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Influence of Human Fibroblasts on Development and Quality of Multilayered Composite Grafts in Athymic Nude Mice

C. Cedidi[1], L. Wilkens[2], A. Berger[3], G. Ingianni[4]

[1]Klinik für Plastische, Rekonstruktive und Ästhetische Chirurgie –
Klinikum Bremen Mitte gGmbH / Universität Witten/Herdecke, Germany
[2]Institut für Pathologie - Universität Bern, Switzerland
[3]International Neurological Institute Hannover, Germany,
[4]Klinik für Plastische und Handchirurgie - Helios-Klinikum Wuppertal Universität Witten/Herdecke, Germany

Abstract
In patients after extensive burn injury the lack of split thickness skin graft donor sites, and consecutive delay in wound closure are critical factors of morbidity and mortality. In addition limited functional and aesthetic results after transplantation of split thickness skin grafts present a socioeconomic problem. For improved wound closure the aim of this study was the development of a one stage technique for the establishment of a multi layer composite graft, existing of a collagen-GAG-matrix with silicon layer of a two layer synthetic dermal equivalent (DE) with integrated fibroblasts, and ceratinocytes.

In 64 athymic nude mice the evaluation of the multi layer skin grafts potential to re-establish a human epidermis, and high quality dermal structure was performed. In addition to clinical investigations we measured wound contraction, and analyzed histomorphologic, immunohistologic, "*in situ* hybridisation", and electro microscopic data.

Our results show, that the seeding of DE with human fibroblasts and ceratinocytes as a composite skin graft reproducible enabled a wound healing with an organised human dermis and epidermis within 10 - 15 days. The histological studies of the grafted composite skin grafts in this model showed morphologically a characteristic dermal-epidermal skin structure with a cornifying epithelium, being of human origin ("*in situ* hybridisation"). Through the co-cultivation of fibroblasts and ceratinocytes in the DE the generation and structural morphology of collagen fibres, and inflammatory reaction in the neodermis is positively influenced, and as a consequence wound contraction significantly reduced. In regard to the early preparation of composite grafts, and the minimal requirements for donor sites – with dependable stable reconstruction of the integument – this technique may present a step forward in the treatment of patients with extensive burns.

Introduction

The healing of full thickness skin includes complex regeneration processes. The contraction of a wound, and scarring are inevitable consequences of deep tissue injuries. Scar tissue is of minor quality, and less elastic then normal skin, and may induce aesthetic and functional disfigurements. Wound contraction, and scarring are believed to be based on an inadequate dermal regeneration, extensive re-modeling of the extra cellular matrix, and differentiation of fibroblasts in myofibroblasts [1, 2]. The processes of cellular differentiation, dermal regeneration, synthesis and re-modeling are regulated through cytokins, cell-cell-contacts, and cell-matrix-interactions [3, 4, 5]. A detailed understanding on these mechanisms, and interactions are yet missing.

The progress in the therapy for severely burned patients with full thickness skin burns is limited through a lack of appropriate skin substitutes. While today early excision, and skin grafting is the gold standard in burns [6], there exists no perfect solution. The quality of split thickness skin grafts is inferior, and harvesting of grafts is associated with substantial donor site morbidity and mortality. The successful development of a durable and qualitative acceptable skin substitute will have a significant impact for the progress of burn care.

Skin substitutes can be categorized in temporary, permanent, epidermal, dermal or composite, and in biologic or synthetic. Biologic components are xenogenic, allogenic, or autologous. Several groups are involved with investigations on skin substitute materials. Rheinwald and Green reported 1975 an effective technique for the cultivation of epithelial cells from a simple biopsy [7, 8]. The application of this technique in burns was published shortly later by Gallico et al. [9, 10]. Since then this technology was widely used in patients with extensive burns [11, 12]. With frequent use of epithelial grafts the drawbacks of this technology became apparent [13, 14, 15]. After excision of the full skin thickness, and coverage with epithelial grafts, the healing rates, and long time stability / quality were inacceptable. Nevertheless – in extensive burns - the epithelial wound closure is an important adjunct of modern patient management.

In the 80ies a working group developed the synthetic dermal substitute Integra™ artificial skin (SDSI). This membrane was to be applied on clean fresh excised wounds, and represents a temporary barrier for moisture and bacterial invasion. This membrane pre-

sents - after placement on a wound - a matrix for the generation of a neodermis. The inner layer of this material is a 2 mm thick combination of collagen fibers out of bovine tendons and glycosaminoglycan-chondroitin-6-sulfate. After extensive investigations the pore size ranges between 70 and 200 micron, and allows an ingrowth of fibroblasts and neovascularisation; after this the matrix is degraded [16-18]. The outer silicon membrane serves as a placeholder for an autologous epithelial graft after an interval of at least 2 weeks [16, 19]. First clinical results of this material were promising [20-30].

Bell and coworkers were the first group, to develop a biologic composite skin graft, and producing a completely allogenic dermal-epidermal product [31]. In this model with unilayered collagen GAG-matrices, and following other models in nude mice a successful ingrowth of cells was seen [32]. A clinical consequence in burns was not established so far. A further example of this concept is a composite graft (CG) consisting of allogenic ceratinocytes, and fibroblasts seeded in an opposing bilaminary bovine collagen matrix [33]. This material (Ortec international - New York, NY) is used as a dressing in partial thickness burns.

As a consequence the optimal skin substitute must replace the lost dermis as well epidermis. The reconstruction of dermal as well as epidermal elements are prerequisites of a high quality functional and aesthetic result. In experimental settings, and in case reports on severely burned patients, the possibility of seeding of ceratinocytes in synthetic CAG-collagenmatrices was reported, and in part compared with results of conventionally grafted cultured epithelial autologous grafts [30, 34-52]. The variable experimental and clinical results mostly showed better epidermal confluence, histomorphologic improved regeneration, stable dermal structure, and as a consequence better functional results.

The successful clinical use of a collagen matrix with integrated allogenic fibroblasts as primary wound coverage, and second stage grafting with autologous cultivated ceratinocytes was reported in experimental settings, and clinical cases [50, 21]. In this 2stage procedure, the primary application of collagen-GAG-matrix seeded with allogenic fibroblasts proved advantageous in respect for the quality of the generated skin - compared with cultured epidermal autografts (CEA). The dermal-epidermal linkages in the investigated patients in the group with the 2stage reconstruction with allogenic fibroblasts in the collagen GAG-Matrix, and secondary transplantation with CEA were twice as fast present than in the group with onstage grafting of CEA. The limited clinical results, and the 2stage concept of this model with a delay wound closure make this technique ineffective for clinical use.

In patients with extensive thermal trauma the lack in skin graft donor sites presents a critical factor for morbidity and mortality. In addition, limited functional and aesthetic results after grafting of widely expanded split thickness skin grafts presents a socioeconomic problem.

The results in literature with the use of SDSI for the 2staged reconstruction in patients after burns show clear advantages regarding functional, and aesthetic results [20, 21, 24-26, 53]. In this technique a 2staged operation is necessary, with grafting of the synthetic dermis equivalent first. In a second stage after 2-3 weeks, and development of the neodermis, a grafting of split thickness skin is performed. A prolonged immobilization, hospitalization of the patient, and requirement for skin grafts are drawbacks of this technique. Despite this, the functional and aesthetic pleasing results with this technique do justify further development of this idea.

Wound contraction with inadequate scarring are triggered through mistaken dermal regeneration, extensive remodeling of the extracellular matrix and differentiation of fibroblasts in myofibroblasts [1, 2, 54, 55]. The processes of cellular differentiation, dermal tissue degradation, synthesis, and remodeling are influenced through cytokines, cell-cell-contacts and cell-matrix-interactions [3-5]. Detailed knowledge about these mechanisms, eliciting scarring, are pending.

The integration of allogenic fibroblasts in a collagen-GAG-matrix, and application for primary wound coverage, and secondary transplantation with CEA was reported [56]. Again the two staged operative concept is a major drawback of this interesting idea.

For this reason, the aim of this study was the development of a 1staged grafting technique with a cell composite skin graft, being a synthetic silicon covered two layered collagen-GAG-matrix dermal equivalent (Integra™) with integrated fibroblasts and ceratinocytes. An efficient use of limited autologous skin graft donor sites in patients with extensive thermal injuries, and a 1stage operation can result in earlier wound closure, and reduced mortality. An improved mechanical quality of the grafted skin with better elasticity and stable surface reconstruction may also have a beneficial influence on long term life quality of severely burned patients, and costs.

