Hypertrophic scar model in the rabbit ear: a reproducible model for studying scar tissue behavior with new observations on silicone gel sheeting for scar reduction

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ABSTRACT

Hypertrophic scarring poses a clinically relevant problem as it can be cosmetically disfiguring and functionally debilitating. A lack of animal models has hindered an understanding of the pathogenesis and development of new treatment strategies therefore has largely been empiric. Our group has developed a unique hypertrophic scar (HS) model in the rabbit ear. The model has been reproducible, quantifiable, and measurable over a time period of 1 month. We describe the development as well as the reliability and responsiveness of this model to different therapeutic agents, such as TGF-β blockade, silicone occlusion, and application of collagen-synthesis inhibitors. Moreover, it has given insights into the mechanism of action of silicone sheeting occlusive treatment and ultimately suggests that the epidermis plays a critical role in the development of HS. Additionally, we will present new data supporting the importance of the epidermis and further clarify the mechanism of action of silicone sheeting. When a semi-occlusive polyurethane film was left in place for an additional time period, scar formation was reduced. HSs of this model covered with silicone sheets and five layers of Tegaderm® showed a significant scar reduction by 80% compared with wounds with only one layer of Tegaderm®. The HS model in the rabbit ear is a highly reliable, responsive, and practical model for studying scar tissue behavior. Furthermore, our data suggest that the degree and the duration of occlusion are most important for reducing scar tissue formation.

The normal response to injury is to heal with scarring. Excessive scarring in the skin is disfiguring but can also produce restriction of motion. Excessive scarring in other organs such as the liver (cirrhosis), kidney (renal failure secondary to glomerulonephritis), and lung (pulmonary fibrosis) can be fatal. In dermal wound healing, the initial stage of healing is cell migration and inflammation followed by cell proliferation, angiogenesis, and matrix synthesis, followed by resolution. In the resolution phase of wound healing, inflammatory cells and mesenchymal cells undergo apoptosis with a resolution of inflammation, reduction in vascularity, and a drop in collagen synthesis so that collagen production is balanced by collagen breakdown.

In analyzing the etiology of hypertrophic scarring in humans, three causes stand out: genetic predisposition, delayed epithelialization, and wound tension. In many patients there is a genetic predisposition to a prolonged imbalance in collagen synthesis vs. breakdown resulting in an increased incidence of hypertrophic scars (HSs). In general, patients with some pigment in their skin (e.g., African, Asian, Mediterranean, Latin American) have a greater incidence.[1,2] Another important factor in humans that lead to HSs is delayed epithelialization (such as partial thickness injuries like burns or in response to surgical injury, e.g., laser resurfacing or dermabrasion). Until a wound is fully epithelialized, there is persistent inflammation and in partial thickness injuries, when epithelialization is delayed beyond 10–12 days, the incidence of HSs goes up dramatically.[3] The other important factor in the incidence of excessive scarring is the persistence of tension on wound closure. Fibroblasts respond to mechanical forces with signal transduction resulting in proliferation and collagen synthesis. In some areas of the body (e.g., central chest) there are significant lateral forces with a tendency to make the wound gape. In such areas, the incidence of HS formation is quite high as opposed to the face or perineum where the skin is more mobile and healing occurs by contraction with a subsequent low incidence of HSs.[3]

Unlike humans, with some exceptions, animals do not heal with excessive scar. The lack of suitable animal models has resulted in a lag in the understanding of the mechanisms of HSs. The reasons for the lack of scarring in animals come in part from an understanding of the major contributing factors in humans discussed above. In response to surgical incisions, animals have a very short resolution phase with a rapid resolution in inflammation and drop in wound cellularity in comparison to humans (unpublished observations). Second, most animals are loose skinned with a panniculus carnosus in the deep dermis, so that in response to an open wound, healing occurs

largely by contracture with rapid epithelial closure. Third, the loose skin means that there is an absence of wound tension. Finally, in partial thickness injuries, the high hair density in animals results in rapid epithelialization.

