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The use of Keratinocytes: Things we should keep in mind!

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Summary

Background Cultivation of keratinocytes and their clinical application was an essential step towards the development of new treatment concepts for patients suffering from severe burns and chronic cutaneous wounds. The aim of this review is to give a current overview of keratinocyte cultivation and keratinocyte application under experimental and clinical conditions as well as to discuss their limitations, complications and future perspectives.

Methods Pubmed and Medline was systematically searched for correlative publications.

Results Promising at first, but over time its limitations became evident: demanding infrastructural requirements, high costs, lack of "ex-vitro" stability, additional requirement for dermal support, and the absence of other skin cell types or appendages have limited the introduction in daily clinical routine. Nonetheless, continuous efforts have been made in the past decades in order to improve the application of keratinocytes. Novel techniques of keratinocyte harvest and cultivation have simplified the clinical application, improved stability, and consequent outcomes.

Conclusions The herein presented efforts despite their drawbacks during the development process and resultant clinical outcomes of keratinocyte culturing bear a solid foundation for the future. The ultimate challenge however remains, bringing any of these efforts into clinical practice while avoiding the failures of the past.

Keywords: Keratinocytes, Tissue engineering, Epidermal replacement, Dermal substitutes, Wound healing

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Overview

The skin is the body's largest organ representing approximately one-tenth of the body mass. It is the body's first line of defence against infection and dehydration and has a considerable role in temperature control. The relative outermost external exposition of the skin as the body's cover makes it extremely vulnerable to frequent external assaults. The ability of skin to repair itself following minor injuries is remarkable, but in more severe destructions of the skin's integrity, medical intervention is frequently required in order to protect the body from further fluid loss and infection, and also to accelerate recovery.

Thermal injuries, chronic ulcerations secondary to diabetes mellitus, venous stasis and trauma have been the main causes for loss of skin integrity and severe morbidity [1].

Over the past decades an enormous progress in the treatment of cutaneous injuries has been achieved, predominantly by the development of methods and techniques like keratinocyte culturing, tissue engineering, skin substitution and wound dressing materials [2].

There are just a few types of tissue being able to propagate their cell types, bearing the potential of stem cells. The epidermal layer is one of them and is predominantly made of keratinocytes, which under in-vitro conditions can differentiate into another stratified structure [3].

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The technical achievements of the last 20 years have made it possible to analyse the growth and differentiation of pathological epidermal keratinocytes in culture, and offered a new way of testing strategies for therapeutic intervention [4].

The aim of this review is to give an overview of different keratinocyte cultivation protocols, of skin tissue engineering and its application in experimental and clinical research as well as to discuss their limitations, complications and future perspectives.

Skin anatomy and histology

The skin is an organ with a complex structure and function consisting of two mutually dependent layers, the epidermis and dermis, which rests on a fatty subcutaneous layer, the hypodermis. Each layer is derived from a distinct embryonic origin, epidermis originates from embryonic ectoderm; dermis and subcutaneous tissue develop from mesoderm [5, 6].

Epidermis is the outermost layer of the skin, an avascular, stratified squamous layer, which consists of various distinctive layers. The majority of the epidermis is made up of keratinocytes (about 90 % of the epidermal cells). Keratinocytes are responsible for the cohesion of the epidermal structure and the barrier function. It also contains melanocytes (responsible for pigmentation and originating from the neural crest), Langerhans cells (immune response cells of mesodermal origin,) and Merkel cells (responsible for sensibility, notably pressure and originating from the neural crest).

The dermis is connective tissue that is responsible for the mechanical properties of the skin. It is composed of fibroblasts of mesodermal origin plus extracellular matrix proteins. Collagens are interwoven with elastin, proteoglycans, fibronectin and other components. The epidermis and dermis are connected by a basement membrane that is composed of various integrins, laminins and collagens. The superficial papillary dermis is arranged in a ridge-like structure called the dermal papillae, which contains microvascular and neural networks and extends the surface area for these epithelial mesenchymal interactions. Many appendage structures are present throughout the skin; the most important of them are the sebaceous glands, eccrine glands, apocrine glands and hair follicles, all of ectodermal origin and developed as down growth of the epidermis into the dermis. In addition, the dermis also contains blood vessels and lymphatic vessels of mesodermal origin, and sensory nerve endings of neural crest origin.