Material and Methods

As SDSI we used Integra™ artificial skin (previously Johnson & Johnson, Hamburg). The 2stage surface reconstruction system Integra™, consists of a dermal, and an epidermal element. The technical details of the production have been reported by Yannas [57, 58]. The membrane acts - after fixation on the wound - as a matrix for the generation of a neodermis. The inner layer of this material is a two millimeter thick combination of collagen fibers, from bovine tendons, and glycosaminoglysan chondroitin-6-sulfate. The outer layer is a 0.009 inch polysiloxan polymer (silicon) with moisture transmission-characteristics, similar to normal epithelium.

After taking Integra™ out of the package (70% Isopropylalkohol) multiple Incubations in PBSdef (Gibco BRL Corp.), Dulbecos Modified Eagle Medium (DMEM, Gibco BRL Corp.), and Incubation in DMEM with 10% bovine calf serum were performed. After incubation in ceratinocyte-culturemedium (Rheinwald and Green [8]) over 12 hours at 37 °C, the seeding procedure for the required sizes were prepared.

Full thickness skin biopsies were harvested from

human skin (excess skin from dermolipectomy). After cultivation of ceratinocytes an incubation for 3h in 0.2% trypsin at 5° - 8 °C was done, followed by trypsination (0.1% Trypsin / 0.01% EDTA 37 °C for 0.5 h).

After sequential trypsination the cells (5 x 10^5 cells/75cm^3 culture medium) were cultivated with the "feeder-layer"- technique (Rheinwald and Green). The medium was changed after 2 days. The cells were brought to 60% confluence in the primary culture, and stored in liquid nitrogen. Before seeding in the synthetic collagen-GAG-matrix, the cells were brought in a secondary culture phase, and after approaching the required cell count, resuspended with trypsin.

For the seeding of the synthetic collagen-GAG-matrix of the synthetic dermal equivalent, the ceratinocytes were resuspended out of the secondary culture phase at the desired cell count (10^5 cells/cm^3) in ceratinocyte medium, and dropped on the collagen-GAG-matrix. The cells were integrated in the C-GAG-Matrix by centrifugation at 500 rpm (10 min). The inoculated matrix was incubated in ceratinocyte medium for 12 h, and the medium changed 2-3 days later. Before grafting an incubation in Leibovitz L 15 (Gibco BRL Corp.) was done. For the additional integration of fibroblasts a quantitative extraction from human skin dermis - up to the epidermis-rete border - was performed. Then the dermal extracts were brought in a spinner container.

Following an incubation over 12h in 0.1% collagenase at 37 °C - until dermal elements were soluble - the enzymatic solution is taken, and centrifugated at 1500 rpm. After this the pellet is resuspended with fibroblast medium (DMEM / 10% bovine calfserum, Gibco BRL Corp.). The seeding, and primary culture was done in a cell count of 5 x 10^4 cells / 75cm^3. After preparation of the synthetic C-GAG-Matrix, and the ceratinocytes as described above, the composite grafts with the fibroblasts were harvested, and resuspended with 0.1% trypsin / 0.1% EDTA. In the next step 10^4 cells/cm^3 were overgrafted, and centrifuged in the C-GAG-Matrix with the previously integrated ceratinocytes at 200rpm for 5 min. The composite grafts were used the following day.

We used athymic nude mice to follow the human cells over the experimental course [85, 86, 88, 109, 135]. The "*in situ* hybridisation" permits analysis of origin and development of cells in the grafted areas. The SDSI with seeded human fibroblasts, and ceratinocytes was grafted over the proximal back of the nude mice. The athymic mice were Balb/c-nu/nu with a mean weight of 21 g. In 64 athymic mice 4 groups were divided, and a segmental defect was created under anesthesia. After 5, 10, 15 or 30 days the investigation was ended.

Group I: In this group (n = 12) a segmental defect of 20 x 15 / 20 x 20 mm was created. After the standard procedure grafting of the SDSI alone was performed.

Group II: In this group (n = 12) a segmental defect of 20 x 15 / 20 x 20 mm was created. After the standard procedure grafting of the SDSI – with seeded fibroblasts - was performed.

Group III: In this group (n = 20) a segmental defect of 20 x 15 / 20 x 20 mm was created. After the standard procedure grafting of the SDSI – with seeded ceratinocytes - was performed.

Group IV: In this group (n = 20) a segmental defect of 20 x 15 / 20 x 20 mm was created. After the standard procedure grafting of the SDSI – with seeded ceratinocytes in coculture with fibroblasts - was performed.

The grafting of the composite grafts was performed in aseptic technique (Laminar Air Flow), Ether-ketanest anesthesia 0.001 mg/gKG. After excision of skin in the proximal back (20 x 15 / 20 x 20 mm) grafting was performed and secured with 6/0 Prolene (Ethicon).

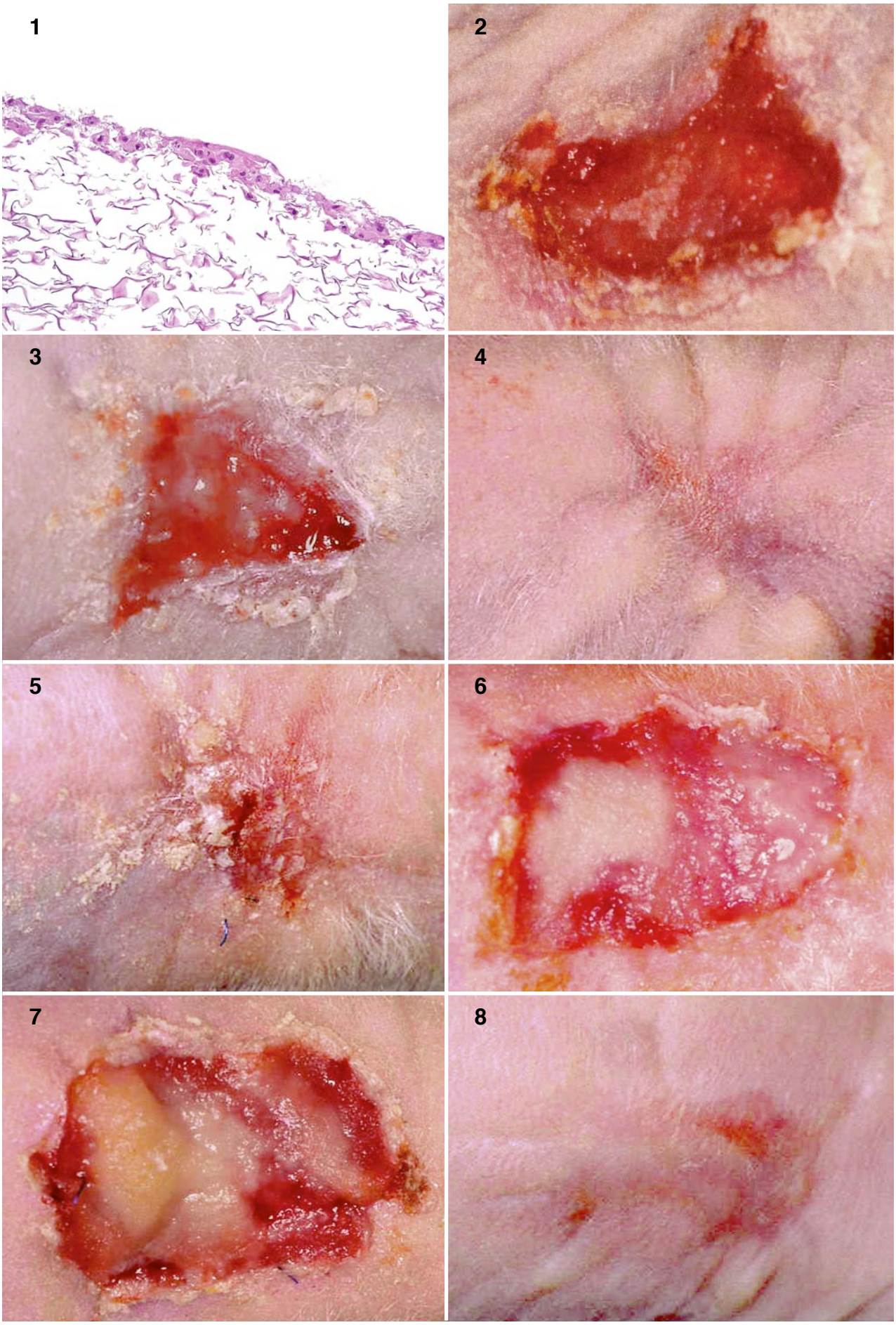
We documented the clinical course, photographic documentation / measurements, histomorphology (microthom Ultracut S, Fa. Reichert - hematoxylin-eosin or toluidin-blue stains), inflammatory cells in the grafted zones, immunhistology, "*in situ* hybridization", and electro microscopy. The daily weights of the animals were documented. For clarification of origin, and persistence of individual cells in the new skin, the immunhistology failed due to cross reactions (rat-anti-human-BER EP 4, CK 56, AE1/3, Dako Diagnostics, Inc.). For this we analyzed the persistence of human cells through "*in situ* hybridization". The demonstration of the human X chromosome was done with a chromogenic "*in situ* hybridisation" (CISH) with a specific probe. The ultra structural analysis of the healed in multilayered composite-grafts was done with a transmission electromicroscop (EM 10CR, CARL ZEISS). The clinical parameters, photographic documentation, histomorphologic, immunhistologic, and electro microscopic analysis were descriptively analyzed. In the semi-quantitative microscopic analysis of the inflammatory reaction, and proinflammatory cells, a grading or biometric analysis was not possible.