Borrowing from an observation first made by Laura Bolton (personal communication), we utilized our experience with the dermal ulcer model to create a reproducible quantifiable model in the rabbit ear.[4,5] Because the wounds do not heal by contraction, epithelialization is delayed and a raised scar is created, which from both appearance and histologic analysis, resembles human HSs (Figures 1 and 2).[4] As we have gained experience with this model we have found that it resembles human HSs in many important characteristics. Like human wounds, rabbit wounds delayed epithelialization result in more scar and old rabbits heal with less scar.[6] In response to therapies, rabbit wounds behave similar to human wounds: (1) steroid treatment reduces scar,[4] (2) silicone gel sheeting (SGS) results in reduced scar[7] (Figure 3), (3) treatment with a collagen synthesis inhibitor results in reduced scar.[8] Finally, blockade of TGF-β results in less scar,[9] which is consistent with current knowledge about the pathogenesis of excessive scarring from other model systems and observations in human scars. We will present in detail our experience with this model.

MATERIALS AND METHODS

Description of the HS model

We use adult New Zealand White female rabbits for this model. Animals are anesthetized with an intramuscular injection using ketamine (60 mg/kg) and xylazine (5 mg/kg). Four wounds are created down to the bare cartilage on the ventral side of each ear using a 7-mm dermal biopsy punch. Unpublished data of our group have demonstrated that 5-mm punch biopsy wounds fail to generate HSs, and 6 mm wounds are less hypertrophic due to faster epithelialization. The cartilage needs to be meticulously

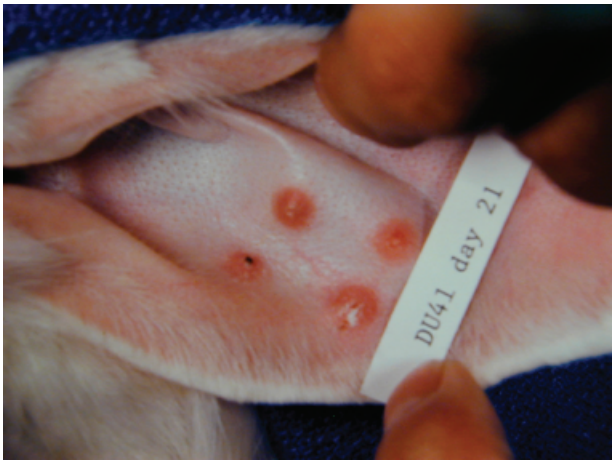

Figure 1. Four hypertrophic scars at the ventral side of the rabbit ear at POD 21. Although scars are most hypertrophic around POD 28, a bulky hypertrophic scar tissue formation is already palpable and visible on gross examination.

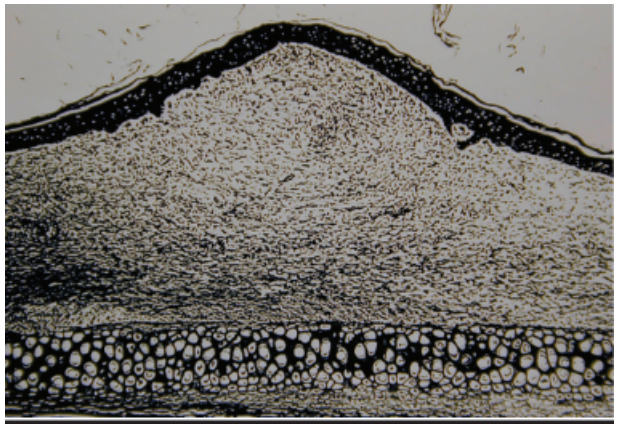

Figure 2. A representative histologic cross-section of a hypertrophic scar at POD 28 (hematoxylin & eosin stain, ×100 magnification).

nicked without full dissection, as the latter would cause undirected granulation tissue and epithelial ingrowth resulting in noninterpretable histology. Epidermis, dermis, and perichondrium are thoroughly removed using a dissecting microscope. Complete removal of the perichondrial layer is mandatory as it delays epithelialization for 8–14 days in 7-mm punch biopsy wounds. Delayed epithelialization increases the degree of hypertrophic scarring and causes persistence of scar elevation beyond POD 48 as demonstrated in a previous study of our group.[4] Occasional bleeding is treated by manual compression and electro-cauterization should only be considered in unappeasable bleedings as it increases the risk of wound necrosis with subsequent alteration of the wound-healing

