Allogeneic fibroblasts in dermal substitutes induce inflammation and scar formation

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In the present study, we compared the use of autologous versus allogeneic fibroblasts in dermal skin substitutes in a porcine wound model. The allogeneic fibroblast populations were isolated from female and a male pig (allo-1, - 2 and - 3) and the controls, autologous fibroblasts, from female graft-recipient pigs (control). The histocompatibility of the three donor pigs with the recipient pigs was determined with a mixed lymphocyte reaction. In two pigs, full-thickness wounds were treated with the fibroblast-seeded dermal substitutes ($n = 5$ per animal) and immediately overgrafted with meshed split-skin autografts. After 6 weeks, wound contraction was measured by planimetry and scar formation was scored. At 2, 4, and 6 weeks biopsies were taken and evaluated for the presence of inflammatory reactions, myofibroblasts, and scar formation. The mixed lymphocyte reaction of both recipient pigs showed the highest responses on peripheral blood mononuclear cells of the allo-3 donor pig, and was low or negative for allo-1 and allo-2. In all ``allogeneic'' wounds, more inflammatory cells were observed over time along with inflammatory foci consisting of a mix of lymphocytes and granulomatous cells. After 4 weeks, myofibroblasts were absent in the control wounds, whereas in ``allogeneic'' wounds, myofibroblasts colocalized with inflammation foci. The final scar tissue of the ``allogeneic'' wounds showed granulating areas with thin, immature collagen bundles. In contrast, the control wounds showed a dermal tissue with mature collagen bundles organized randomly like in normal skin. The wounds treated with allo-3 fibroblasts showed in both pigs a significant increase in scar formation and wound contraction when compared with control wounds. In conclusion, for optimal restoration of dermal skin function with minimal scar formation, skin substitutes containing autologous fibroblasts are preferred over skin substitutes with allogeneic fibroblasts. **(WOUND REP REG 2002;10:152–160)**

Since the creation of the first living bilayered skin substitute by Bell et al.,[1] much effort has been put into creating an "off-the-shelf" skin replacement. The most practical choice would be a replacement using allogeneic cells. The advantages of allogenic cells over autologous cells are reduced patient donor sites, decreased operating time, and avoidance of a delay in wound treatment caused by the time required for autologous cell isolation and multiplication.[2,3] During the past two decades, attention was mainly focused on cultured keratinocyte grafts and their application on burns and chronic ulcers.[4] The results from these studies showed that keratinocyte allografts could accelerate healing and decrease pain of both split-thickness skin graft donor sites and chronic ulcers.[5,6] In

DMEM	Dulbecco's modified Eagle's medium
FCS	Fetal calf serum
H&E	Hematoxylin & eosin
HLA	Human leukocyte antigen
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buered saline solution

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addition, it was shown that allogeneic keratinocytes are replaced by autologous keratinocytes.[7,8] However, controversy still exists as to what length of time allogeneic keratinocytes survive.[3] In immune-competent animals, keratinocyte survival appeared to be relatively short (<1 week)[9] whereas in patients with large burns, a degree of immunosuppression exists that could favor prolongation of allogeneic cell survival.[10,11] For adequate skin substitution, both skin components, dermis and epidermis, are needed. Previously, we have demonstrated that a dermal substitute in combination with split-skin mesh grafts is able to improve the quality of scar tissue compared with split-skin mesh graft treatment alone.[12,13] In developing a dermal equivalent, there are several reasons to include fibroblasts. The presence of fibroblasts in a dermal equivalent not only stimulates keratinocyte outgrowth, differentiation, and basement membrane regeneration,[14–16] but fibroblasts also accelerate and improve the quality of dermal tissue regeneration.[17,18] The autologous fibroblasts seeded in our dermal substitute survived and proliferated after in vivo transplantation,[19] and we have shown the existence of a correlation between the number of fibroblasts present in the dermal substitute and the prevention of scar tissue formation.[20] Important questions remain to be answered, e.g., Can allogeneic fibroblasts replace autologous fibroblasts, and is the quality of the regenerated dermal tissue as good as when autologous fibroblasts are used? This is clinically also important because skin substitutes containing allogeneic fibroblasts have become more commercially available.[21]

In the literature, data are not conclusive regarding immunogenicity of human allogeneic fibroblasts,[21–25] and it is unclear whether they are able to persist in the newly regenerated skin or are replaced by host cells without adverse effects on tissue regeneration.[2] In most clinical studies, it is difficult to evaluate the effects of allogeneic fibroblasts due to the small number of patients evaluated, the multiple wound variables, and the lack of histological data and controls. Therefore, we used the porcine full-thickness wound model to compare the use of allogeneic fibroblast populations in dermal substitution with the use of autologous fibroblasts. For the allogeneic fibroblast populations, we chose two cell populations of female and male origin (allo-1 and -2, respectively) genetically closely related to the recipient animals and one fibroblast population of female origin (allo-3) that was less closely related. As an indication, the genetic relation between donors and recipients was investigated with mixed lymphocyte reactions (MLRs). We investigated inflammatory reactions in the granulation tissue, stained for the presence of myofibroblasts, and assessed the quality of the regenerated dermal tissue.