Finally, the hypoderm is primarily composed of adipose tissue and separates the dermis from the underlying muscle fascia [5, 6].

In case of severe skin damage like third degree burns with total loss of all skin layers and skin structures, it is more difficult to obtain fast wound coverage and thereby good functional and aesthetic results. In these cases, it has become common practice to perform a combined reconstruction (combination of dermal substitutes with cultured keratinocytes or split-thickness skin grafts (STSG)). It has become possible to harvest and cultivate almost all skin cell types (e.g. keratinocytes and melanocytes etc.), but it is not possible to cultivate an entire full thickness skin patch containing all cell types [7].

Development of the clinical use of cultured keratinocyte

In 1953, Billingham and Reynolds have examined the principal potential of epidermal cell suspensions or epidermal sheet grafts and thereby their potential use for wound or defect coverage [8].

In the 1970s, Rheinwald and Green [9, 10] were the first scientists who were able to culture keratinocyte under laboratory conditions; their method has led to the production of cultured epidermal autografts (CEA), which were then applied in daily clinical routine. CEAs normally consist of 3–5 cell layers (Fig. 1). Their method has been applied in numerous patients suffering from deep burns during the 1980s, and also found its way to

Fig. 1 Histological cross section of a cultivated keratinocyte sheet graft (keratinocytes cultivated on a fibroblasts containing fibrin matrix)
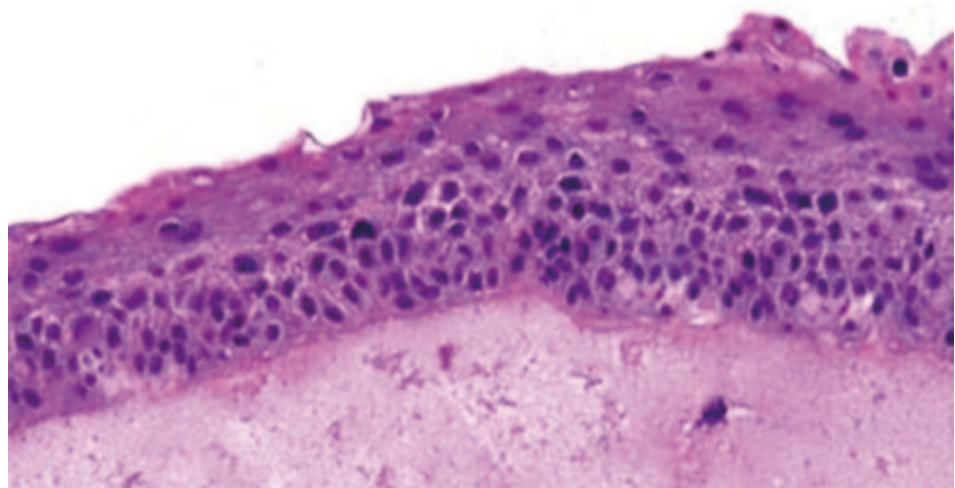
Fig. 2 Long term result (12 month after operation–tangential excision and grafting with keratinocytes) of a deep dermal hand burn

the treatment of chronic skin ulcers as observed in diabetic ulcers and various other skin diseases [11–13] (Fig. 2). This technique made it possible to culture large amounts of CEA sheets from a tiny skin biopsy. The obvious problem of these CEAs was the inherent absence of vascularised dermal support, which resulted in a fragile wound coverage and thereby often in severe wound contraction and hypertrophic scar development. In sum, the overall long time results for the treatment of deep burns were rather disappointing [14, 15].

In 1981, Yannas and Burke [16] developed the first dermal substitute, commercially better known under the name Integra® consisting predominantly of bovine collagen and shark chondroitin sulphate covered by a silicon membrane as a temporary epidermal replacement and coverage. Following its application onto a wound, the outer silicone layer was removed after its complete vascularisation and then covered with a split-thickness skin graft or CEA and other related tissue engineered materials [16].

In the beginning, cultured cells were directly applied to the substitute but these techniques mostly failed due to lack of cell adherence to the dermal substitute. This lead to the development of skin tissue-engineered autologous keratinocytes, fibroblasts and bovine collagen sheets, which improved adherence to the substitute covered wound bed [17, 18].