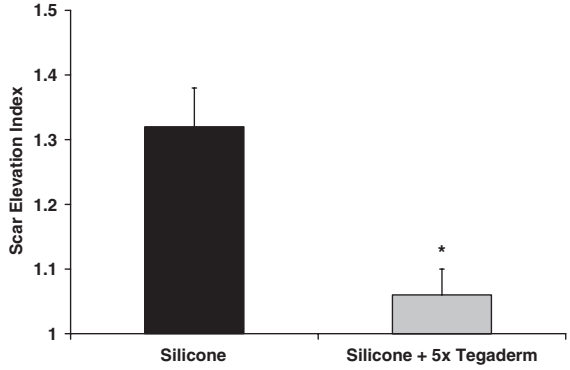

Figure 3. Influence of total occlusion on hypertrophic scar formation in silicone-occluded wounds. One layer of occlusive silicone dressing (Cica-Care®) was directly applied to all healed full-thickness wounds. Then, either one or five layers of Tegaderm® were attached on top of the silicone dressing until POD 30. Wounds sealed with five layers of Tegaderm®, permitting no oxygen transmission, showed an 80% decrease in scar tissue formation compared with wounds covered with one layer of Tegaderm® (*$p < 0.05$, $n=15$). Our data suggest that occlusion and not oxygen transmission might be the key mechanism in explaining the scar-reducing properties of silicone gel sheeting.

process. Finally, a liquid adhesive (Mastisol®, Ferndale Laboratories Inc., Ferndale, MI) is applied to the surrounding skin followed by wound coverage with a polyurethane dressing (Tegaderm®, 3M Health Care, St. Paul, MN). Wounds are examined every day for signs of infection as well as for epithelialization progress on gross examination. The polyurethane dressing must remain attached at all times to ensure a moist wound environment and can be removed when wounds are fully epithelialized. Treatment starts when HS formation appears most prominent. Wounds are hematoxylin & eosin or trichrome stained and harvested with the cross-section passing through the most elevated scar portion (Figure 2).

The scar elevation index (SEI) is employed for histomorphometric analysis (Figure 4).[4] The SEI measures the ratio of total scar connective tissue area to the area of underlying dermis. The height of the underlying dermis is determined based on the height of the adjacent unwounded dermis. All measurements are taken within the confines of the wounded area and the epithelial height is *not* considered in SEI calculations. It follows from the above that an SEI of one indicates no newly formed hypertrophied dermis whereas and index greater than one denotes HS formation. Epithelial parameters, such as epithelial gap or epithelial ingrowth, can also be measured in this model. If not stated otherwise a Student's *t* test is employed for statistical analysis and results are considered significant at $p < 0.05$.

Silicone occlusive treatment and the HS model

SGS is a mainstay in the treatment of HSs in the daily practice of plastic surgeons and dermatologists. A previous study of our group investigated a possible scar-diminishing effect of SGS (Cica-Care®, Smith & Nephew, Largo, FL) as well as Tegaderm®, Op-Site® (Smith & Nephew) and a two-layer paper-strip (Steri-Strips®, 3M Health Care) in the HS model.[7] Wounds were occluded on POD 28 and harvested 4 weeks later. Uncovered wounds on the opposite ear in each animal served as controls and a total of 40 wounds were enrolled for final analysis.

Of all different occlusion types, only SGS occluded wounds showed significant scar reduction in comparison to control wounds as assessed by the SEI ($p < 0.001$,

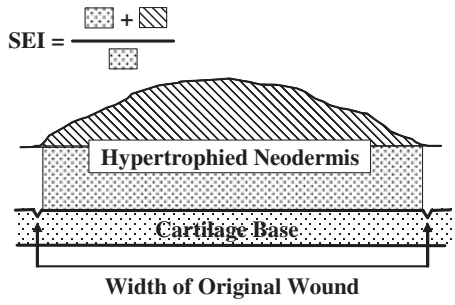

Figure 4. The scar elevation index (SEI) is an accurate and reproducible instrument to evaluate hypertrophic scar formation. The SEI is the ratio of the total dermal area, including the newly formed hypertrophied dermis, to the area of the underlying dermis.