MATERIALS AND METHODS

Heparinized peripheral blood from pigs, from which the different fibroblast populations were isolated, was layered on a density gradient (Lymphoprep, Nycomed, Oslo, Norway) and centrifuged to remove erythrocytes and granulocytes. The interface was washed three times with phophate buffered saline solution (PBS) containing 1% bovine serum albumin (BSA) to remove thrombocytes. The time-point of isolation was different for each pig. Peripheral blood mononuclear cells (PBMCs) were cryopreserved in fetal calf serum (FCS) containing 10% dimethyl sulfoxide in liquid nitrogen until used. To measure the MLR response, 2×10^4 allogeneic PBMCs were incubated with 2×10^4 autologous PBMCs for 6 days in round-bottom 96-well plates. Cells were cultured in 200 μl Dulbecco's modified Eagle's medium (DMEM) media containing 10% FCS and 50 μM β-mercaptoethanol. The number of proliferating cells was measured by addition of 0.3 μCi ^{3}H-thymidine (Amersham, Aylesbury, UK) to each well 16 hours before harvesting. Incorporation was measured by liquid scintillation counting in average disintegrations per minute. As controls, 2×10^4 PBMCs of each animal were incubated alone to measure background proliferation.

Preparation of dermal substitute grafts

The allogeneic and autologous fibroblasts were isolated from skin biopsies taken from the backs of the Yorkshire pigs (for the autologous fibroblasts 3 weeks before transplantation). Two allogeneic fibroblast populations were obtained from a female and male pig (allo-1 and allo-2) of the same breeding strain as the recipient animal from which the autologous fibroblasts (control) were isolated. The third allogeneic fibroblast population (allo-3) was isolated from a female pig of a different breeding strain. From all skin biopsies, the epidermis was cut off and dermal tissue was minced and digested for 1–2 hours at 37 °C in PBS containing 0.25% (w/w) collagenase A and dispase (Boehringer Mannheim, Mannheim, Germany; 1 ml/biopsy). The digest was sieved over a 70-μm cell strainer (Falcon, Becton Dickinson, Mountainview, AL), washed in culture media, and subsequently cultured in DMEM supplemented with 10% (v/v) FCS, 1 mM L-glutamine and antibiotics (penicillin [100 IU/ml] and streptomycin [100 μg/ml]), all from Life Technologies, Breda, The Netherlands. Fibroblasts were passaged with PBS solution containing 0.25% trypsin and seeded in the dermal substitute the day before operation at an identical density (5×10^5 fibroblasts/cm^2) between passage 4 and 6. All fibroblast populations used had been frozen and thawed from passage 2 to 3. The dermal substitutes (4 × 4 cm) were seeded separately by inoculating the upper side of premoistened matrices with culture medium containing

the fibroblasts (1 ml/10 cm^2 of substitute). The seeding volume was absorbed by the substitute. The dermal substitute was a freeze-dried non-cross-linked native bovine collagen matrix (isolated from bovine skin) coated with a 3% (w/w) -elastin hydrolysate (80% MW 60 kDa and 20% MW 200 kDa from bovine ligamentum nuchae) and was kindly provided by Dr. Suwelack Skin & Healthcare (Billerbeck, Germany).[12] For each group extra substitutes were prepared, which were fixed on the day of operation in 4% paraformaldehyde PBS solution for 16 hours at room temperature.

Operation, grafting, and bandaging procedures

The Committee of Animal Welfare of the University of Amsterdam approved the protocol and two female Yorkshire pigs (A and B; 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 (3–5 L/min). Anesthesia was maintained with 1–2% isoflurane and the same air mixture. If necessary, postoperative pain was treated with a subcutaneous injection containing flunixine 50 mg/50 kg/day (Finadyne®, Schering-Plough, Segré, France).