The term tissue-engineered skin comprises the production of a wholly new skin layer by combining keratinocytes with a scaffold or even culturing techniques to form high-quality skin "out of the in-vitro box" for replacing damaged or diseased tissues [19].

Current methods of harvesting and cultivating human keratinocyte

Methods of keratinocyte isolation and cultivation can be divided into the following main categories:

1. Detaching a purely epidermal sheet from a healthy (unaffected) donor site [20, 21] and culturing a single cell culture or co-culture of keratinocyte in combination with 3T3 cells as a feeder layer.
2. Cultivating the keratinocytes isolated from the outer root sheet (ORS) of plucked scalp hair follicles.

3. Differentiating keratinocytes from stem cells resources, such as umbilical cord blood (UCB), bone marrow (BM) and embryonic stem cell (ESC).

The degree and type of cutaneous damage usually determines the most suitable method for cultivation. Almost all of the methods mentioned can theoretically be applied in the treatment of cutaneous wounds, but in reality just a few of them have been used in clinical trials. The most commonly used method is to take a skin biopsy of 2–4 cm^2 from an appropriate area, to separate the epidermis from the dermis, and to bring the basal epidermal cells into suspension. The cell suspension is plated on the flasks, which can be pre-seeded e.g. with mouse fibroblast (3T3 cells) as a feeder layer. Mouse fibroblasts must be pre-treated with Mitomycin-C or gamma-irradiation to overcome the overgrowth of fibroblasts by converting them into an irreversible post-mitotic state [22]. Human dermal fibroblasts can also be applied as feeder layer, as supported by previous studies demonstrating good clinical application in the presence of non-irradiated human dermal fibroblast [23, 24]. The culture medium commonly contains fetal calf serum (FCS) and other supplements such as hormones and growth factors [9]. This kind of culturing may lead to preparation of the keratinocytes as a cell suspension or as epithelial multilayer sheets (Fig. 1).

Releasing the keratinocyte sheets from flasks is often achieved by the enzyme Dispase®, which is hypothesized to remove some surface proteins from keratinocytes, therefore reducing the cells' adhesion potential [25]. These with Dispase® treated keratinocyte sheets are very fragile and difficult to handle, so they should be placed onto a supportive material such as petrolatum gauze, fibrin layers or other carriers during transport and before actual grafting in a clinical setting [19, 26, 27] (Fig. 1 and 2).

Therefore, keratinocyte suspensions have become easier to handle, especially as spray grafts [26].

In some countries, specialised (in-hospital) skin banks provide the logistics for cultured epithelial keratinocytes [28–30], but also commercially available kits for autologous cell transplantation have become available (e.g. Epicel®, Laserskin®, Cellspray®, Bioseed® and ReCell®) [1, 19].

The second method, ORS harvesting from hair follicles, has been a readily available source of keratinocytes

with a high proliferative potential even in donors of age [31]. The ORS is the external layer of the hair follicle that maintains contact with the surrounding basement membrane [32]. Hair follicle stem cells (SCs) are typically quiescent, but they can be stimulated and have the ability to form colonies under in-vitro conditions. These SCs have been found to be located in a discrete location in the hair follicle called the bulge [6]. The bulge SC is a reservoir of pluripotent SCs, which can be recruited during wound healing and epidermal repair. Therefore, ORS SCs can be regarded as precursor cells for interfollicular epidermal keratinocytes [32, 33].

Plenty of studies have been planned to determine the optimal way of isolation and cultivation for ORS SCs, some of them have even become available as commercial products like EpiDex® [34, 35].

The methodology, in brief, consists of plucking hair follicles from the scalp, selecting cells in the anagen phase under a dissecting microscope and finally explanting them onto a microporous membrane of cell culture inserts with post-mitotic dermal fibroblasts as feeder layer. Approximately, after 2 weeks the confluent ORS keratinocytes and can be detached with trypsin. The culture medium is normally Dulbecco's modified Eagle's medium DMEM/F12 (3:1) supplemented with fetal calf serum or human AB serum, growth factors and hormones [33, 34].

The third method or source of keratinocytes are SCs, well known for their substantial preserved capacity of "self-renewal" and ability to differentiate into more than one cell type, representing an inexhaustible source of donor cells [36].

Keratinocytes can be derived from haematopoietic SC (HSC) of peripheral blood flow or UCB as well as ESC, BM mesenchymal stem cells (MSC) and fat or adipose tissue derived stem cells (ADS) [37–39].