For analysis of wound contraction the primary aimed variable was the change of surface in mm^2 between the days 0, 15 and 30. The wound contraction was measured as the difference in reduction of surface in mm^2 between day 0 and day 15 - 30. The changes between group IV with the multilayer composite graft, and the control groups was analyzed, and compared with the student T-test to the level 0.05%.

1 : no / minimal infiltration
(0 - 5 mononuclear cells per visual field)
2 : low infiltration
(6 - 20 mononuclear cells per visual field)
3 : moderate infiltration
(21 - 60 mononuclear cells per visual field)
4 : intense / diffuse infiltration
(> 60 mononuclear cells per visual field)

RESULTS

With this technique of cocultivation of fibroblasts, and ceratinocytes in the collagen GAG-matrix of a SDSI we reproducibly produced a multilayered CG. In vitality tests with trypan blue this constantly was over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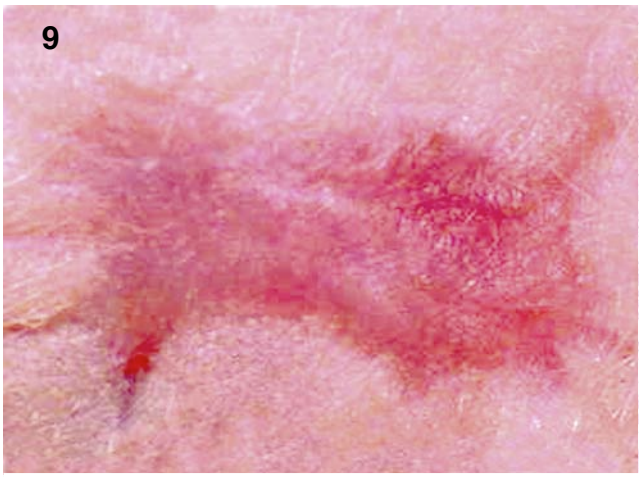


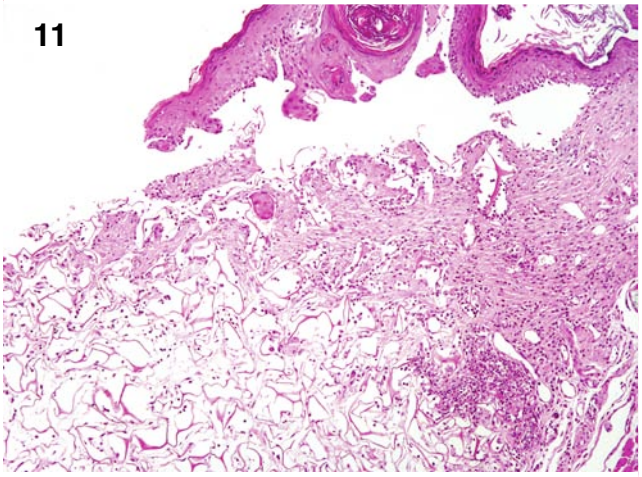


Fig. 1. The SIDS seeded *in vitro* with fibroblasts, and ceratinocytes (100x, HE).

Fig. 2. Macroscopic aspect day 15 after grafting (SDSI as control).

Fig. 3. Macroscopic aspect day 15 after grafting (SDSI with fibroblast control).

Fig. 4. Macroscopic aspect day 30 after grafting (SDSI as control).

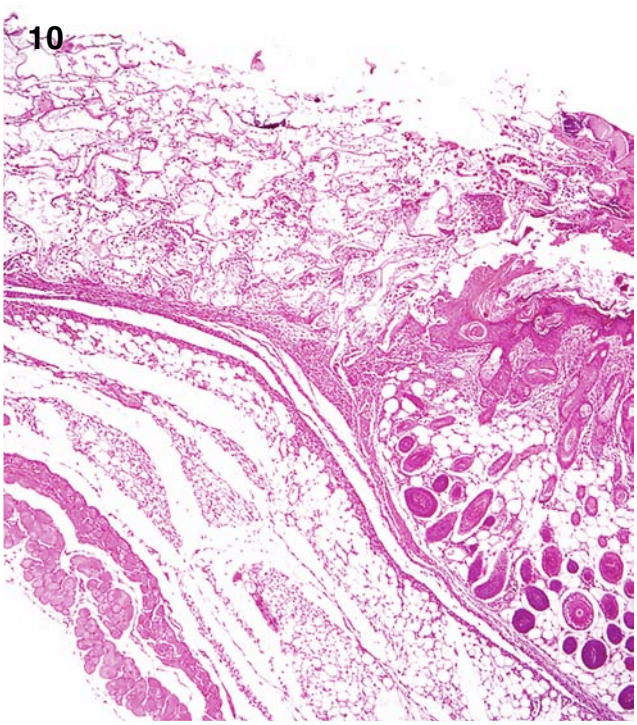
Fig. 5. Macroscopic aspect day 30 after grafting (SDSI with fibroblast control).

Fig. 6. Macroscopic aspect day 15 after grafting (SDSI with ceratinocytes).

Fig. 7. Macroscopic aspect day 15 after grafting (SDSI with fibroblasts cocultured with ceratinocytes).

Fig. 8. Macroscopic aspect day 30 after grafting (SDSI with ceratinocytes).

Fig. 9. Macroscopic aspect day 30 after grafting (SDSI with fibroblasts cocultured with ceratinocytes).



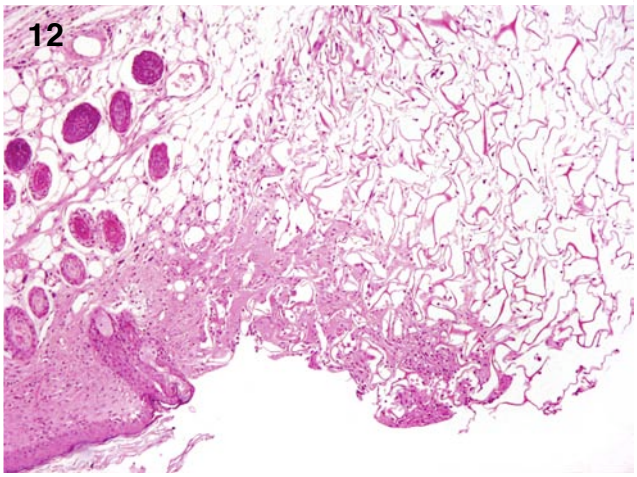


Fig. 10. Histomorphologic study day 5 after grafting (SDSI as control). In the border zone between SDSI, and mouse skin a slow ingrowths of cells in the matrix is present, and limited cellularity (50x, HE).