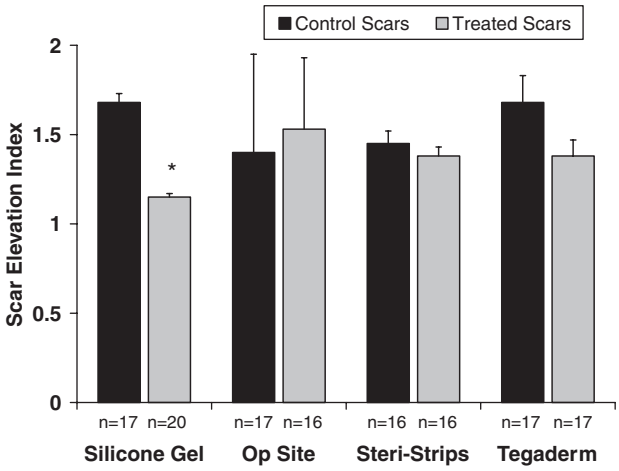

Figure 5. Healed full-thickness wounds in the rabbit ear model were treated for 4 weeks (postwounding days 28–56) with different occlusive dressings. Only silicone-treated wounds demonstrated a significant scar elevation index (SEI)-reduction vs. control scars of the (*$p < 0.001$). In contradistinction, Op-Site®, Steri-Strip®, and Tegaderm® failed to show a significant scar-reducing effect. Corresponding *p*-values are 0.64, 0.51, and 0.11, respectively. The SEI expresses the ratio of elevated scar tissue to unwounded tissue underneath. An index > 1 indicates hypertrophic scarring whereas an index of 1 displays no scar formation (with permission of *Aesthetic Surgery Journal*).

Figure 5). Histologic evaluation of the dermal compartment, comparing each occlusive treatment with nonoccluded wounds, revealed no significant differences in overall cellularity, collagen fiber organization, vascularity, inflammation and total amount of mast cells, lymphocytes, multilobulated cells, and eosinophils. In addition, cellularity and mitotic activity were not altered in the different epidermal layers of occluded vs. nonoccluded wounds.

In contrast, SGS-occluded wounds demonstrated remarkable differences compared with control scars, most prominently in the basal cell layer on an ultrastructural level, when examined by transmission electron microscopy (Figure 6). SGS-treated wounds were more akin to unwounded control skin in terms of dermal collagen structure with fewer but more flocculent basal cell vacuoles and a more distinct, though more irregular, basal lamina. Conversely, the basal cells in control scars and Op-Site®-treated wounds revealed multiple vacuoles, morphologically different from endosomes, lysosomes, peroxisomes, or secretory vacuoles with loose and unstructured collagen bundles and a less distinct basal lamina compared with unwounded control skin.

Previous studies employing the HS model

In another study, we used the HS model to investigate the effect of Mederma® (Merz Pharma & Co., Frankfurt, Germany) on scar tissue formation.[10] Mederma® is a popular and quite prevalent over-the-counter topical gel with a proposed scar-improving effect yet with a lack of scientific

