 10-DS50 had significantly less dermal area containing myofibroblasts compared to the ADS treated wounds (p<0.04). SD is indicated by bars.
Histological observations after six weeks of healing

After 6 weeks of healing, tissue (1 cm in width) was dissected from the middle of the wound, to give a complete view of the regenerated tissue in between the wound edges. The observed acceleration of ECM deposition in the wounds treated with 10-DS10, 10-DS50 and 0-DS50 dermal substitutes at 3 weeks resulted in a regenerated tissue with thicker collagen bundles organised with a basket weave pattern as in normal skin at 6 weeks (Fig. 5B, D). This regenerated dermal tissue could only be distinguished from normal skin by a slightly increased cell density and the presence of more vascular structures. In the wounds treated with ADS and 0-DS10, a granulating area in the middle of the regenerated dermal tissue was still present. This area (Fig. 5A (in between arrowheads), C) was characterised as immature granulation tissue with a high cell density and with thin collagen bundles having a preferential orientation parallel with the epidermis. This is typical of scar tissue.

Figure 5. Regenerated dermis of wounds treated with ADS (A, C) and 10-DS50 (B, D) substitutes, 6 weeks post-wounding. Regenerated dermis of the ADS-treated wounds contained more cells and especially in the middle of the dermis (area between arrowheads) had less remodelled ECM than the 10-DS50-treated wounds. (C) and (D) 2.5-times higher magnification of insets in A and B, respectively, clearly demonstrating in the ADS treated wound preferential organisation of cells and ECM parallel with the epidermis. Original magnification of figure A and B: 35x
From investigations with polarized light on the maturation of the collagen bundles, it was evident that this area contained little to no mature collagen bundles (Fig. 6A, between arrowheads). The regenerated dermis of the wounds treated with 0-DS50, 10-DS10 and 10-DS50 (Fig. 6B) showed almost only mature collagen bundles.

Figure 6. Polarized light image identifying mature collagen bundles in the regenerated dermis of wounds treated with ADS (A) and 10-DS50 (B) substitutes, 6 weeks post-wounding. In the middle of the regenerated dermis of the ADS-treated wounds, an area with immature collagen bundles was present. This area was absent in the dermis of the 10-DS50 treated wounds, indicating faster regeneration and remodelling of the ECM in these wounds (bar = 160 μm). (C) For each group, the average percentages of dermal area containing mature collagen bundles is shown. The wounds treated with 0-DS50, 10-DS10, and 10-DS50 had significantly more dermal area with mature collagen bundles than the ADS-treated wounds ($p<0.05$). SD is indicated by bars.
Elastin staining also identified an area in the middle of the regenerated tissue negative for elastin (Fig. 7A and 7B, area between arrowheads), which was larger for the ADS treatment (Fig. 6A) than for the 10-DS50 treatment (Fig. 6B). The treatments 0-DS50, 10-DS10 and 10-DS50 showed significantly higher percentages of dermal area with mature collagen bundles and elastin in the regenerated tissue than the ADS treatment (Fig. 6C and 7C).

Figure 7. Elastin staining of the regenerated dermis of wounds treated with ADS (A) and 10-DS50 (B) substitutes, 6 weeks post-wounding. In the area between arrowheads elastin staining is absent. For the ADS treated wound this area was clearly larger than for the 10-DS50 treated wounds (bar = 260 μm). (C) For each group the average percentages of dermal area containing elastin staining is shown. The wounds treated with 0-DS50, 10-DS10, and 10-DS50 had significantly more dermal area with elastin staining than the ADS-treated wounds (**p<0.05, unpaired t-test; *p<0.05, paired t-test). SD is indicated by bars.
**Fibroblast density measurements in cultured dermal substitutes**

Additional prepared dermal substitutes with fibroblasts, which were fixed on the day of the operation (n=5 for each treatment group), were stained for nuclei with propidium iodine. The total fibroblast numbers were estimated with image analysis. The average number of fibroblasts found for the 0-DS10 substitute \(1.05 \times 10^6\) (SD±0.35) corresponded to the number of fibroblasts seeded in the substitute 4 to 16 h earlier. However, for the 0-DS50 substitutes a lower number \(4.1 \times 10^6\) (SD±0.75) was found than was seeded initially. Of the cultured substitutes, the 10-DS10 substitutes contained \(4.9 \times 10^6\) (SD±1.54) fibroblasts and the 10-DS50 substitutes \(7.5 \times 10^6\) (SD±1.26). Figure 8 illustrates the cellular distribution throughout the different dermal substitutes. The 0-DS10 and 0-DS50 showed fibroblasts attached to matrix fibres throughout the dermal substitute but with higher densities in the upper part. In the cultured dermal substitutes, fibroblasts were distributed more evenly indicating that they had migration into the dermal substitute. Staining of the dermal substitutes for α-smooth-muscle positive myofibroblasts showed variable percentages of positive fibroblasts which varied more between the different pigs than between the different dermal substitutes. The percentages of myofibroblasts were never higher than 30%. In addition, the cultured dermal substitute surface was somewhat reduced by contraction, i.e. 3% (SD±3%, n=12) for the 10-DS10 substitutes, and 6% (SD±4%, n=12) for the 10-DS50, and some deposition of ECM molecules was observed (not shown).

