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INVITED REVIEW

Keratinocytes in the treatment of severe burn injury: an update

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Key words

Artificial skin; Burns; Cultured keratinocytes; Epidermal autograft; Skin substitutes

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Abstract

Burns are among the most life-threatening physical injuries, in which fast wound closure is crucial. The surgical burn care has evolved considerably throughout the past decennia resulting in a shift of therapeutic goals. Therapies aiming to provide coverage of the burn have been replaced by treatments that have both functional as aesthetic outcomes. The standard in treating severe burns is still early excision followed by skin grafting. The use of cultured keratinocytes to cover extensive burn wounds appeared very promising at first, but the technique still has several limitations of which the long time to culture, the major costs, the risk of infection and the need for an adequate dermal layer limit clinical application. The introduction of dermal substitutes, composite grafts, tissue engineering based on stem cell application have been advocated. The aim of this review is to assess the use of cultured keratinocytes in terms of technical aspects, clinical application, limitations and future perspectives. Cultured keratinocytes are expected to keep playing a role in wound healing, especially in the field of chronic wounds. In severe burns, despite its limitations, keratinocytes can be beneficial if implemented as one of the elements in a broader wound management.

Introduction

Burns are one of the most complex acute wounds to treat (1). The severity and the prognosis of survival strongly depend on the total burned surface area (TBSA), the age of the patient and the association of inhalation injury (2,3). The treatment of burn wounds is primarily based on TBSA and burn depth.

Recent progress in the management of shock, infection control and organ dysfunction combined with better nutritional support lead to an important decrease in mortality after severe burns. Nowadays most patients survive the initial phase, resulting in a clear shift of the therapeutic focus from 'survival' to 'quality of survival' (4,5). In addition to rapid wound closure, the aesthetic aspect of the resulting scar has become increasingly important (6).

Depending on the need for surgery, burns are classified as 'superficial' or as 'deep'. In the so-called deep burns, the

dermis is damaged to such an extent that the wound cannot heal spontaneously without extensive scarring, and therefore surgical treatment is required (7).

The gold standard for deep burns is early excision followed by wound coverage (8–10). This is usually achieved using autologous split-thickness skin grafts (STG), harvested

Key Messages

- the technique of cultured keratinocytes is prone to several limitations such as the long culturing time, the uncertain take rate and the high cost
- cultured keratinocytes results in more significant scarring in deep burn injuries because of the lack of dermis; therefore a combination with a dermal layer might be preferred

- skin substitutes combined with cultured keratinocytes may contribute to a better treatment of burns, chronic wounds and congenital skin diseases
- incorporation of endothelial cells, melanocytes and/or cells originating from appendages or even stem cells in keratinocyte cultures might improve functional and esthetical outcomes after burn surgery

elsewhere on the not burned part of the body of the patient (1). However, the limited number of donor sites in severely burned patients and the risk of contraction, due to the lack of dermis, remain the biggest obstacles. Skin expansion techniques (e.g. mesh, meek) result in even more scarring (7,9). Full-thickness skin grafts (FTG) give a better result because of the inclusion of dermal tissue, but the indications are restricted to small wounds on functionally important places (hands, face) since the donor areas have to be closed primarily and are therefore scarce (4). Skinflap-surgery can be executed when treating burns because an optimal result in terms of aesthetics and function can be accomplished. However, the complexity of the procedure and the substantial donor morbidity limits its use (4).

Mainly because of the lack of donor skin, alternative techniques to obtain rapid wound closure have been explored, such as cultured keratinocytes. The objective of this article was to provide a review on the application of cultured keratinocytes in burn care, covering the development and optimisation of the culturing technique, the advantages and the limitations of the clinical application as well as its future perspectives. The availability of evidence for each of these items is assessed.

Historical overview of the development and the initial use of keratinocytes

One of the most important milestones in the history of burn care is the introduction of skin grafting (STG and FTG) initially described by Reverdin in 1869. He observed epithelial islands during the healing process of burned skin: confluence of those epithelial islands resulted in accelerated wound healing, even when these islands were artificially grafted on the granulating tissue. Numerous clinical experiments showed that healing is more rapid in a clean wound bed and that the thickness of the graft is important (the thinner the graft, the faster wound closure was realised). Complete coverage of the wound with grafts seemed to result in better scar quality, suggesting the need for larger grafts (7,9).