For the operation, in which the full-thickness wounds were created, 0.001 mg/kg atropine was included as additional sedative. After complete anesthesia was induced, animals were intubated and artificial respiration was applied. During the procedure, vital functions were monitored and fluid loss was compensated by an intravenous infusion with Ringers solution. This 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 dorsal 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 to the subepidermal depth and applying tattoo paste. On the day of operation, the skin was disinfected as described above and full-thickness wounds (2.5 × 2.5 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 the subcutaneous fat layer. The wounds were grafted with the fibroblast seeded dermal substitutes and covered with the split-skin mesh grafts. Each treatment group was applied five times on both animals in a randomized fashion. The wounds were protected against dehydratation 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 gauze (5 × 5 cm) fixed with adhesive tape and two layers of hydrophilic gauze (20 × 20 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 30–35 kg and after 6 weeks, 70–75 kg.

Macroscopic evaluation of wound healing

Wounds were evaluated weekly for 6 weeks. Wound contraction was measured by computerized planimetry, 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 to asses the quality of the scar. Two independent experienced observers scored three parameters. They scored on a 1–5 scale for wound color (pink to purple/red), smoothness and height of the scar tissue, and scar suppleness or stiffness (elasticity/pliability). The overall score ranged from 3 (like normal skin) to 15 (excessive scarring).

Histological analysis of wound healing

After 2 and 4 weeks, a 4-mm punch biopsy was taken from each wound from an identical location at the interstices of the meshed split-skin graft and after 6 weeks, a cross biopsy from the middle of the wounds was also taken (0.5 × 3–4 cm). The biopsies were fixed in 4% formaldehyde PBS solution for at least 16 hours at room temperature, and subsequently embedded in paraffin according to standard procedures. Hematoxylin and eosin (H&E) stains were used to visualize cell infiltration and dermal architecture (microscopic anatomy). In each biopsy, the degree of inflammatory response was scored on a 0–5 scale (0 indicating a few lymphocytes/granulocytes and 5 high numbers of inflammatory cells and presence of localized foci of mixed lymphocytes and granulomatous cells).

Myofibroblasts were identified with immunostaining for α-smooth muscle actin. Staining of the sections was performed according to standard immunohistochemical methods as previously described.[20] Six weeks postwounding, dermal architecture was analyzed for the presence of nonremodeled amorphous extracellular matrix, cellularity, and collagen bundle orientation (random like in normal skin or parallel with the epidermis as in scar tissue). The maturation of collagen fibers was investigated with polarized light in H&E stained wound sections.

Statistical analysis

The different treatments were applied five times on each animal in a randomized controlled design. In both animals, the treatments were tested for significant differences ($p < 0.05$) intra-individually with a paired two-tailed Students t-test, apart from the scar evaluation for which significance was tested with the nonparametric Wilcoxon signed rank test.

RESULTS

Figure 1 shows the MLR response of the PBMCs of the recipient pigs (A and B) on the different allogeneic PBMC populations. The allo-3 pig, which was genetically the least related to the recipient pigs, showed the highest response, i.e., 4–6 times higher than background values. The MLR response with the allo-1 and - 2 PBMCs was relatively low. Only the MLR with PBMCs of pig A and the allo-2 pig induced some response, almost three times higher than background values. The figure shown is a representative example of three independent experiments.

Scar evaluation and contraction six weeks postwounding

The wound appearance of each treatment group ($n = 5$) is shown 6 weeks postwounding (Figure 2). The wounds treated with the dermal substitute seeded with autologous fibroblast, control treatment (A), were most similar to normal skin. The wounds treated with the dermal substitutes seeded with allogeneic fibroblasts populations (allo-1, B; allo-2, C; allo-3, D) had a mixed red/pink color and a more uneven skin level with stiffer fibrotic wound areas. The wound treated with the substitute seeded with allo-3 fibroblasts (D) had contracted the most. Scar evaluation in both animals showed that the allo-3 fibroblast seeded substitutes ($n = 5$ per animal) significantly worsened the cosmetic outcome of wound healing (Figure 3A). In Figure 3(B), the average wound contraction of each group is shown in both pigs. In pig A, both substitutes seeded with allo-2 and - 3 fibroblasts and in pig B, substitutes seeded with the allo-3 fibroblasts induced significantly more wound contraction compared with treatment with the dermal substitute seeded with control fibroblasts.

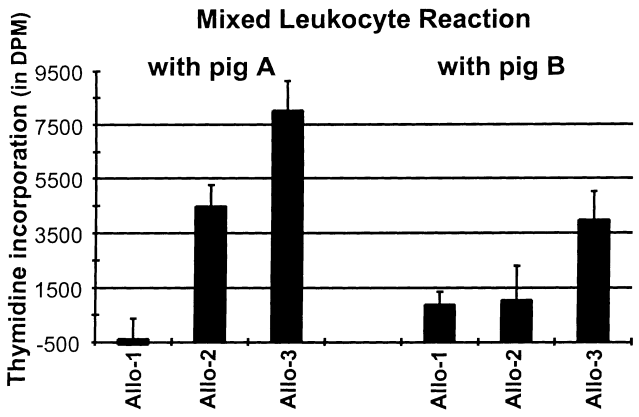

FIGURE 1. Mixed lymphocyte reaction responses of PBMCs of pig A and B on PBMC's of the allogeneic pigs, allo-1 to -3. The data shown are a representative example of 3 independent experiments and the mean values ($n = 4$) were corrected for the sum of the background counts of the two corresponding single PBMC populations and were never higher than 1500 dpm.