**Discussion**

Only a few studies investigated the influence of fibroblasts in skin substitutes on dermal regeneration in a comparative fashion taking into consideration wound contraction and other histological evaluation criteria. Murphy *et al.* (1990)\(^{34}\) showed various positive effects on wound healing and dermal regeneration with a cultured collagen-glycosaminoglycan substitute containing autologous fibroblasts and keratinocytes in a guinea pig model, but the authors used open non-treated wounds as controls. Boyce *et al.* (1991)\(^{35}\) used athymic nude mice to compare collagen-glycosaminoglycan substitutes with cultured human keratinocytes with and without human fibroblasts. For the wounds treated with substitutes with fibroblasts, they found positive effects on epidermal regeneration, but they did not see any improvement
on wound contraction and they did not investigate the quality of the regenerated tissue. In a guinea pig dermal wound model, Marks et al. (1991) showed that the addition of fibroblasts to a collagen sponge (2x10⁴ cells/cm², cultured for 1 week) accelerated the ultimate tensile strength of the wounds and the fibroblasts significantly retarded the collagen sponge biodegradation. However, up to the last time point of evaluation (30 days) they did not observe any positive effects on epithelialization or on collagen fiber diameter and orientation. In addition, the animal models used might not be very relevant to the human situation, since rodent skin does not have the same architecture as human skin. The porcine model we used is a more suitable model to study dermal regeneration.

Figure 8. Distribution of fibroblasts on the day of operation within the seeded and pre-cultured dermal substitutes stained with propidium iodide to identify cell nuclei. Original magnification: 45x.
In the past, the amplification of keratinocytes was considered to be the limiting step for a cultured skin graft. Improved culture methods have made this more realizable. In addition, thin split-skin harvest methods have reduced the risk of creating cosmetically unattractive donor-sites. However, if for the creation of a cultured composite graft high numbers of fibroblasts are also required, the need for more donor-site tissue is likely to be associated with deeper donor-site defects. To overcome this problem, we investigated subcutaneous fat as a fibroblast source. In a single-stage operation, subcutaneous fat was harvested, fibroblasts isolated, disposed of vascular fragments, and seeded at a density of 1x10^6 cells/cm^2 in our dermal substitutes before they were implanted in full-thickness wounds. These dermal substitutes were able to improve tissue regeneration and healed with significantly less wound contraction (24.5% (SD=3.4%, n=13)) than to the wound contraction using acellular dermal substitutes (35.5% (SD=4.8%, n=15)). In spite of these promising results, augmenting the number of freshly isolated subcutaneous fibroblasts in the dermal substitute instead of cultured dermal fibroblasts reversed the positive effects observed. This was probably caused by impurities in the freshly isolated subcutaneous fibroblast population, e.g. inflammatory cells and collagen fragments (unpublished data).

The concept of a dermal equivalent seeded with fibroblasts seems to be a simple one. However, one has to consider that the fibroblast environment in a dermal equivalent may result in phenotypic changes, which might negatively influence the dermal regeneration process after transplantation. Fibroblasts can differentiate into a contractile phenotype, the myofibroblast, capable of rapid matrix contraction, or they might exhibit increased proteolytic activity once seeded in a dermal substitute. In general, these processes are undesirable at the moment of skin grafting but may also be used to advantage. In the case of Dermagraft, the pre-culture of fibroblasts in the vicryl mesh caused degradation and replacement of the substitute material with fibroblast-synthesized ECM. This resulted in a reduction in the inflammatory reactions occurring in vivo with hydrolysis of the vicryl material. Another point to consider is that prolonged culture periods could also result in a filling of the pores of the dermal substitute. This could eventually inhibit graft vascularisation and/or interfere with epidermal graft survival due to limited wound-fluid exchange and reduced diffusion.
of nutrients to the keratinocytes. The acellular dermal substitute used in the present study allowed fast vascularisation\textsuperscript{42} and the pre-culture of fibroblasts in the dermal substitute did not impede this. In comparison to the total amount of dermal substitute, the amounts of ECM deposited by the fibroblasts in the 10-DS10 and 10-DS50 substitutes were low and the contribution of these proteins to the observed accelerated dermal regeneration is likely to be small. Moreover, in vitro studies with substitutes cultured under similar circumstances but seeded with $1\times10^6$ fibroblasts/cm$^2$ and cultured for 7 days showed a decrease of 9% in total hydroxyproline content, indicating some degradation of the substitute.\textsuperscript{43} In addition, in vitro we observed some reduction in the areas of the 10-DS10 and 10 DS50 substitutes (3% and 6%, respectively). Staining for $\alpha$-smooth-muscle actin positive myofibroblasts in the dermal substitute showed a variable percentage of positive cells which varied most between animals and not so much between instantaneously seeded or pre-cultured dermal substitutes. This was previously observed by Desmoulière \textit{et al.} (1992)\textsuperscript{44} in different fibroblast populations cultured on plastic (passages 4 and 5). Normally, the myofibroblast characteristics are lost during culture\textsuperscript{45}, but the number of passages used in our present study (p4-p6) is probably too low to achieve this.