In the meantime, epithelial 'cell-seeding' was explored. After scraping the skin of a patient's forearm, the resulting pulp was applied on the wounds. This resulted in less donor-site morbidity and a more regular healing pattern since cell clusters seemed to attach better to the wound than conventional skin grafts (7,9).

The next step in skin grafting was the use of STG harvested with a dermatome or a scalpel. These grafts rapidly became established as the gold standard in surgical burn care (7). Later on, the FTG were introduced (11). In 1964, Tanner unleashed

a revolution by developing the technique of meshing a skin graft: a simple procedure to expand the autograft to a bigger surface. Combination of meshed grafts with cadaveric skin, the so called '*Sandwich-technique*' first advocated by Alexander in 1981, became the standard treatment of severe burn patients (7). However, expansion techniques result in more visible scarring because of the slower healing and persisting grid image caused by contraction. Moreover, the meshing technique cannot deal with the shortage of donor sites in the most extensive burns. Because of these limitations, techniques have been developed to multiply individual skin cells, more particularly the keratinocytes (9).

A first step towards expansion of keratinocytes was the possibility to separate dermis and epidermis using trypsin without compromising cell viability. The next milestone was the finding that keratinocytes could survive in tissue culture. Driven by these discoveries, Rheinwald and Green were the first to describe serial cultivation of strains of human epithelial cells in 1975. Differentiation of confluent cells resulted in the formation of cultured epidermal autograft (CEA) (12–14). Using this technique, it became feasible to culture large sheets of keratinocytes starting from a small skin biopsy (7,13). Clinical application in a burn patient was realised for the first time in 1981 (7,15–17). Since then, several studies reported on the use of CEA grafts in the treatment of burns, various skin diseases and difficultly healing skin ulcers (18).

However, because of significant shortcomings, the initial enthusiasm was quickly tampered. One of the main problems was the development of scars and contractures due to the lack of dermis (19). This resulted in further research concerning the development of dermal substitutes. Yannas and Burke defined the conditions for a perfect skin substitute which eventually resulted in 1981 in the development of the first and probably best known dermal analogue, Integra® (7,11,14,20).

The concept 'Tissue Engineering' has been defined in 1987 by the US National Science Foundation (21). This concept comprises the construction of new tissue by combining cells with a scaffold and growth factors (10,21). Nowadays research focuses on further improvement of culturing techniques and the combination of keratinocytes with a scaffold to form skin equivalents.

Current use of keratinocytes

The culturing technique

A small skin biopsy of 2–4 cm² is taken from the scalp, the axillar, inguinal or retro-auricular area (9,22). After separation of the epidermis of the underlying dermis, further trypsinisation breaks the bonds between the keratinocytes which results in a cell suspension. This suspension was traditionally plated on a feeder layer of (mouse) fibroblasts, pre-treated with radiation or mitomycin-C to overcome the overgrowth of fibroblasts by converting them into an irreversible post-mitotic state (23). The cells were cultured in a medium containing fetal calf serum enriched with growth factors, spore-elements and hormones (9,24,25). Newer culturing techniques realise graftable epithelia with excellent clinical application by using non-irradiated (human) fibroblasts or by using coated culture

flasks (26). *In vitro* keratinocytes are stimulated to divide after 22–24 hours, a process which takes much longer *in vivo*. At 'preconfluence', cells are separated from the culture flask with trypsin and subsequently sub-cultured in a secondary and tertiary culture. At 'confluence', the cells are detached of the culture dishes with dispase, and placed onto petrolatum gauze or another carrier, which makes them ready for clinical application. An alternative is the production of spray grafts, in which a concentration of cells is applied in a solution (9,27–29). In some countries, the autologous cultured keratinocytes are provided by a skin bank or a specialised keratinocyte bank of a (university) hospital. Skin biopsies are also processed by commercial enterprises resulting in autologous cell products with trade names such as Epicel[®], Laserskin[®], Vivoderm[®], Cellspray[®], Bioseed[®] and Epidex[®] (30).