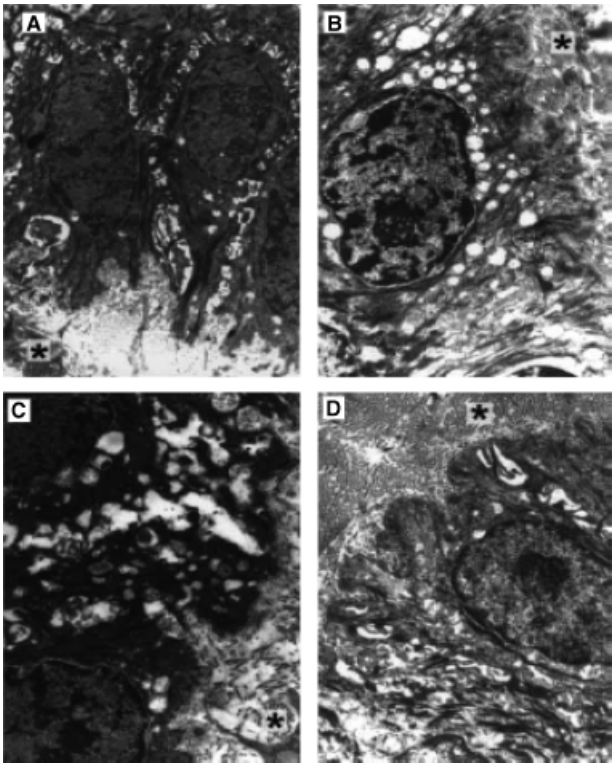

Figure 6. We investigated the ultrastructure from samples of unwounded skin, untreated scar tissue, and treated scar tissue by electron microscopy: (A) unwounded, control skin; (B) untreated scar tissue; (C) Op-Site® treated scar; (D) silicone-gel-treated scar. Silicone gel-treated scars demonstrated similarity to unwounded control skin in terms of an improved collagen structure and fewer basal cell vacuoles. *Dermal site of micrograph (with permission of *Aesthetic Surgery Journal*).

support. Treatment with Mederma® was initiated at POD 28 for a period of 4 weeks. Histologic analysis revealed no significant reduction in the SEI for Mederma®-treated wounds. In addition, no significant changes regarding dermal vascularity, inflammation, or scar erythema were observed. A prospective double-blinded placebo-controlled study in humans confirmed our results, in which posttraumatic scars were treated either with Mederma® or placebo.[11] A significant improvement in terms of softer and less visible scars was only observed by patients after 2 months but was not confirmed by the evaluating physicians.

We also sought to investigate the applicability of our model in aged rabbits addressing the clinical observation of many surgeons that elderly patients show less scarring.[6] The underlying mechanisms influencing the different wound-healing stages in aged patients are barely elucidated,[12] and an animal model that parallels human scar behavior in the elderly would be of significant relevance to researchers. Twelve aged (44–62 months) and twelve young (3–6 months) rabbits were wounded and the wounds of each 4 animals harvested on POD 15, 21, and 28, respectively. Evaluation for HS formation (SEI) was performed on POD 28. On histologic analysis, the SEI confirmed a significant scar reduction in the aged vs. young rabbits ($p < 0.01$), which was consistent with gross macroscopic findings. Moreover, epithelialization was significantly delayed in aged animals, which correlated positively with the apoptotic indices assessed in this study. Previous studies of other groups described a correlation between apoptosis and epithelialization,[13,14] which is coherent with our findings in the HS model.

Furthermore, we investigated the effect of collagen synthesis inhibitors. In one study, the phenanthrolinone derivate FG-1648, a prolyl 4-hydroxylase inhibitor, was topically applied for the first 7 days postwounding.[8] Either 0.5% (low dose) or 1.0% (high dose) FG-1648 gel solution was applied to each wound on a daily basis and each animal served as its own control. Wounds were harvested on POD 28 and scar elevation was measured using the SEI. The low dose group failed to show a reduction in scar formation whereas the high-dose group demonstrated a significant decrease by 26% in average ($p < 0.001$). However, collagen organization was not altered by FG-1648 treatment.

Subsequently, we investigated the influence of intradermal application of anti-TGF-β 1, 2, and 3 monoclonal antibodies on HS formation.[9] Treatment was started at different stages of wound healing (early, middle, and late) to address the varying role of TGF-β throughout the wound-healing process. Early (days 0, 2, and 4 postwounding) antibody treatment resulted in delayed wound healing without alteration in scar tissue formation. In contradistinction, middle and late onset of TGF-β 1, 2, and 3 blockage (between days 7 and 13) caused a significant reduction in HS formation. These findings confirmed our hypothesis that the beneficial effects of anti-TGF-β treatment in the setting of wound healing are time-dependent and that premature TGF-β blockage negatively affects wound healing.

