Artificial skin and tissue regeneration
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Summary

Tissue engineering and artificial skin substitution is a rapidly progressing field in the centre of the attention of dermatologists, plastic surgeons, burn wound specialists, and pharmaceutical companies and many research groups. The objective of most skin substitutes is stimulation of wound healing, especially in chronic wounds, and diminishment of the time needed for wounds to close (1-3). Surprisingly, little attention was given to effects of artificial skin substitutes on dermal tissue regeneration and to the role of fibroblasts. The experiments described in this thesis focus on these aspects in the healing of full thickness skin defects.

New treatments can only be tested in patients after efficacy and safety have been proven in appropriate animal models. It is unrealistic to think that wound healing studies in animal models can be completely replaced by in vitro models. In wound healing, a multitude of factors is involved, forming a complex interplay of signals that wound cells are receiving. The complexity of signals during early wound healing is reviewed in chapter 1 combining the scientific fields of molecular biology, biochemistry and tissue physiology.

The architecture and complexity of human skin is unique. In non-primates, the skin of the pig seems to be most appropriate model to study skin tissue regeneration, wound contraction and scar formation (4). Important is that new treatments which are analysed in an animal or clinical setting should be evaluated with objective methods which not only allow measurement of the degree of scarring, but preferably also visualize the scar tissue formed. Wound healing can be easily analysed from the exterior, but wound histology seems to be even more important (5).

Scarring not only becomes more severe when the wound depth increases, but also becomes permanent (6,7). Scarring is a problem of dermal molecular architecture and not so much of epidermal regeneration or epidermal-dermal attachment. The latter often occurs in scars from wounds which were treated with cultured epidermal sheets.
The lack or excess of specific proteins and architecture of collagen bundles will determine the quality or severity of the scar.

The Department of Dermatology, Wound Healing Research Group, Academic Medical Centre, Amsterdam, Burns Centre, Red Cross Hospital and Dutch Burns Foundation, Beverwijk have developed a dermal substitute, which consists of a native collagen 3D scaffold coated with 3% elastin hydrolysate (9,10). It provides a suitable substrate for the ingrowth of cells and the deposition of extracellular cellular molecules (ECM). In a pig model, full thickness wounds treated with these substitutes showed a stimulation of dermal tissue regeneration, and a reduction of wound contraction and scar formation (9).

The objectives of the experiments in this thesis were to optimize the healing of full thickness skin defects so that they would heal without scar formation. This was performed by the application of the artificial dermal substitute containing fibroblasts populations and topical dressings. In chapter 2, the healing of wounds treated with the collagen/elastin dermal substitutes combined with split-skin mesh grafts were compared to wounds treated with split-skin mesh grafts without dermal substitutes. Healing processes were investigated and followed by immuno-histochemistry. Results indicated that the dermal substitute accelerated the deposition of chondroitin sulphate basement membrane proteins in the basement membrane zone, induced a faster remodelling of the ECM in the sub-dermal area, and stimulated the regeneration of elastin in this area. This reduction in scar formation was furthermore evidenced by the reduced presence of thin collagen bundles organised parallel with the epidermis.

The presence and localisation of chondroitin sulphate basement membrane protein (probably bamacan) in the basement membrane zone proved to be a good marker to study basement membrane regeneration. Other basement membrane proteins studied in relation to basement membrane regeneration, e.g. laminin, collagen IV and VII, are present within the basement membrane zone already within 14 days after wounding (5,11). However, the presence of these proteins or the presence of hemidesmosomes is related to the formation of anchoring fibrils. Since the regenerated epidermis has to resist to shearing forces, the presence of rete ridges and fibrils extending much deeper in the dermis is more vital (12). Elastin fibrils and the chondroitin sulphate basement membrane protein, bamacan, are likely to have an
important function in the firm attachment of the epidermis to the dermis (13-15). Apparently, the induced stimulation of dermal tissue by the dermal substitute positively influenced the regeneration of a functional basement membrane. In addition, the immunohistochemical parameters described in this chapter proved to be very useful for the evaluation of consecutive wound healing studies. In addition, patient studies investigating scar formation by means of wound histology were often limited to the dermal tissue directly underlying the epidermis (16-19). This study also demonstrated that proper scar evaluations should include the entire reticular dermal layer.