The major drawbacks

Time to culture

Normally it requires 3 weeks to expand a 2 cm² biopsy to a surface, sufficient to cover the whole body-surface (1.73 m²). Some studies showed that culturing skin of the elderly (>60 years) takes still more time to reach confluence (25,31).

This delay presents a serious limitation to the clinical use because it increases the risk of complications like malnourishment and infection (18,32). When infection occurs, the process of engraftment of cultured skin is jeopardised. Therefore, the excised wounds have to be covered temporarily with available allografts and/or xenografts.

In spite of progress in preparation and culturing techniques, it still takes quite some time to carry out the numerous, delicate and time-consuming manipulations, in extremely sterile circumstances (9,27,33,34).

Take ratio

Graft take is a complex process depending on revascularisation and survival of the epidermal cells. Reported take ratios of CEA vary from 0% to 100%. The take ratio is related to factors like wound bed preparation, the presence of dermal elements, manipulation of the CEA, sepsis and post-grafting complications like infections, mechanical pressure and graft displacement (7,24,32,35). Early graft failure is mainly attributed to a bad general condition of the patient with the presence of infection (36). Younger age is associated with higher take rates, probably due to the higher proliferative potential of young keratinocytes (22). Several previous studies also showed a smaller TBSA in children (37). Combined with the shorter culture time of young keratinocytes, this makes patients younger than 15 the preferred population for production of CEA (15).

Scar formation post-grafting

Scar formation and disfigurement have a negative influence on the self image, the self-confidence and on quality of life in general (9,38,39).

In all deep burns, scar formation is problematic, because clinical pictures ranging from chronic ulceration to

hypertrophic scarring are seen (40,41). The presence and degree of hypertrophic scarring is directly correlated to the time needed to heal. The long healing time with cultured keratinocytes results in an excessive accumulation of extracellular matrix (ECM) and important scarring (41). As scar formation and contraction are inversely proportional to dermal thickness of the graft, the CEA results in more significant scarring because its lack of dermis (19,42). Therefore, the use of cultured keratinocytes should be limited to wounds with a sufficient dermal wound bed. Factors like the graft bed, the TBSA, the patient's age, the location of the graft and even the inter-individual variation need to be considered too (43).

Costs

The culturing of keratinocytes has to be performed by highly skilled staff in specialised laboratories with clean room facilities, resulting in considerable costs (27,44). The additional cost, especially in comparison with other treatment modalities, is difficult to quantify. The total cost of burn care, in general, is difficult to assess due to the combination of both direct and indirect costs. Direct costs comprise hospital care, primary care, emergencies, medicines and material costs. Indirect costs contain the loss of productivity and social costs because of premature death or inability. As the number of patients per year is relatively small and CEA are considered to be a lifesaving procedure, the (additional) costs are accepted by the society (22).

Graft site malignancy

Malignant transformation is known to occur in scars of burn wounds. Squamous cell carcinoma is the most common histological type (45). Chronic ulcerations, caused by increased trauma or tension of the scar tissue, are thought to be responsible for the development of malignancy. To our knowledge, only one case of graft site malignancy has been reported in a patient treated with CEA (46). In this case, the additional stress placed upon the cultured skin overlying the joint could have contributed to the repeated ulceration, which eventually led to malignant transformation. Another theoretically possible contributor to the malignant degeneration of the grafted epithelium is the use of mitogenic growth factors, during the *in vitro* expansion phase of the cultured epidermal autografts, which could be a reason for concern (46). However, the low occurrence of malignancy in wounds treated with CEA makes the latter less likely.

Long-term fragility

The long-term stability of CEA is less than a standard STG (34). Electron microscopy showed that the complete absence of a dermal layer in the CEA causes fragility on long term (47). The lack of dermis leads to a delay in wound bed adherence, which combined with a delay in rete ridge formation results in easy bruising and blister formation within the first months after application (17,47). Careful early mobilisation, moisturizing and the use of compression garments in

the early phase are therefore strongly recommended. Besides mechanical and infectious causes for graft loss, some investigators also describe an immunological component to be responsible for blistering and ulceration of initially healed wounds (18).