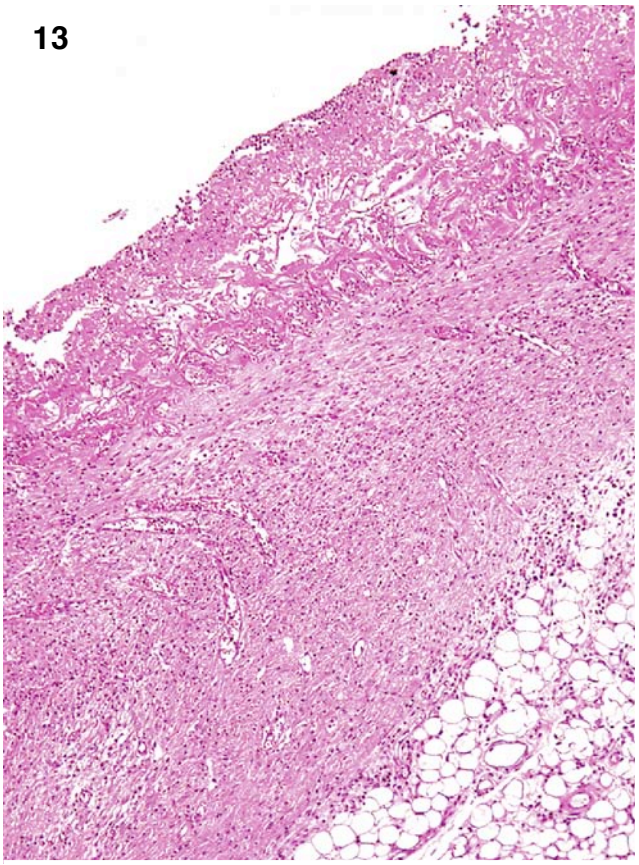
Fig. 11. Histomorphologic study day 15 after grafting (SDSI as control). In the border zone between SDSI only few cells are in the matrix (50x, HE), slowly invading the matrix. No cellular presence is seen distant from the transition zone.

Fig. 12. Histomorphologic study day 15 after grafting (SDSI with fibroblasts as control). Also here in the transition zone only little expansion of cellularity, and limited cellularity distant from the border zone is seen (50x, HE).

80%. The distribution of ceratinocytes, and fibroblasts in the Integra™-matrix was homogenous, with an accumulation of fibroblasts and ceratinocytes underneath the silicon membrane. The fibroblasts and ceratinocytes were adherent to the pores of the collagen-GAG-matrix of the SDSI (Fig. 1).

Four mice in the control groups I and III died from an early postoperative infection. Limited wound infections were documented. The operative procedure was well documented. Two mice in the control group I did not tolerate the operative procedure, and were replaced. No other relevant behavioral changes, ulcerations or mutilations were noted over the experimental course in any group.

In all mice with the control- or CG, an adherence to the wound bed was seen over the experimental course.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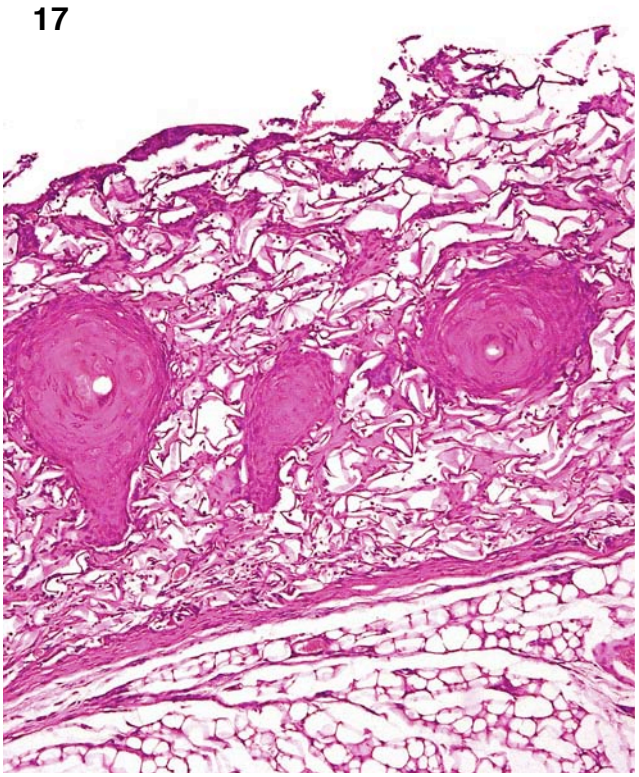
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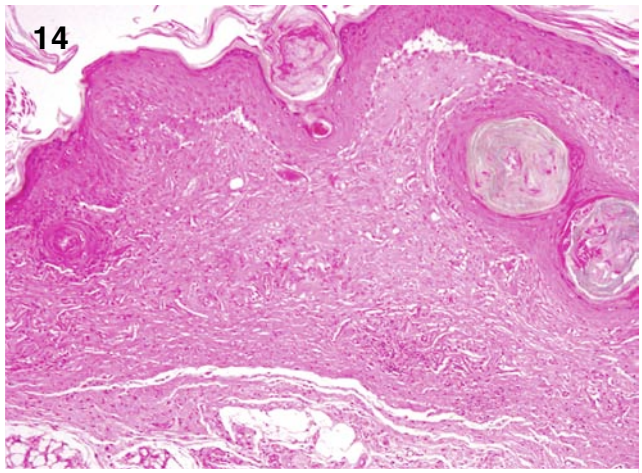
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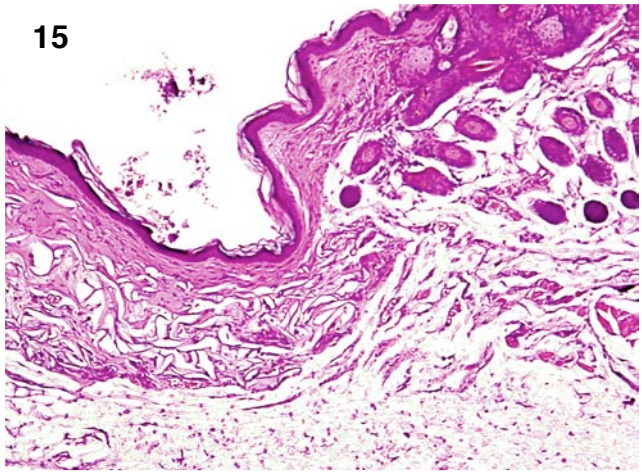
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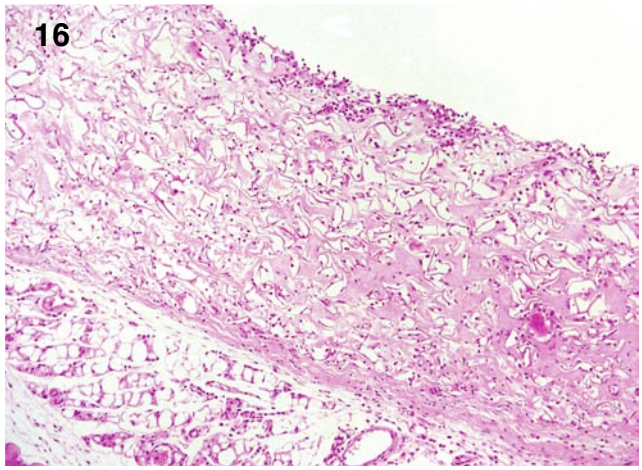
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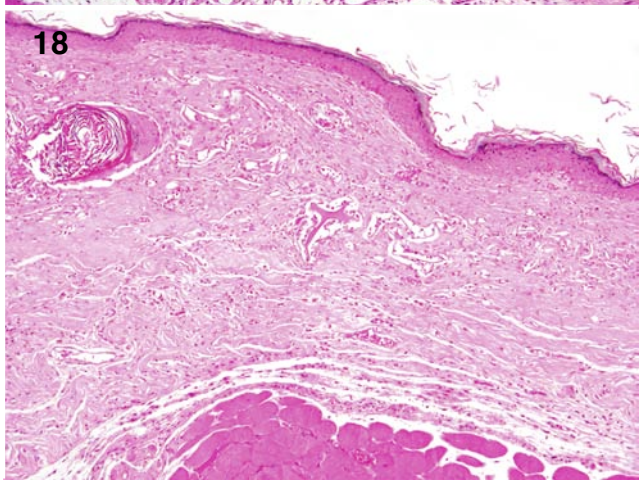
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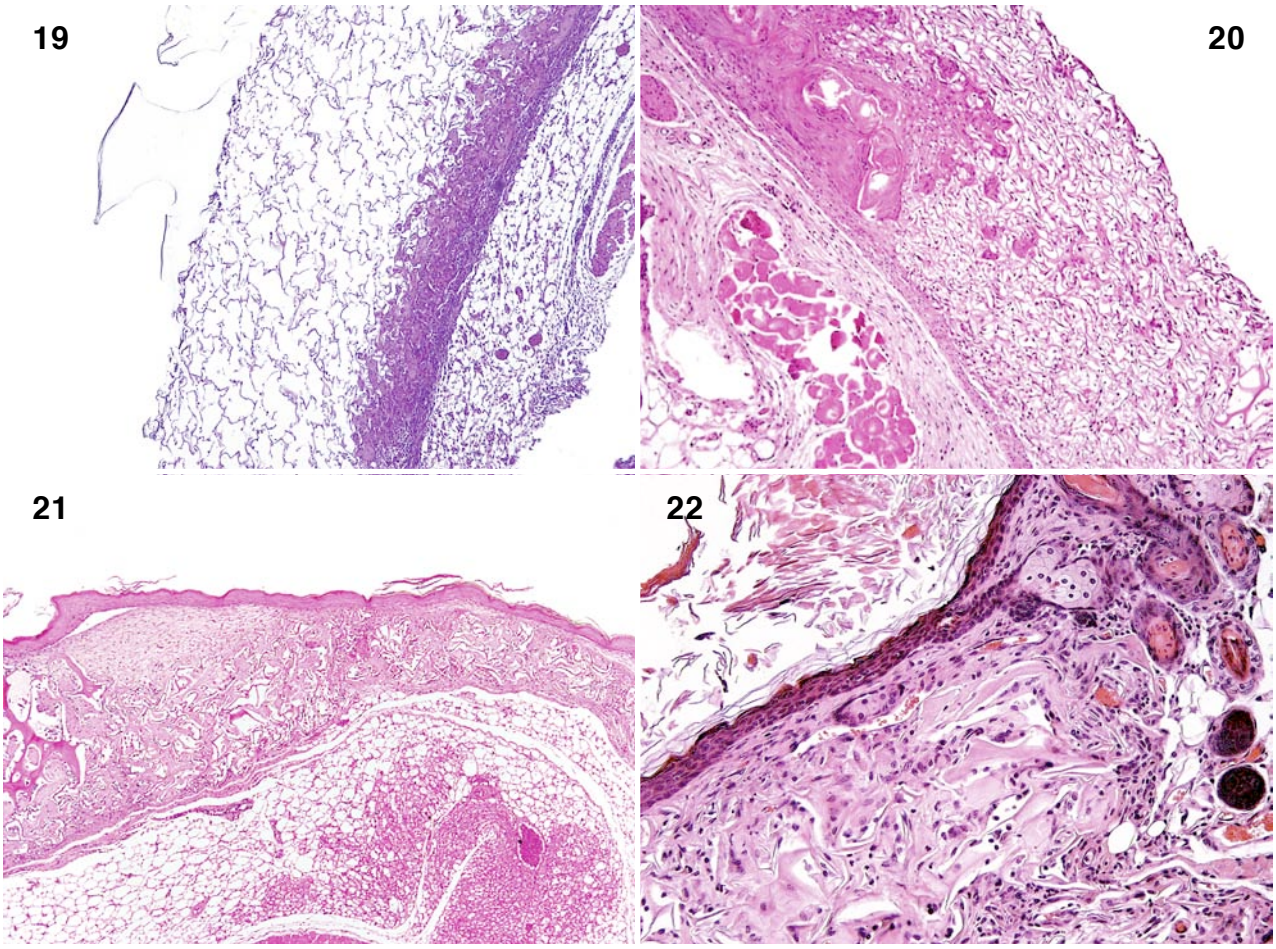