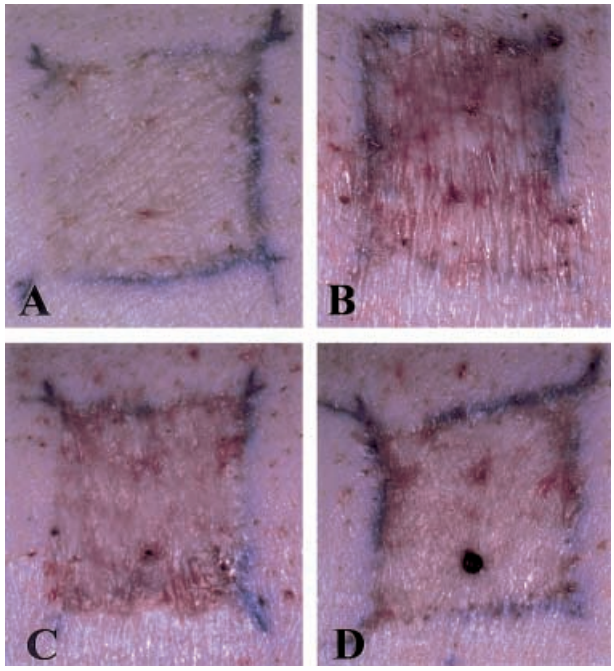

FIGURE 2. Cosmetic appearance of wounds 6 weeks after grafting. The wound treated with a substitute seeded with autologous fibroblasts, control treatment (A) most resembled normal skin. The wounds of the other groups, allo-1 (B), allo-2 (C) and allo-3 fibroblasts (D), still had red and fibrotic wound areas. The wound treated with the substitute seeded with allo-3 fibroblasts (D) had contracted the most.

Histological observations and inflammatory responses during healing

In Figure 4 a representative example of the granulation tissue of each treatment group is shown 2 weeks postwounding. The granulation tissue of the wounds treated with substitutes seeded with control fibroblasts (Figure 4, A and B) showed the presence of some diffusely distributed inflammatory cells, which is normal for wounds in this stage of healing. In contrast, the wounds treated with substitutes seeded with allo-1 (Figure 4, C and D), allo-2 (Figure 4, E and F) and allo-3 (Figure 4, G and H) fibroblasts showed a marked increase in the number of inflammatory cells and the presence of localized mixed lymphocyte and granulomatous cell responses (inflammatory cell foci).

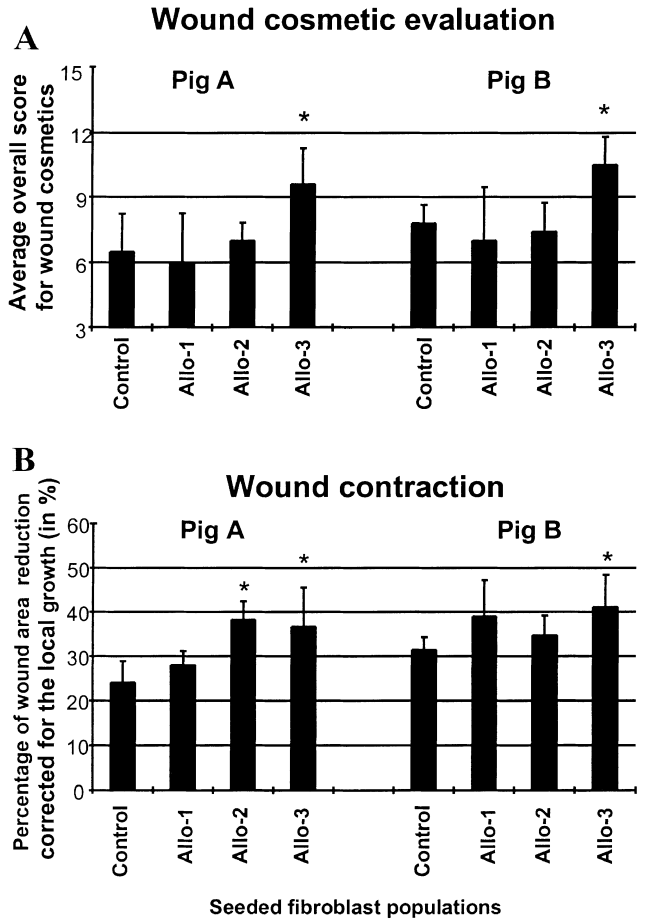

FIGURE 3. Average scar quality scores (A) and wound contraction (B) of the different treatments ($n = 5$) 6 weeks after grafting. The allo-3 fibroblasts significantly increased scar formation compared to control fibroblasts (*$p < 0.05$, Wilcoxon signed rank test). The allo-3 fibroblasts induced in both pigs and the allo-2 fibroblasts induced in pig A significantly more wound contraction when compared to treatment with control fibroblasts (*$p < 0.05$, paired *t*-test).