The improved tissue regeneration with the cultured dermal substitutes seems to be correlated with the higher numbers of fibroblasts present in the dermal substitutes at the moment of transplantation. This is reinforced by similar fibroblast numbers found for the 0-DS50 and 10-DS10 treatments and their comparable wound-healing results. For the 10-DS50 treatment the fibroblast numbers only doubled during the 10-day culture period. Moreover, after the seeding of $5\times10^5$ fibroblasts/cm$^2$ (0-DS50) almost 20% of the initial cell numbers was lost and during the culture period fibroblasts migrated out of the dermal substitute. Therefore, it is not advisable to culture such high cell numbers in a dermal substitute for 10 days. In order to minimize the loss of fibroblasts, one could seed lower cell numbers several times and culture them for a shorter period. In addition, it is also possible that higher cell numbers than found in the 10-DS50 substitutes improve dermal tissue regeneration even more and reduce wound contraction further.

The culture period could be important for the fibroblasts to adapt to their new environment and to firmly attach to the substrate. In this, the elastin coating of the
dermal substitute might be important. Fibroblasts are able to adhere strongly to elastin and receptor binding alters the cell metabolism. Furthermore, elastin is capable of modulating proteolytic activity and elastin peptides stimulate the growth of skin fibroblasts. Moreover, we previously showed that the elastin component and fibroblast seeding retarded biodegradation of the substitute.

Three weeks after healing, the wounds treated with 10-DS50 showed almost no staining for myofibroblasts positive for α-smooth-muscle actin. At this time point, the 10-DS50 wounds already had contracted by 20% and it can not be excluded that myofibroblasts were present during the first two weeks of healing. However, our definition of wound contraction is the percentage of original wound area after being corrected for local growth of animal. If the latter correction was not made, the 10-DS50 wounds showed no contraction of the original wound area and the 20% wound contraction could then also be considered to represent retarded growth of the wound tissue. The remarkable inhibition of fibroblast differentiation might be caused by several factors, e.g. reduced inflammatory signals and proteolytic wound activity. Nevertheless, apart from several growth factors, it is still not clear how components in the direct surroundings of fibroblasts induce myofibroblast differentiation. We believe that the early presence of fibroblasts in the dermal substitute might reduce the need of fibroblasts to migrate into the wounds from the subcutaneous tissue. Concurrently, it might be that migrated fibroblasts encounter more activating signals which render these fibroblasts more susceptible to differentiation.

After three weeks, a typical area appeared in the middle of the granulation tissue of wounds which healed with more wound contraction. This area was characterized by high cell densities, preferential cell organisation parallel with the epidermis, and being positive for α-smooth-muscle actin. The existence of this area seemed to correlate with thicker layers of granulation tissue, which in part can be explained by the increased contraction of the wound tissue. This typical area remained present until six weeks after wounding, by which time most cells had disappeared and were replaced by thin immature collagen bundles, again organised parallel with the epidermis. This area remained negative for elastin and might evolve into scar tissue. With the 10-DS50 treatment, this typical area was not observed, and after 6 weeks the regenerated dermis consisted almost completely of randomly organised mature
collagen bundles. Although significantly more elastin was present in the regenerated tissue of the 0-DS50, 10-DS10 and 10-DS50 wounds than in the ADS wounds, the elastin fibres were not yet completely regenerated. However, this observation may be due to the relatively short period of our evaluation.

In conclusion, we demonstrated that the cosmetic results of wound healing correlated with histological observations. Wounds with more myofibroblast formation healed with more contraction, showed more scar formation, were not supple, and remained more reddish in colour. The best healing was observed with a dermal substitute seeded with a high number of fibroblasts and cultured for 10 days prior to grafting. Furthermore, the accelerated and improved regeneration of dermal tissue was correlated to the higher number of fibroblasts present in the dermal substitute at the moment of grafting.

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