Fig. 13. Histomorphologic study day 30 after grafting (SDSI as control). The SDSI matrix is mostly necrotic, and a granulocytary inflammation is seen with a broad collagen wall (50x, HE).

Fig. 14. Histomorphologic study (100 x, HE) day 30 after grafting (SDSI with fibroblasts as control). The SDSI matrix is almost completely destructed, and a granulocytary inflammation is in the basal neodermis.

Fig. 15. Histomorphologic study day 10 after grafting (SDSI with ceratinocytes). In the border zone an early formation of a moderate neodermis, and epidermis is present (50x, HE).

Fig. 16. Histomorphologic study day 10 after grafting (SDSI with ceratinocytes). In the transition zone cellularity is markedly elevated with intradermal/intraintegral proliferation clusters, and an orthoceratosis. In distant zones the cellularity is superior compared to the first 2 groups (50x, HE).

Fig. 17. Histomorphologic study (150x, HE) day 15 after grafting (SDSI with ceratinocytes). Within the matrix clearly ceratinocyte clusters are present, being autonomous without relation to the wound edge.

Fig. 18. Histomorphologic study (150x, HE) day 30 after grafting (SDSI with ceratinocytes). A new dermis, and epidermis has formed with textural disturbances through ceratinocyte clusters in the basal dermal zone. Only residuals of the SDSI are left.

Fig. 19. Histomorphologic study day 5 after grafting (SDSI with cocultured fibroblasts, and ceratinocytes) in a neodermal matrix is formed (50x HE).

Fig. 20. Histomorphologic study day 10 after grafting (SDSI with cocultured fibroblasts, and ceratinocytes). A homogeneous celluar population, and epithelialisation is present (50x HE).

Fig. 21. Histomorphologic study day 15 after grafting (SDSI with cocultured fibroblasts, and ceratinocytes). In contrast to the previous probes a clear differentiation of epithelial, and mesenchymal elements in the SDSI, being present as such, is present. Also in distant areas to the wound edge, a clear differentiation of new dermal elements is seen (50x HE).

Fig. 22. Histomorphologic study day 30 after grafting. (SDSI with cocultured fibroblasts, and ceratinocytes). An advanced differentiation of epithelial, and mesenchymal elements in the SDSI is seen, with an intact matrix (150x, HE).

On day 15 the silicon was removed without difficulties. In the first 2 control groups no epithelialisation was found on the wound on day 15 (Figs. 2 and 3), but a hard active granulating wound with significant contraction. After 4 weeks an almost complete wound closure through secondary wound contraction, and epithelialisation from the wound edges (Figs. 4 and 5).

In the groups III and IV with the ceratinocyte- and multilayered composite grafts a clear epithelialisation of the wound surface was seen (Figs. 6 and 7). On day 30 in all mice with the ceratinocyte / fibroblast CG's a homogenous wound closure, and epithelialisation