Furthermore, in these wounds the epidermis appeared to be overstimulated, which is illustrated by thicker and larger rete-ridges as compared with control wounds. In time, the granulation tissue progressively matured and the number of lymphocytes and granulocytes diminished. In the wounds treated with substitutes seeded with control fibroblasts, inflammatory cell foci were never observed, however. A semiquantitative evaluation (not shown) showed that a number of inflammatory cells in the wounds treated with allogeneic fibroblasts never subsided to the level found in control wounds.

Presence of myofibroblasts in the granulation tissue

Two weeks after grafting, myofibroblasts were present in the granulation tissue of all wounds. In wounds treated with the control fibroblasts, localized areas with myofibroblasts were present in the middle of the granulation tissue. In wounds treated with the allogeneic fibroblast populations, areas with myofibroblasts were found throughout the granulation tissue and often colocalized with inflammatory cell foci. Four weeks after grafting, the myofibroblasts positive for α-smooth muscle actin were no longer detected in the granulation tissue of the control wounds (Figure 5A). However, in the allogeneic wounds, areas with myofibroblasts were still present, mainly colocalized with inflammatory cell foci (Figure 5B).

Dermal tissue regeneration six weeks postwounding

In the wounds treated with control fibroblasts, the regenerated dermal tissue at six weeks showed randomly organized collagen bundles as in normal skin (Figure 6, A and B). Moreover, using polarized light, in which mature collagen bundles reflect the light but immature bundles do not, all collagen bundles turned out to be of a mature nature (Figure 6C). In contrast, in the allogeneic fibroblast-treated wounds the regenerated tissue contained areas that were still granulating (Figure 6, D–L). These areas were characterized by higher cell numbers (Figure 6B, E, H, and K [higher magnification of insets]) and thin, immature collagen bundles with a tendency to be organized parallel with the epidermis, which is typical for scar tissue (Figure 6 F, I, and L). In addition, after 6 weeks the allogeneic wounds contained inflammatory cell foci (arrowheads in Figure 6D, G, and J).

DISCUSSION

The present study was designed to establish whether allogeneic fibroblast populations are able to stimulate dermal tissue regeneration without inducing adverse inflammatory reactions. Although the number of animals used in this study was low, in one animal the number of applied treatments per group was five, allowing adequate intra-individual evaluation. We consistently found that all allogeneic fibroblast populations used induced an inflammatory response that was not observed in the wounds treated with control fibroblast-seeded substitutes. These specific inflammatory responses could easily be observed, because the dermal substitute used consisted of native non-cross-linked collagen coated with elastin-hydrolysate and the bio-absorption of the material occurs with minimal inflammatory reactions.[13,26] In addition, dermal substitutes were implanted 1 day after fibroblast seeding to exclude differences in cell density in the substitute, which could arise after in vitro culture of substitutes. Histological evaluation of all substitutes prior to seeding showed an identical fibroblast distribution in the dermal substitutes