Another study, focusing on inhibition of collagen synthesis as well, investigated the effect of blocking procollagen C-proteinase (PCP) with a specific inhibitor (Pfizer Inc., Sandwich, UK).[15] This enzyme catalyzes the cleavage of the C-terminal propeptide from its precursor molecule to generate collagen fibrils. PCP-inhibitor was injected subcutaneously at four different timepoints at an early or late stage of wound healing. Wounds were harvested on POD 28 and scar tissue formation assessed by SEI. Early treatment with the PCP inhibitor demonstrated no decrease in scar hypertrophy whereas late treatment significantly reduced the SEI ($p < 0.01$, $n=40$).

Finally, we employed the HS model to study the effect of a retroviral-delivered dominant negative TGF-β-receptor-II.[16] The HS model proved its scientific value and confirmed our hypothesis that antagonizing TGF-β-receptor II leads to mitigation of scar tissue formation.

RESULTS

New insight into the therapeutic mechanisms of SGS: semi-permeability or occlusion?

The semi-permeable nature of SGS allows different interpretations whether the scar-reducing properties derive from its oxygen-permitting feature or are exclusively based

on occlusion. We therefore sought to employ the HS model to investigate possible differences in scar tissue formation in silicon gel-sheeted wounds additionally occluded with either one or multiple layers of Tegaderm®. As soon as wounds were fully epithelialized, a single sheet of silicone (Cica-Care®) was applied to each wound with an additional coverage of either one or five layers of Tegaderm®. The latter were considered fully occluded, lacking semi-permeable properties. Wounds were harvested on POD 30 and subsequently evaluated using the SEI. Intriguingly, wounds covered with five layers of Tegaderm® demonstrated an approximately 80% reduction in scar tissue formation compared with wounds covered with only one layer of Tegaderm® ($p < 0.05$, Figure 3). These findings strongly suggest that occlusion, rather than the semi-permeable properties, is a key mechanism in the scar-reducing features of silicone treatment.

Besides the degree of occlusion, duration is another important factor in occluded wounds. In a new set of hypertrophic wounds in the rabbit ear, a single layer of Tegaderm® was left in place until either POD 12 or POD 18. An SGS was not applied in this experiment. The POD 18 group demonstrated a reduction in scar tissue of 18% compared with wounds treated until POD 12 ($p < 0.05$, $n=35$, Figure 7).

DISCUSSION

Scar tissue formation poses a relevant clinical problem to surgeons in different fields not only from an aesthetic viewpoint but also from a functional perspective. The endeavor to generate new scar-improving therapies has often been hindered in the past due to a lack of reliable animal models.

The rabbit dermal ulcer model was first described in 1966 by Joseph and Dyson[17] and subsequently improved upon by Morris.[4] Currently, our model represents a genuine approach to study hypertrophic scarring in a reproducible and controlled fashion. Complete removal of the epidermis, dermis, and perichondrial layer causes delayed epithelialization, which results in hypertrophic scarring. Additionally, the 7-mm punch wounds of our model show no signs of contraction. Therefore, wound healing in this model is carried out exclusively through granulation tissue formation and reepithelialization. The inability of the wounds to contract, which exposes fibroblasts within the wound to tension, and the delay in epithelialization of approximately 2 weeks, result in predictable scar elevation and a histological appearance of an HS (Figures 1 and 2).

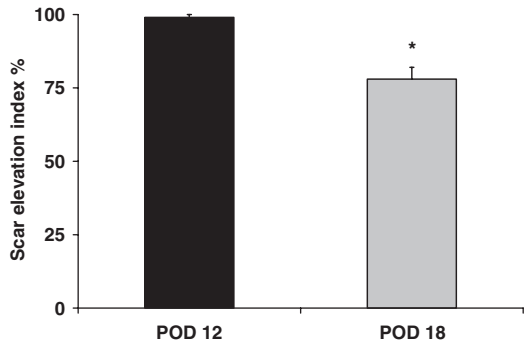

Figure 7. Wounds were only covered with Tegaderm® either until POD 12 or 18. An extended duration of occlusion significantly reduced hypertrophic scar formation by 18% (*$p < 0.05$, $n=35$).