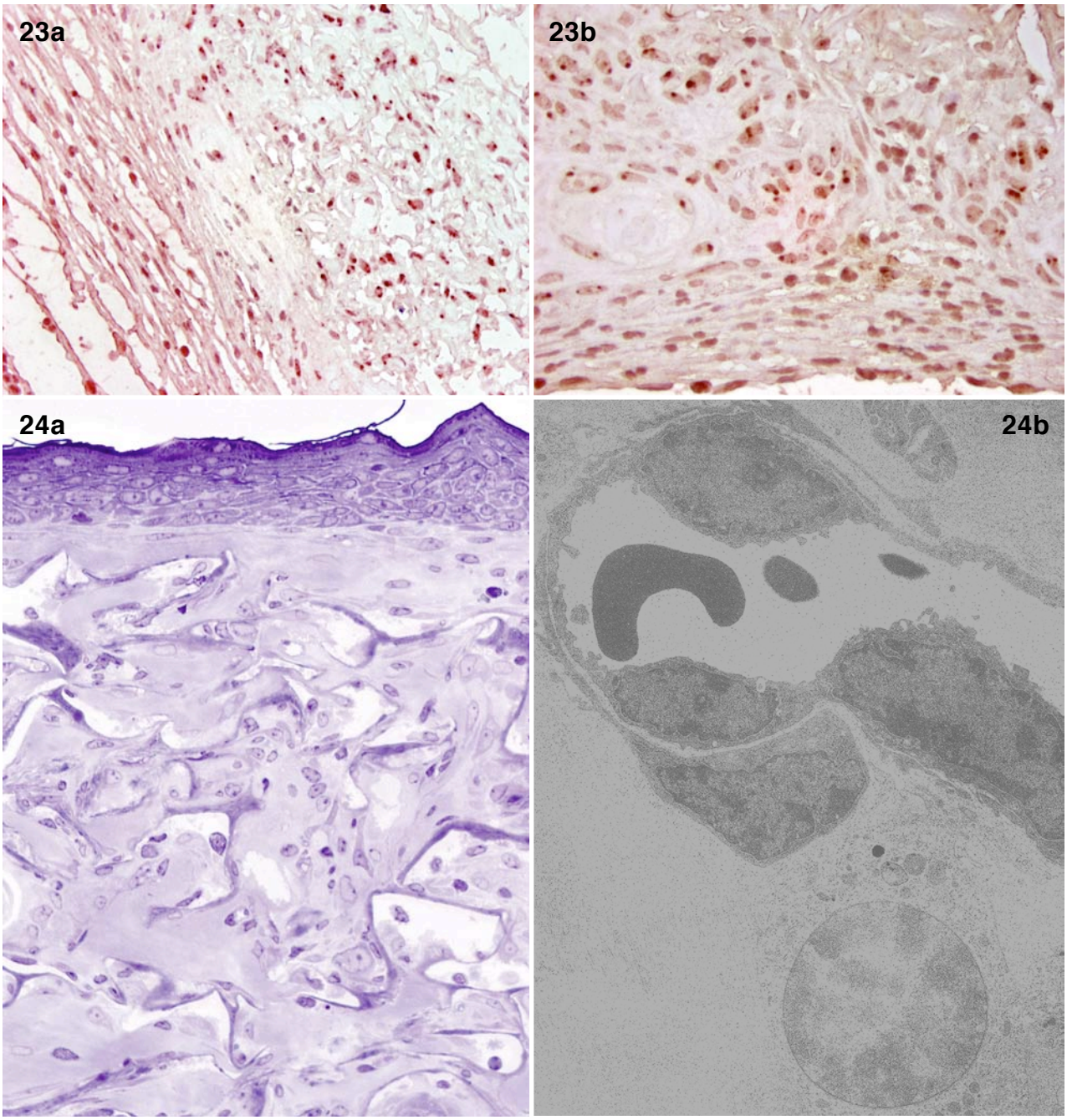
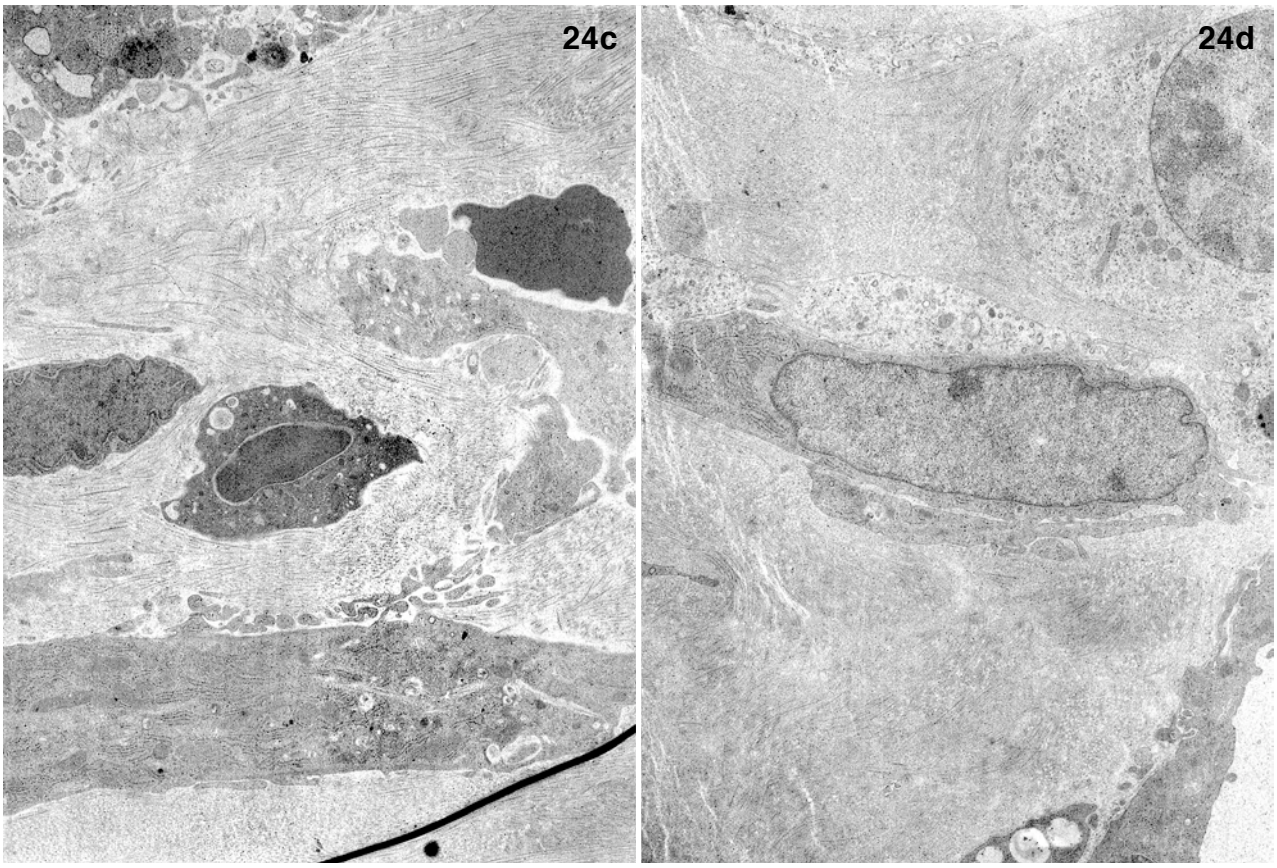


Fig. 23a, b. The "*in situ* hybridisation" day 15 after grafting (SDSI with cocultured fibroblasts, and ceratinocytes), (a: 400x; b: 630x) with verification of the human X chromosome.

Fig. 24 a-d. The toluidin blue-stain in the overview shows a multilayered CG with integrated fibroblasts, and ceratinocytes on day 30 with advanced differentiation of epithelial, and mesenchymal elements in the SDSI (a, 400x), being clearly intact. In the EM (2000x, marking 10µm) a normal skin structure with structured collagen fibers formation is shown (b), with active neoangiogenesis (c), and predominance of fibroblasts or proinflammatory cells (d).

(Figs. 8 and 9) was seen. The completely healed in CG was soft and supple.

In all animals a wound contraction was documented in variable intensity beginning day 10-15. In average, in control group 1 a wound contraction on day 15 of 41.4 % (SD = 9.5) was seen, of 36 % (SD = 7.1) in the control group 2, the wound contraction in control group 3 was 28.4 % (SD = 5.1), and the wound contraction in the experimental group 4 measured 17.6 % (SD = 4). On day 30 an average wound contraction of 59.8 % (SD = 3.5) was found in the control group 1. In control group 2 the wound contraction was 51.8 % (SD = 4.8), in control group 3 this was 38.7 % (SD = 2.9) respectively, and the wound contraction in the experimental group 4 was 28.1 % (SD = 4.7). In the statistic analysis with the student t-test the wound contraction on day 15 ($p < 0.0005$), and day 30 ($p < 0.0008$) in the experimental group 4 was significantly



reduced. The analysis of 40 animals weights reflected no irregularities in body mass, and showed a continuous gain of weight during the individual groups courses, without significant differences in the groups

Histomorphologic Analysis

The histolomorphologic investigations of the composite grafts in the first two control groups revealed a similar morphologic picture. On day 5 the synthetic collagen matrix in the control groups 1-2 was hardly remodeled. In the contact zone an inflammatory infiltrate was present (Fig. 10). The border zone of SDSI-mouse skin revealed an active destructive inflammatory reaction with granulocytes. At day 15 the infiltrate further in the synthetic DE also showed increased ingrowths of fibroblasts, and endothelial cells (Figs. 11, 12). In the border zone only few new cells are detectable, slowly invading the DE. In zones distant from the edge no cellular activity is detectable. The epithelialisation of the wound surface derived from the wound edge – in terms of secondary healing. On day 30 the SDSI was widely replaced through collagen fibers, and the epithelialisation completed by ingrowths of ceratinocytes from the wound edges. (Figs. 13, 14). The integra matrix was necrotic, and a granulocytary inflammation present.