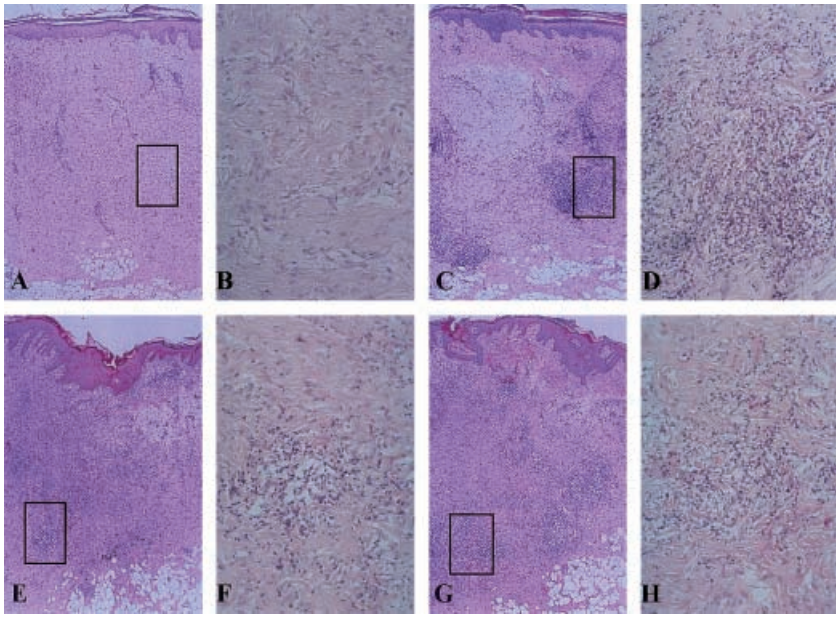

FIGURE 4. Granulation tissue of the wounds 2 weeks after grafting. The wounds treated with substitutes seeded with control fibroblasts (A, B) resembled a normal healing wound containing few granulocytes and lymphocytes. In contrast, the ''allogeneic'' wounds with allo-1 (C, D), allo-2 (E, F), and allo-3 fibroblasts (G, H), showed increased numbers of inflammatory cells and the presence of mixed granulomatous and lymphocytic inflammatory foci. A, C, E, and G original magnification 20×; B, D, F and H enlargements of insets 5×.

similar to what has previously been reported.[20] In the latter study, we showed that the quality of dermal tissue regeneration was dependent on the number of autologous fibroblasts present in the dermal substitute. The number of fibroblasts seeded in this study is identical to the lowest number of fibroblasts for which statistical beneficial effects were previously found.[20] Normally, to achieve optimal dermal tissue regeneration, it would have been preferable to seed higher cell numbers. However, the seeding of more allogeneic fibroblasts will most likely induce an even stronger inflammatory response, whereas lower numbers do not significantly improve the regeneration process when compared with substitutes without fibroblasts. Actually, in wounds treated the allogeneic fibroblast-seeded substitutes, the quality of scarring seems to be lower or similar to that of wounds treated with the acellular substitutes in previous studies.

The MLR performed gives a good indication of the suitability of donors for the transplantation of organs or cells. The haplotype of the recipient and donor pigs was not investigated because not all antibodies are available for all swine leukocyte antigen subregions, making it impossible to get the complete serological tissue type of the pigs used in this study. In reality, a perfect human leukocyte antigen (HLA) match is often not possible due to the limited availability of tissues. In skin tissue engineering, early wound treatment with allogeneic fibroblasts is likely to only allow a close HLA matching as displayed in this study. The allo-3 allogeneic fibroblast population used was genetically the least related to the recipient pigs, which was shown by the highest MLR response in both pigs. The allo-3 fibroblasts also induced significantly more wound contraction and scar formation when compared with control fibroblasts in both pigs. The PBMCs of pig A induced some response when mixed with the PBMCs of the allo-2 pig, and in pig A the wound contraction induced by allo-2 fibroblasts was also significantly higher when compared with control fibroblasts. The other MLRs were comparable to background levels, but despite the negative MLR responses, an immunological response was observed in the granulation tissue of all wounds treated with the different allogeneic fibroblast populations. This is not surprising knowing that even after HLA matching and negative MLR responses, allogeneic skin grafts containing the epidermis were ultimately rejected.[27] In our study, rejection of the allogeneic dermal skin substitutes was not observed. However, the observed inflammatory and immunological responses did affect dermal tissue regeneration and induced more scar tissue.

In a similar porcine wound model, Reagan et al. compared the uses of acellular and cellular allogeneic dermal grafts.[22] They found that the cellular dermal grafts elicited a drastic inflammatory response, which seemed to be directed against epithelial follicular elements but not against fibroblasts or extracellular matrix elements. Although these grafts also showed no signs of rejection, the inflammatory responses worsened the cosmetic outcomes of wound healing and increased wound contraction similar to in our study. In renal transplantation, rejection increases the presence of myofibroblasts and tissue fibrosis.[28] Myofibroblasts play an important role in wound contraction,[29] and we have found that in the porcine wound