The main factors, which drive these SCs to differentiate into keratinocytes, is the presence of culture supplements or special factors released by keratinocytes [37].

BM and subcutaneous tissues are the two main sources of human MSCs [40]. Facilitated accessibility of ADS in comparison to BM SCs makes it a alternative source for pluripotent stem cells [38]. Recent research demonstrated the capability of these type of MSCs to differentiate into keratinocyte and dermal fibroblasts under in-vitro conditions, and even lead to improved cutaneous wound healing and neo-angiogenesis in chronic wounds in mice and rats [38, 40–43]. But further, even clinical research is needed in order to prove its feasibility in future.

Pluripotent ESC, e.g. from the umbilical cord blood, are another possible source [19, 44]. Their high potential for proliferation and differentiation derive from the preserved length of their telomeres (the region of repetitive nucleotide sequences at each end of a chromosome) and put these cells into focus of research initiatives, notably in regenerative medicine. Nevertheless, ethical hurdles have limited the application of human ESC in clinical trials [19, 36, 45].

Clinical and laboratory application of various culturing methods

The most commonly applied clinical method, notably in burns or diabetic ulcer treatment, is transplantation of cultured autologous keratinocytes either as a spray (keratinocyte cell suspensions) or as a sheet graft (CAE). Keratinocyte cell suspension sprays (e.g. ReCell® and Cell spray®) are a fast and reliable method, particularly useful in the treatment of superficial dermal burns, moderate wounds, scar revision surgery, skin resurfacing following dermabrasion and vitiligo [1]. Cultured keratinocyte sprays have also become available and were used therapeutically in severe burns and chronic wounds, either alone, or in combination with meshed skin-autografts [46] or on a fibrin matrix [47].

The CAE method was developed before autologous keratinocyte sprays were available. The processing takes atleast 3–5 weeks in order to culture adequate amount of epidermal cells from a skin biopsy. Regardless of their source, CEA or sprays have a lack of dermal structures, making them fragile and thereby not optimal for the coverage of full thickness defects [15, 48, 49].

ORS keratinocytes were also used for the treatment of leg ulcers and chronic wounds, and demonstrated closure of chronic wounds within 8 weeks after transplantation in almost 50 % of the cases, and almost 40 % reduction in wound size in the rest of the wounds [31, 33]. As previously mentioned, the reason for the limited use of stem cells under clinical conditions is mainly based on ethical considerations and legal restrictions, although first results were very promising [41, 50, 51].

Advantages and disadvantages

The main advantage of the application of cultured keratinocytes in order to improve cutaneous wound healing is that there is almost unrestrained possibility of cultivation; and possibility to cover a large body surface area with cells harvested from a small skin biopsy, from ORS or even from a SC resource within a short period of time (almost 3–5 weeks).

Nevertheless, all of these methods and their clinical applications have limitations. The lack of a vascularised dermal support (in case of isolated keratinocytes transplantation and in case of full thickness defects), can lead to a fragile wound coverage and thereby to wound contraction and hypertrophic scars. This factor was one of the previously mentioned drawbacks or limitations for its clinical usage. This problem has been partially solved by the development of dermal substitutes, and by the technique of combined reconstruction (Fig. 3).

Dermal re-establishment is fundamental for the long time results, and thereby for the overall outcome. Dermal matrices are predominantly composed of extracellular matrix components such as hyaluronic acid, collagen and glycosaminoglycans or synthetic polymers [52, 53]. Dermal reconstruction can be performed in a one- or
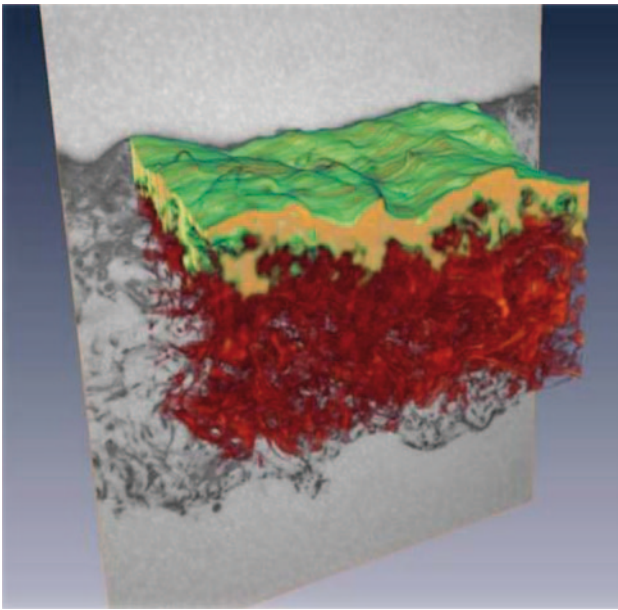
Fig. 3 3D image (HREM): keratinocytes cultivated on a collagen–elastin matrix