A completely different morphologic picture was seen in control group 3, in which only ceratinocytes were integrated in the SDSI. On day 10 ceratinocytes were detectable in a homogenous distribution, and having epithelial clusters to the surface (Fig. 15). A moderate infiltrate of mononuclear cells, primarily in the healing zone, between the multilayered CG and the wound base. An organized dermis, and epidermis is detectable in the specimen. On day 15 an increased cellularity is found in the border zones, and intradermal / intraintegral proliferation clusters with an orthoceratosis (Fig. 16). In zones distant from the wound edge the cellularity is clearly higher compared to the first two control groups. A higher frequency of ceratinocyte clusters, opening to the surface, is found here (Fig. 17). In addition a predominance of fibroblasts, and neovascularisation within the matrix infiltrate is visible. At day 30 a new dermis and epidermis is formed with ceratinocyte clusters (Fig. 18). Only residual parts of the integra matrix are found, and few inflammatory infiltrates.

A different morphologic picture is seen in the experimental group with the cocultured ceratinocytes and fibroblasts in the SDSI. Within the multilayered composite graft both cell types are present in an equal distribution (Fig. 19) with accumulation of ceratinocyte clusters in the healing zone, and migration to the surface. A moderate infiltrate consisting of mononuclear cells, mostly in the healing zone, and the wound is seen. On day 10 an epithelial layer has formed on the SDSI with ceratinocyte accumulations, again opening to the surface (Fig. 20). As in control group 3 a predominance of fibroblasts, macrophages, and lymphocytes with intensive neovascularisation within the matrix. The border zone between the seeded SDSI, and the wound is becoming unclear. On day 15 a distinct differentiation of epithelial, and mesenchymal elements in the SDSI, is seen. The SDSI is still detectable (Fig. 21). Also in distant zones, a clear

differentiation of the new dermal elements is found. In the analysis on day 30, The CG`s show a highly developed, multilayered, cornifying epithelium (Fig. 22). Few infiltrates in the CG are seen with a predominance of fibroblasts, macrophages, and lymphocytes. An advanced differentiation of epithelial and mesenchymal elements in the SDSI is detectable, and as such still present. A broad and even collagen network in the remodeled SDSI is found.

Semi-quantitative Analysis of the Inflammatory Reaction

The histomorphologic investigations of the CG`s - under the aspect of semi-quantitative analysis of mononuclear cells - in 5 non-overlapping light microscopic fields in 40 mice was analyzed. While in the initial healing time only small differences between the groups were found, while a shift in the intensity of the inflammatory reaction was seen with proceeding experimental time. On day 10 a higher inflammatory reaction was seen in the control groups I-III, becoming even clearer on days 15 and 30 – as a granulocytary infection. The least intensity of inflammatory reaction was found in the group 4 with the co-cultured fibroblasts, and ceratinocytes co-cultivated within the SDSI.

Immunhistologic Investigations and "*in situ* Hybridisation"

Through a immunhistologic study with a variety of antibodies a clear differentiation to the origin of seen cells was not possible, due to cross reactions. For this reason, we used an "*in situ* hybridisation" of the healed in, and epithelialised multilayered CG`s. The investigations of ceratinocytes, and fibroblasts in the CG`s prove the human origin of the epithelial wound closure. In the border to normal skin a mixing of human cells, defined through one or two red signal points, and murine cells. In zones distant from the border zone the transition of the SDSI to the underlying tissue is visible. In deeper parts of the SDSI epithelial cells are found, creating layers. The missing of some signals in the human cells is an artifact, explainable through partial cutting of the nucleus of cells (Fig. 23).

Elektromicroscopic Analysis

The exemplary electron microscopic investigations of the multilayered CG`s on day 30 gives additional information. In a toluidin blue view of the biopsies (Fig. 24a) the matrix of the SDSI is prominent, with an advanced differentiation of epithelial and mesenchymal elements within the neodermis. The electro microscopic investigation gives a detailed ultra structural picture of the dermal compartment. Within the dermal matrix-architecture an active neoangiogenesis with numerous capillaries is present (Fig. 24b). In the investigation numerous bundles of parallel oriented, structured collagen fibers are seen over the complete dermal matrix (Fig. 24c). As in the toluidin blue overview, (Fig. 24 a) also here a predominance of fibroblast cells, and of pro-inflammatory cells is found (Fig. 24d). The fibroblast cells show a close association to the matrix collagen, and are surrounded by numerous structured collagen fibers.

Discussion

The synthetic production of permanent skin substitutes presents a potential source for technologic advanced therapy of acute and chronic wounds. In significant acute or chronic full thickness wounds the surgical excision, and grafting with either skin grafts or vascularized flaps is indicated. Despite modern transplantation techniques the wound closure of extensive wounds is an unsolved problem. In patients with extensive full thickness the integumental reconstruction remains difficult. The wound closure with meshed skin grafts in burn patients aggravates burn illness by the increase of the wound surface.

To date materials based on collagen appear to be promising for the synthetic dermal replacement. An important criterium hereby is the biocompatibility [16, 18]. The adherence, proliferation and turnover of collagen through fibroblast is also a decisive factor in the choice of the synthetic matrix for a composite skin graft. The cytotoxicity of the material, the degradation of the dermis equivalent, and the de novo synthesis have been investigated [59]. Here three different collagen materials have been used: 1. None reconstituted, none cross-linked collagen, 2. Reconstituted collagen being cross-linked with glutaraldehyd, aluminum-alginate or acetate and 3. Native collagen with or without extra cellular matrix molecules (elastin hydrolysate, hyaluronic acid, or fibronectin). The reconstituted and not cross linked collagens were rapidly degraded by fibroblasts. The chemical cross linked materials were cycotoxic; the native collagen fibers were stable. In the absence of ascorbic acid, the addition of elastin hydrolysate induced a reduction of collagen degradation. Elastin hydrolysate, and fibronectin reduced a fibroblast-associated contraction. Hyaluronic acid was not effective in reducing collagen degradation, and fibroblast associated contraction was seen in the native collagen fibers with elastin-hydrolysat or fibronectin. Ascorbinic acid enhanced collagen synthesis in the native collagen matrix, and in the material with elastin hydrolysate, but not with hyaluronic acid.

Integra™ artificial skin is a two-layered membrane-skin-reconstruction system, used for replacement of skin defects after deep dermal or full thickness burns. This system was used for reconstruction of flexible, and stable skin coverage in patients with full thickness burns or for secondary reconstruction after burns [21, 24-26]. In one series [21] the healing in rate in 22 patients of the surface reconstruction system was 87 – 95%, with 3 partial infections and 2 complete losses. The generation of a neodermis took at least 14 days. Hypertrophic scarring or contractures were not seen at follow up 12 months later. Biopsies of Integra™ grafted zones showed a normal dermal structure, without skin appendages, a predominance of fibroblasts, intense capillary network, and collagen fibers. Also the immunhistologic differentiation with a monoclonal antibody against vimentin proved a predominance of fi-

broblasts in the remodeled Integra™ matrix.

This use of the SDSI Integra™ for improved reconstruction requires a 2staged procedure, and skin graft donor sites, and prolonged immobilization until wound closure. These draw backs necessitate the search for a new 1staged, efficient but high quality reconstruction of a flexible and stable skin surface.

The advantage of dermal fibroblasts in an *in vitro* constructed composite-skin graft was reported [47, 60]. Here a SDSI in a modification [31] was seeded with dermal fibroblasts, and used as a one layered CG. In a second operation the transplantation of cultured epidermal grafts was performed. The mechanic, and cosmetic aspects of this 2staged procedure were advantageous to acellular SDSI or other techniques. After 3 – 4 months a normal dermo-epidermal junction was seen, and elastic fibers after 9 months. The efficient regeneration of mechanic stability was attributed to the presence of cultivated fibroblasts in the SDSI.