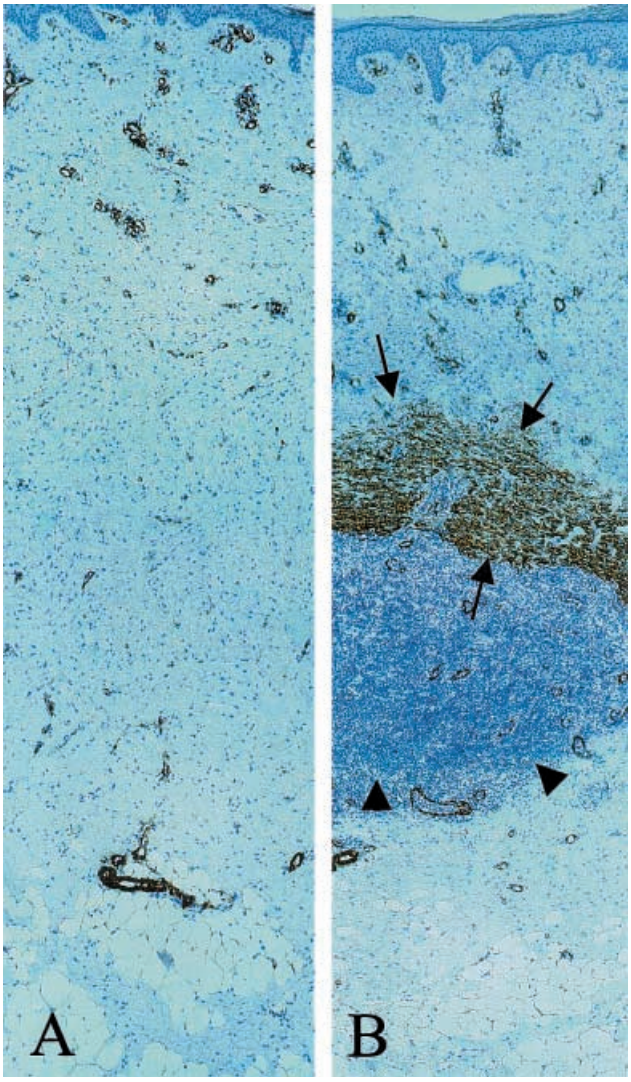

FIGURE 5. Alpha-smooth muscle actin staining identifying smooth muscle cells and myofibroblasts (arrows) in the granulation tissue 4 weeks after grafting. Wounds treated with substitutes seeded with autologous fibroblasts (control treatment) (A) and allo-2 fibroblasts (B). The area positive for myofibroblasts is just above inflammatory foci consisting of a mix of lymphocytes and granulomatous cells (arrowheads). Original magnification (A) 50×; (B) 40×.

model the number of myofibroblasts found 2 weeks after healing correlated with the percentage of wound contraction measured at 6 weeks.[20] In this study, it appears that the observed inflammatory reactions stimulated the fibroblast differentiation into myofibroblasts and prolonged the presence of these myofibroblasts. This could explain the observed increase in wound contraction and scar formation in the wounds treated with the "allogeneic" substitutes.

In many studies, fibroblasts were included in a dermal equivalent for their positive actions on epidermal regeneration. In the present study, differences in outgrowth or closure of the meshed split-skin graft interstices could not be observed because the wounds completely epithelialized in between the evaluation time points of 1 and 2 weeks. Hansbrough et al. showed that allogeneic fibroblasts allowed rapid epidermal regeneration of meshed epidermal interstices xenografted to nude mice in a vicryl mesh dermal substitute.[30] Treating burn patients with this "allogeneic" dermal substitute (Dermagraft®), they did not observe evidence of immunological rejection to the allogeneic fibroblasts or to the vicryl fibers 14 days after grafting.[31] Unfortunately, the study design only allowed comparison to treatment with split-skin mesh grafts and not to a dermal substitute with autologous fibroblasts. Another commercialized skin substitute, Graftskin®, now called Apligraf®, contains both male allogeneic fibroblasts and keratinocytes. This substitute has been applied on patients with surgical wounds and chronic ulcers without clinical signs of rejection.[21,32,33] The immunocompatibilty is claimed to be based on several factors, such as the lack of antigen-presenting cells bearing major histocompatibility complex (MHC) class-II molecules; the cytokine environment, which negatively influences maturation of unprimed T-cells; and the limited access of host immune cells to grafted cells. Apligraf® is a modification of the organotypic collagen gel skin substitute originally described by Bell et al.[1] These authors also reported that these grafts populated with allogeneic fibroblasts were accepted across the barriers of histocompatibility.[23,24] Persistence of the allogeneic fibroblasts was, however, only investigated by karyotyping of allogeneic cells in isogeneic transplanted rats. More recently, Otto et al. also detected male cellular markers in a female patient up to 2.5 years after grafting using a more sophisticated molecular biological technique.[11] Although in these studies the quality of the regenerated dermis was not investigated, the long survival of these cells seems to indicate that in these types of skin substitutes, allografted cells may survive longer than expected.