In full thickness wounds, there is a delay of at least 3 days before fibroblasts start to migrate into the wound (20,21). The addition of fibroblasts to a dermal substitute is likely to stimulate dermal tissue regeneration, since fibroblasts are the cells responsible for the synthesis and remodelling of the newly deposited ECM. Indeed, the addition of fibroblasts to the collagen/elastin dermal substitute has been found to stimulate dermal tissue regeneration and reduce wound contraction of full thickness wounds more than the acellular substitute did (22). In chapter 3, the survival of fibroblasts seeded in the dermal substitute was investigated. A relatively low number of fluorescently labelled fibroblasts was seeded and was shown to proliferate after being transplanted in the wound. After 5 days, their cell number had almost tripled. This study proved that fibroblasts seeded in a dermal substitute and transplanted in wounds within a few hours after seeding had survived. This is important, because vascular structures are absent in the dermal substitute (20) and the supply of nutrients and removal of waste products are impaired during the first days after transplantation. In addition, the presence of fibroblasts in the dermal substitute also seems to inhibit the migration of other mesenchymal cells and significantly retards substitute degradation. Cell migration is correlated to proteolytic activity necessary for tissue invasion (reviewed in chapter 1). It is likely that the apparent reduction of mesenchymal cell migration also correlates with the retarded degradation of the dermal substitute. Moreover, this also supports the idea that the control of fibroblast cell migration from the subcutis into the wound bed and their role in fibroplasia could be crucial for the control of scar formation (6). How fibroblasts present in the dermal substitute influenced tissue regeneration and scar formation at
later time points after wounding was investigated in chapter 4.

In chapter 4, living dermal substitutes were created in different ways and compared in the pig wound model. The seeding density of the fibroblasts was varied and instantaneous seeding was compared to a preculture period of 10 days. The length of the preculture period was chosen as the longest period still feasible in a clinical setting (23). Fibroblasts were precultured in the substitute to increase fibroblast deposition of the ECM molecules in the substitute. The best wound healing was observed with the precultured substitutes seeded with the highest fibroblast densities. Three weeks after wounding, this treatment almost completely abolished the presence of myofibroblast positive for alpha-smooth muscle actin. The wounds with the presence of more myofibroblast in the granulation tissue healed with more contraction and scar formation, were not supple, and remained more reddish in colour. Six weeks after wounding, the precultured substitutes seeded with the highest fibroblast densities showed a dermal tissue which consisted of only mature collagen bundles oriented in a basket weave pattern. This was never observed in previous studies.

The improved healing observed with the precultured substitutes seeded with the highest fibroblast density could be related to three factors: the increased deposition of ECM molecules, the numbers of fibroblasts in the substitute or alterations in the phenotypes of the fibroblasts in the cultured substitutes. The increased deposition of ECM molecules is unlikely to be responsible for the observed improvements in tissue regeneration. Histologically, a substantial deposition of ECM molecules was not observed in the substitutes. Furthermore, the substitutes seeded with the highest fibroblast density transplanted instantaneously and the precultured substitute seeded with the lower fibroblast density showed comparable wound healing results. The number of fibroblasts present in the substitute at the moment of grafting did correlate with the improved regeneration of dermal tissue, whereas the percentages of α-smooth muscle actin positive myofibroblasts did not correlate. Fibroblast populations in cell culture always seem to contain a variable percentage of α-smooth muscle actin positive myofibroblasts (24).

These results look very promising for treatment of patients. However, autologous fibroblasts were used in this study and this means that for the treatment of large wound surfaces substantial numbers of autologous fibroblasts would be
required. In this respect, it is also important to note that the seeding of fibroblasts in the dermal substitute at high density resulted in the loss of about 20% of the fibroblasts. Apparently, they leaked out of the substitute. Furthermore, fibroblasts also migrated out of the substitutes during the 10 days of preculture. Future studies should investigate which method is most optimal to obtain the highest fibroblasts densities in the dermal substitute in the shortest culture period. In addition, new developments to improve fibroblast isolation methods and culture conditions would help in the generation of more fibroblasts in the same or shorter period.