The combination with dermal components and the use of composite grafts has been explored as possible solutions for the above mentioned post-grafting problems (15,34,35). Adding a dermal substitute underneath a meshed STG or cultured keratinocytes makes the grafting procedure more complex but results in significantly less scarring since the dermo-epidermal junction is reconstructed (30,42,48–50). Nowadays, several dermal substitutes are available (4). There are acellular substitutes on one hand and cellular ones that contain allogeneic or autologous fibroblasts on the other hand (10,13,20,21,51,52). The available dermal substitutes have been discussed extensively elsewhere (4). Yet, the advantages of the combination of CEA with dermal analogues led to the development of composite grafts: full-thickness skin substitutes consisting of cultured keratinocytes, dermal matrix components and fibroblasts. The production process of composite grafts usually comprises several phases: in a first step, the cellular elements of a dermal allograft are removed until a relatively inert acellular dermis is left, this is followed by the addition of cultured keratinocytes (42,52). Studies show that this approach improves CEA-take, but up till today there is no consensus about the most optimal product on long term (13,38). The first clinical results have been rather disappointing because of the higher density of keratinocytes needed to inoculate the surface of the scaffold and the high cost related to the expensive production process and quality assurance (17).

Another possibility to circumvent some of the problems listed above is the delivery of preconfluent cultured keratinocytes (29,32). These cultures show evidence of high mitotic activity and subsequently efficient take to the wound bed (29). Confluence and differentiation are achieved *in vivo* at a later stage (32). The advantages of using preconfluent cultures are the larger number of more proliferative keratinocytes that can be applied to the wound, after a shorter culture period (5–7 days) (18). Furthermore, the potentially damaging enzymatic separation from the culture vessel is avoided which results in a better clinical outcome (32). To transfer preconfluent keratinocytes to a wound, a delivery system is required. Numerous delivery systems have been described (fibrin glue, a collagen-glycosaminoglycan matrix, an aerosol spray) but it is not readily apparent as to which method is the most efficient, because most studies today are limited to animal wound bed models (32).

Recent developments and future perspectives

Although the primary goal in patients with burns is survival, the ultimate goal in wound healing is to repair all skin functions like sensitivity; elasticity; normal structure and function of appendages, pigmentation and at the same time to obtain optimal cosmesis (53). As most skin substitutes only consist of 1 or 2 cell types, they are not able to repair every function of authentic skin. Therefore, several research groups

have tried adding other cell types to the substitutes. Although these advancements will especially be beneficial for other skin conditions like pigmentary abnormalities, they may in the future also contribute to better burn management.

Addition of melanocytes

Application of CEA often results in absent or irregular pigmentation of the skin, which is associated with deficiencies in solar protection and with an unaesthetic colour match (30,54,55). It has been postulated that co-culturing of keratinocytes and melanocytes could solve these problems; while the melanocytes guarantee the pigmentation, the keratinocytes ensure a fast healing and a smaller risk of scar formation (30). This technique, possibly in an aerosol application, could provide a means to treat patients with pigmentary abnormalities like vitiligo, but could also be used in burn treatment (29,56,57).

Addition of endothelial cells

Cultured keratinocyte sheets have no vascular plexus, consequently vascularisation is slower than with a STG. Owing to the absence of blood supply and the consequential lack of nutrients, cultured skin is more susceptible to microbial contamination and takes failure. A possible solution is to incorporate human endothelial cells into the skin grafts to facilitate angiogenesis. Studies showed however that only few endothelial cells survive the culturing process and that the keratinocytes grow even slower in this context (1).

Recently, work has been conducted to promote vascularisation by using genetically modified keratinocytes, which overexpress vascular endothelial growth factor (VEGF) (29).

Addition of appendages

There have been very few reports on human skin equivalents with integrated pilosebaceous units. The majority of skin equivalents with hair follicles and sebaceous glands have been developed as models that can help to understand the transport route of active substances and form a breakthrough in the topical treatment of different skin diseases (21,58).

Skin regeneration with stem cells

Regenerative medicine based on stem cells might further improve the treatment and outcome of burns. Current understanding of stem cell isolation combined with the progress made in tissue-engineering makes this closer to clinical reality (15).