These data lead to confusion in this research field because the majority of the evidence seems to be in favor of nonsurvival of cultured allografted cells, especially for keratinocytes.[3] The expression of MHC antigens is thought to be responsible for transplantation rejection. T cells are capable of recognizing allogeneic MHC antigens without the usual requirement that their peptides are processed and presented by recipient antigen-presenting cells. This immediately elicits a cytotoxic response.[34] On resting fibroblasts, the expression of MHC-I is low and MHC-II is not detectable. After fibroblast activation the MHC-I levels are up-regulated, and in chronic inflammatory loci expression of MHC-II on fibroblasts has been reported,[35,36] especially in the presence of interferon-γ.[37] In vitro,

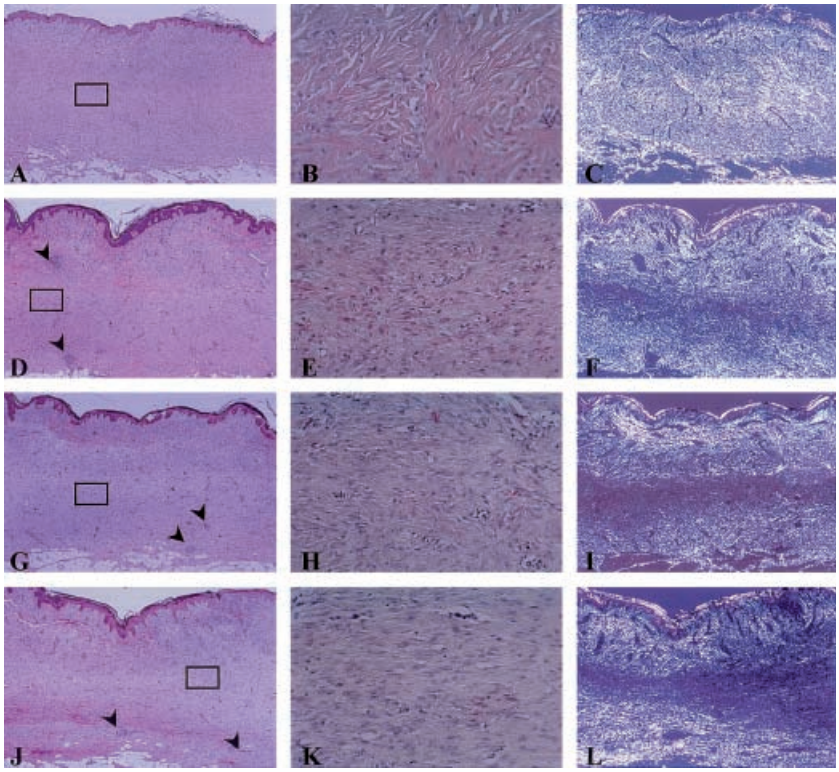

FIGURE 6. Regeneration of dermal tissue of the wounds treated with substitutes seeded with control (A–C), allo-1 (D–F), allo-2 (G–I), and allo-3 fibroblasts (J–L) 6 weeks after grafting. A, D, G, and J give an overview of the dermal tissue; B, E, H, and K are enlargements of insets illustrated in A, D, G, and J, respectively; and C, F, I, and L are images of the regenerated tissue under polarized light, which highlights mature collagen. The regenerated tissue of the ''control'' wounds resembles normal skin, whereas the ''allogeneic'' wounds still had granulating areas in the middle of the regenerated tissue with increased cell numbers and nonremodeled ECM with immature collagen bundles. In addition, the ''allogeneic'' wounds still contained mixed granulomatous and lymphocytic inflammatory foci (arrowheads). A, D, G, and J, original magnification 13×; B, E, H, and K 120×; and C, F, I and L, 15×.

interferon-γ–induced MHC expression on fibroblasts did not appear able to stimulate, or only moderately stimulated, unprimed allogeneic T-cell or lymphocyte responses.[38,39] However, when mixed with primed T cells, the allogeneic fibroblasts did induce T-cell proliferation.[40] Moreover, the coculture of keratinocytes with 3T3-fibroblasts induced the expression of both MHC-I and II on 3T3-fibroblasts,[41] and the 3T3 fibroblasts were able to sensitize a graft recipient for accelerated second-set rejection.[42] Moreover, studies with MHC-II knock-out mice provided evidence that graft rejection can also occur due to the presentation of donor antigens by recipient MHC molecules, called "indirect" recognition.[43]

The data presented in our study also seem to support the fact that allogeneic fibroblasts are recognized by lymphocytes and provoke inflammatory responses. We believe, therefore, that in skin transplantation where scar quality is an issue, it is better to use autologous fibroblasts in dermal skin substitution. However, when used as a temporary cover to stimulate wound healing, allogeneic fibroblasts might also play a role in triggering the immune system. This is especially interesting in chronic wounds in which the local immune status is impaired.

In conclusion, for optimal restoration of dermal skin function with minimal scar formation, the use of skin substitutes with autologous fibroblasts is preferred over skin substitutes with allogeneic fibroblasts.

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