However, the model is not ideal. The underlying cartilage is also stimulated to hypertrophy in some instances and scar elevation can be variable. Monica Zepeda from Canji Inc. has modified the model by adenoviral transfection with PDGF to increase scar hypertrophy.[18] The rabbit as a species is obviously more difficult because of the relative paucity of species-specific reagents, such as antibodies or primers. Nonetheless, as the rabbit cornea is a standard model for eye research, gene sequences will become more available in the near future.

Numerous studies of our group validated the clinical reliability and applicability of this model. As such, we have demonstrated a significant scar-reducing response to steroid injection, collagen-synthesis inhibitors, and TGF-β-antibody blockade. In addition, we were able to reproduce the known scar-reducing effects of silicone in the rabbit ear with a significant SEI-decrease in treated wounds (Figures 3 and 5).[7]

The underlying mechanism through which SGS imparts its beneficial effects on scar tissue remains a subject of speculation. Sawada and Sone[19] believe that hydration of the stratum corneum plays a key role in understanding the therapeutic mechanism of SGS. Suetake et al.[20] demonstrated an increased baseline hydration level of the stratum corneum in HSs and keloids vs. normal skin. However, the same group observed a consistently vanishing hydration effect under continuing SGS-treatment with hydration levels on treatment day 7 nearly as low as hydration levels of untreated control skin.[21] Therefore, hydration as a single factor to explain the effects of SGS remains debatable. Furthermore, Quinn et al.,[22] who reported on the scar-softening and scar-reducing properties of SGS, excluded pressure and temperature or a chemical effect due to silicone absorption as possible modes of action. Occlusion finally returned into focus of scientific interest when a study showed reduced fibroblast proliferation in a keratinocyte/fibroblast co-culture system, in which keratinocytes were hydrated with either water or silicone oil, suggesting that hydration rather than silicone is responsible for the alteration in fibroblast/keratinocyte interaction in vitro.[23]

We were able to demonstrate a significant scar reduction of about 80% in silicone-occluded wounds covered with five layers of Tegaderm® compared with wounds with only one layer. Moreover, we showed that the duration of occlusion in itself is a critical factor causing a scar reduction by 18% in this experiment. Our new data presented here indicate that occlusion might play a key role in explaining the therapeutic mechanisms of SGS and that the oxygen transmission through silicone sheeting is, if at all, of much lower relevance. Atkinson et al. treated surgical incisions of 39 patients with paper tape occlusion and found a reduction in scar volume by 0.16 cm^3. In addition, the odds to develop HS tissue formation in this study were 13.6 times greater in nontaped wounds.[24,25] Most recent in vivo experiments of our group (Kloeters and Mustoe; publication in preparation) employing different methods of partial occlusion, such as paper tape and silicone, resulted in a less hyperplastic epidermis, a reduction in the

inflammatory response in the early stages of wound healing and significant cytokine alterations in the epidermal/dermal crosstalk.[26] Our data in aggregate combined with the co-culture results strongly support the theory that the hydration of the stratum corneum plays a critical role and would support other studies that investigated the role of taping and other methods to achieve partial occlusion.[1,24,25] The stratum corneum plays a critical role as a water barrier and in a healing wound the stratum corneum remains immature for a period of time after epithelialization is complete. The ultrastructural evidence on electron microscopy (Figure 6D) would suggest that the SGS re-establishes the immature stratum corneum as a "normal" water barrier with a restoration of the basal cell layer to a quiescent state characteristic resembling that of mature skin.

In summary, given the evidence presented above, we consider the HS model in the rabbit ear to be the most advanced to date, chiefly as it parallels human scar tissue behavior in terms of morphology and therapeutic responsiveness. It serves as a useful tool to researchers in the field of wound healing with an interest in studying cellular and histologic alterations in HS tissue.

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