In our studies we found, that the *in vitro* co-cultivation of fibroblasts with ceratinocytes in a SDSI, and one staged grafting results in improved tissue regeneration, compared to a one-layered cultivation of fibroblasts, ceratinocytes or the pure acellular matrix. This proves, that fibroblasts in co-cultivation within the synthetic DE survive after grating, and play an important role in the regeneration of skin tissue. The "*in situ* hybridization" showed a persistence of human fibroblasts, and ceratinocytes in the multilayered CG. The regeneration of skin coverage was superior regarding contraction of the graft, structured formation of collagen fibers, and inflammatory reaction. Possibly the immediate availability of "allogenic" fibroblasts in the CG reduces the migration or proliferation of own fibroblasts in the wound. The early presence of fibroblasts in the wound together with a reduced migration of own fibroblasts could positively influence the time scale, and intensity of wound healing.

Cellular migration is associated with expression, and consecutive formation of proteolysis enzymes [61, 62]. The inhibition of fibroblast migration could in this context be related with the inhibition of proteolysis activity in the wound. In our studies, we could also find residual parts of the SDSI, in the co-cultivated grafts within the first 4 weeks. The prolonged presence of a matrix structure in the wound could also have a "place holder function" within a neomatrix for the ingrowths or proliferation of cells, and hereby prevent an unstructured wound contraction. On the other side it also seems logic, that the degradation of the DE is enhanced under infectious conditions. An other aspect is that an enhanced proteolysis activity induces a fragmentation of proteins, and consecutive activation of a variety of otherwise inactive growth factors [60], resulting in a higher chemo tactic activity in the wound. For example peptide degradation products of different collagen types [63], fibrin [64] and fibronectin [65, 66] are chemo tactic active for fibroblasts. Some peptides promote an up regulation in the synthesis of proteolysis enzymes in fibroblasts [67]. As a result, the binding of integrins to fibronectin-peptides, but not on intact proteins results in a generation of matrix-metalloproteinase's [68, 69], while elastin peptides - originating from the degradation of a collagen matrix - enhance elastase activity [70]. In addition, elastin peptides proved to be chemo tactic for monocytes [71].

Various other investigations showed, that the presence of fibroblasts in SDSI positively influence the epidermal differentiation [71, 72, 74, 75]. Probably fibroblasts stimulate the wound healing process by shortening the time interval, in which fibroblasts invade the wound, and create new surface coverage. Lamme and coworkers further investigated on this hypothesis, by analyzing the survival of fibroblasts in a synthetic DE on experimental wounds, and their effects on the formation of granulation tissue, regeneration of skin, and degradation of the SDSI [51, 76]. Here the development of fibroblasts in a synthetic elastin / collagen-SDSI, the influence of the cultivated fibroblasts on cell migration, and degradation of the SDSI in an autologous grafting model in a pig model was investigated. In these studies the persistence, and even proliferation of the cultured fibroblasts was proven. These effects of cultured fibroblasts on dermal regeneration appeared to be related to a reduction of subcutaneous fibroblast cell migration, and or proliferation in the wounds, without a migration distortion of monocytes, macrophages or endothelial cells. The degradation of the SDSI was delayed, being a hint for the protective influence of cultured fibroblasts. These results also were supported by findings of de Vries, and coworkers, when they developed an acellular SDSI, and noted a fibroblast related reduction of myofibroblastary cells, and wound contraction in the matrix [77, 78].

The influence or the effects of fibroblasts, and ceratinocytes in the contraction of human dermal extracellular matrix, and fibronectin production was studied by Ralston, and supported the hypothesis of an advantageous role of fibroblasts during wound healing [79, 80]. In these experiments a fibroblast-ceratinocyte layer was created in an acellular human dermis, and their independent capability to shrink a CG *in vitro*. There were tremendous shrinkages in ceratinocytes alone or in the presence of fibroblasts. Whereas no relevant shrinkage was seen in the presence of fibroblasts alone or the acellular matrix. The presence or the absence of ceratinocytes had no influence on dermal contraction, and was no index of epidermal-mesenchymal interactions. The analysis of the individual culture mediums of CG`s showed fibroblasts alone carrying the highest potential for fibronectin production, followed by ceratinocytes. The lowest fibronectin production was found in the CG`s, in which fibroblasts and ceratinocytes were together, indicating that ceratinocytes have an influence on fibroblasts in the generation of an extracelluar matrix. This supports the thesis on mutual interactions of these both cell types in epidermal-mesenchymal interactions.

These results were also confirmed in organotypic culture systems [81-83]. In these experiments a dynamic interaction, and cooperation of epithelial and mesenchymal ceratinocytes was found for the generation of a basal membrane, and obviously is transmitted via diffunding factors. As a consequence mesenchymal and epithelial interactions are decisive for the regeneration of a tissue specific extra cellular matrix.

With a high presence of fibroblasts during the sequential wound healing phase, different direct or indirect actions on cellular repair mechanisms secondary to growth promoting substances are promoted. There are five growth factors or families of growth factors, known to play an important role during wound healing. These are the epidermal growth factor, transforming growth factor-beta, insulin-like growth factor, platelet-derived growth factor, and fibroblast growth factor [84, 85]. Various authors have also reported on the use of allogenic dermis or allogenic ceratinocytes, as a temporary dressing on chronic wounds, superficial wounds, and split thickness skin graft donor sites [86-88], while existing data show, that the cells are not prevalent longer than 14 days. Apparently growth factors, secreted or excreted from allogenic cells, induce signals promoting wound healing [59, 89].

In a cultivation technique for human fibroblasts, and ceratinocytes in the collagen-GAG-matrix of a SDSI, and our studies in vivo we were able to show, that a reconstruction of an organized human dermis and epidermis within 10 - 15 days is possible. After 5 days the collagen-GAG-Matrix of the composite grafts was filled with proliferating fibroblasts, and ceratinocytes. The inflammatory reaction seemed to be reduced since between the synthetic collagen-GAG-matrix, and the wound bed very few polymorphic cells were seen, but a high presence of fibroblasts were demonstrated. After 10 days, the surface of the SDSI with fibroblasts and / or ceratinocytes showed a well vascularized dermal layer and confluating epidermal layer.

In the control animals with the unseeded matrix or with seeded fibroblasts alone in the SDSI clear signs of enhanced inflammatory reaction - through a higher relation of polymorphic neutrophil granulocytes, and late presence of fibroblasts – were appreciated. An organized neovascularisation was not demonstrated in a comparable period of time, despite presence of endothelial cells.

Possibly fibroblasts, and ceratinocytes can stimulate reepithelialisation, fasten the wound healing process, and quality. This becomes apparent by the early predominance of fibroblasts, and formation of neovascularisation in the composite grafts. In the experimental group 4 – with cocultivation of fibroblasts, and ceratinocytes in the SDSI – on day 30, a superior developed, and structured collagen matrix was found. Our findings support the positive influence of fibroblasts or fibroblast related production on growth factors or quality of wound healing [46, 47, 59, 56, 90].

The histomorphologic described processes in formation of proliferating, and differentiating fibroblasts, and ceratinocytes with migration of cornifying cells to the surface, and development of a structured dermis, and multilayered epidermis can be reproduced in our wound-healing model. The reason for this migration, and differentiation processes are possibly the semi permeable gas exchange over the silicon membrane, or chemo tactic signals within the wound. The reconstitution of an epidermis then loosens the mechanic bindings between the collagen-GAG-matrix, and the silicon membrane within 14 – 21 days.

Our results show that the seeding of SDSI with human fibroblasts, and ceratinocytes as a composite graft, reproducibly enables wound healing with an organized human dermis and epidermis within 10 - 15 days. The histolomorphologic analysis of the multilayered composite grafts showed a characteristic dermal-epidermal structure with cornifying epithelium, being of human origin in the "*in situ* hybridization". The co-cultivation of fibroblasts, and ceratinocytes in the SDSI positively influenced the generation, structural formation of collagen fibers, and inflammatory reaction in the neodermis. As a consequence wound contraction could be significantly reduced.

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Address for correspondence:
C. Can Cedidi MD PhD
Klinikum Bremen Mitte gGmbH
Clinic for Plastic, Reconstructive and Aesthetic Surgery
St. Jürgenstr. 1