The generation of sufficient autologous fibroblasts for dermal substitution implies a delay in wound treatment, which could be solved by the use of allogeneic fibroblasts (25). In chapter 5, the use of allogeneic fibroblast populations for dermal substitution was investigated and compared to the use of autologous fibroblasts. Studies investigating the use of allogeneic fibroblasts for skin substitution in relation to dermal tissue regeneration are presently not published. Most studies only included autologous or allogeneic fibroblasts for their stimulating activity on epidermal regeneration and keratinocyte differentiation (26,27).

The results of this study clearly showed that allogeneic fibroblasts induced specific inflammatory reactions in the granulation tissue, which adversely affected tissue regeneration. After 6 weeks of healing the regenerated dermal tissue of the wounds treated with substitutes seeded with allogeneic fibroblasts showed more scar formation when compared to the wounds treated with autologous fibroblasts. Furthermore, wound contraction was significantly increased in the wounds treated with allogeneic fibroblast populations of which the mixed lymphocyte reactions induced the strongest responses. This study shows that the use of skin equivalents with autologous fibroblasts is preferred to skin equivalents with allogeneic fibroblasts, when the objective is the restoration of dermal skin function without scar formation. For the treatment of chronic wounds, products are already on the market which contain allogeneic fibroblasts (28,29). In those wounds, faster healing rates are considered of more importance than scar formation. Their future marketing strategy is going in the direction of skin substitution and the results of this study is highly relevant for these developments.

In chronic wounds and burns, skin substitution is also accompanied with a high
risk of failure due to bacterial infection (30) and the presence of excess of proteolytic activity (31-33). This could result in the loss of expensive autografts and even reverse the healing process resulting in increased scar formation. In the past, the infection control with antimicrobials has been shown to impair wound healing, which was explained by their toxicity for cells (34,35). The use of antibiotics is less favoured because of the danger of bacterial resistance. In chapter 6, a wound dressing, cadexomer-iodine ointment, containing the strong antimicrobial iodine was applied to full thickness wounds and wound healing was investigated in time. The cadexomer-iodine ointment was compared to two treatments being cadexomer without iodine and saline gauzes. In the past iodine containing wound products proved to be toxic for cells in a dose dependent fashion (36). The cadexomer-iodine dressing contains a slow iodine release mechanism. This mechanism proved to control the iodine concentration in the wound at sufficiently low levels so that adverse effects on wound healing were not observed. In fact, the released iodine of the ointment stimulated epidermal regeneration when compared to both other treatments. The stimulation of epidermal regeneration was concluded after observations showing the presence of a thicker epidermis, more and larger rete-ridges and the deposition of chondroitin-sulphate basement membrane protein. Systemic iodine absorption was observed but remained far below systemic toxic levels and was cleared from the blood stream within one week after treatment cessation.

The development of the artificial dermal skin substitutes has learned us more about wound healing and tissue regeneration. The fibroblasts play a central role in tissue regeneration. The understanding of fibroblast behaviour and differentiation will not only help in development of engineered tissues, but could also result in the discovery of new anti-fibrotic agents. The latter has important implications for the treatment of fibrotic diseases, e.g. scleroderma, and fibrosis associated with inflammatory reactions in organs.

In the next years, the artificial skin will find its way into clinical practice. This clinical implementation will raise new problems. These problems will concern: nutrition of the cells in living skin substitutes after the first days of transplantation; the control of bacterial contamination and proteolytic activity; the harvesting and culture of autologous cells. In the clinic, wound aetiology differs so much that the ideal
wound treatment should be able to interact with the wound environment and respond to it in an adequate manner. This can only be achieved by the use of skin cells in combination with a dressing providing nutrients for the cells, and allowing the control of bacterial contaminations and excess inflammation.

**References**