Wound healing is a complex process that involves coordinated efforts of cells of different origin and various cytokines (59). Therefore, it is neither practical nor realistic to identify every single component to assemble new skin. The advantage of using stem cells lies in its power to simplify the different components needed in the initial stadium and to use the intrinsic engineering capacity of the cells to lead the regenerative process.

Bone marrow and fat are considered to be the richest sources of human mesenchymal *multipotent* stem cells (MSC) (60,61). The combination of MSC and a skin substitute onto deep burns results in increased wound healing and neo-angiogenesis. The healing process of donor sites and chronic venous ulcers also improved spectacularly after topical application of MSC (61,62). The chronic wound changes into an acute wound that heals or can be treated more easily with a skin graft. However, bone marrow mesenchymal stem cells need to be harvested under general anesthesia, which may lead to severe complications (63).

Recent research suggests that adipose-derived stem cells (ASC) represent an alternative source of multipotent cells with similar characteristics to bone marrow stem cells (63). Numerous publications emphasise that the high abundance and easy accessibility of this particular stem cell source, makes its use in tissue-engineering up-and-coming (59,63–65). Additionally, lipofilling might also become a valuable technique to reconstruct the subcutaneous layers.

When combined with (sprayed) cultured keratinocytes, a new bilayered skin substitute can be developed (66). Although the effects of ASC on cutaneous wound healing are not completely understood, recent reports showed that ASC have great promise as a powerful source of skin regeneration, as they do not only provide cellular elements, but also numerous cytokines. (59,63). Lee *et al.* showed that autologous ASC improve the healing of full thickness wounds in mice (63). Further randomised controlled studies in human wounds of different origin (burns, venous ulcers, diabetic wounds) are necessary to further evaluate the performance of the ASC.

Another largely untouched source of stem cells is the *pluripotent* stem cells in embryonic and fetal tissue, for example, human umbilical cord blood. The naive immune status and the unshortened length of their telomers can have an enormous impact on medicine (10,67). Nevertheless, ethical objections put the use of embryonic stem cells to a stop.

As the field of regenerative medicine using stem cells and gene therapy is fairly recent, further evaluation of genetic modified cultured skin and of the use of stem cells is necessary to improve wound healing modalities and to make new treatments possible for cutaneous diseases and systemic deficiencies (67).

Discussion

When treating burn wounds the primordial goal remains survival of the patient, while a good functionality and a decent aesthetic outcome come in second and third place. Increased insights in the pathophysiology of burns and the development of new therapeutic methods have lead to a significantly improved survival of burn patients. One of the biggest challenges remains the development of an ideal replacement of the damaged skin. The gold standard for deep extensive burns remains early excision and coverage with a temporary dressing followed by a more permanent skin replacement. Today no substitute exists that is able to replace all functions of intact skin, and the limited amount of the patient's own skin urges the search for good alternatives.

The technique of cultured keratinocytes has been refined over the last 30 years, but remains prone to several limitations. The prophecy that cultured keratinocytes would lead to fast and easy covering of deep and large burns, is still not fulfilled (10). Especially the long culturing time, the fragility of the grafts, the uncertain take rate, the high cost and the risk of scars and contractures make CEA only an additional tool in the treatment of burn wounds, not an alternative (17). The enormous potential of this technique, however, initiated many attempts to solve the previous mentioned shortcomings. The development of dermal substitutes and composite grafts resulted in a significant improvement of the post-grafting scar formation (7,20). Some of these *in vitro* produced skin substitutes are nowadays combined with cultured keratinocytes and can contribute to an improved treatment of burns, chronic wounds and congenital skin diseases.

Future research has the objective to improve the morphology and physiology of skin substitutes (14,54). Several groups continue to search for a more advanced skin substitute by experimenting with the incorporation of different cell types such as endothelial cells, melanocytes and/or cells originating from appendages or with the incorporation of stem cells, for example, fat-derived stem cells in the skin substitutes (4,14).

To conclude, keratinocytes will probably remain useful in burn care although the focus will probably shift to other substitutes combined with keratinocytes.

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