two-step approach; depending upon whether the cultured keratinocytes or split thickness autografts will be applied within the same operation or in the second one. Commercially available dermal matrices are Integra®, Matriderm®, ProDerm® and Hyalomatrix® [1, 52, 54].

However, the co-application of dermal matrices and cultured keratinocytes still bears its inherent limitations and possible complications.

First of all the factor timing is crucial—it has already been mentioned that it takes atleast 3–4 weeks to culture CAEs suitable for coverage of large defects, and this period can be a crucial time for patients suffering from large burns due to the lack of the skin's first defence system, and therefore having higher susceptibility to sepsis [55].

The second limiting factor is the not yet optimal take-rate, even in the presence of an appropriate wound bed preparation, adequate infection control, and presence of a dermal substitute [19]. These factors are important to prevent early stage graft failures due to infection, and a patient's instable general condition [56].

Another problem is the lack of melanocytes, which in comparison to the previously described methods are only present in non-cultured keratinocyte suspensions. After skin harvesting and digestion of non-cultured keratinocyte suspensions by enzymes, the direct application of the cell suspension contains viable keratinocytes, fibroblasts and also melanocytes (e.g. Recell®) [1, 57]. In contrast to this, the lack of melanocytes leads to the absence of pigmentation in the grafted area resulting in severe photosensitivity and unmatched skin colours in the reconstructed areas [19, 58].

The development of skin malignancies following keratinocyte application must also be considered. Although very rare in occurrence and very scarce in the literature (only one published case report), it is hypothesized that the aetiology is derived from chronic ulceration of the grafted area and the use of mutagen material during the in-vitro cultivation process of keratinocytes [59].

Last but not the least, associated costs or lack of cost-effectiveness has also led to the failure of various commercial endeavours in the market and has restrained the applicability in daily clinical practice—the costs of running a skin bank or developing the logistic infrastructure for some of the available kits to name a few.

Wound treatment especially in burns and chronic wounds is expensive. Relating the cost to the general health expenditure of emergencies, hospital care, medications and material costs, the use of autologous or allogeneic keratinocytes requiring financially burdening costs of research, a lengthy development process, highly skilled staff, requirements for product safety and storage render the application of this methodology especially in restrained economic times burdensome for every health care system. Profit oriented organisations have traditionally been quicker to adopt to these changes as compared with government's, and have shifted their commercially-based interest from cell-based to cell-free materials [2, 19, 35].

Outlook

Nowadays, the main focus of current research initiatives is the development of an optimal protocol for keratinocyte cultivation, and matching the skin's architecture wherever possible. These efforts include co-culturing the keratinocyte in combination with melanocytes, which is predominantly applied in the treatment of vitiligo, and skin pigmentation disorders [19, 58, 60, 61].

All types of tissue engineered combinations so far lack normal skin appendages. A few studies have reported on the ability of co-cultivating hair follicles and sebaceous gland as an integrated pilo-sebaceous unit in an experimental context, with no proof of clinical application yet [19, 60, 62].

The epidermal layer, itself non-vascular, receives its nutrients from dermal vessels; thereby this layer is in the absence of a dermal layer more susceptible to infections. The addition of angiogeneic factors to the dermal substitutes could promote the epidermal development by the increased speed of revascularisation (of the dermal matrix) and modification of the underlying wound bed [63]. Another report suggests the promotion of neo-vascularisation by the use of genetically-modified keratinocytes, which can over-express vascular endothelial growth factors [19].

The three-dimensional tissue-engineered culture of skin is another remarkable effort in this context, and includes among other methods the scaffold model, which is based on synthetic or biological materials mimicking the extra cellular matrix (ECM) for the ingrowth of fibroblasts, followed by the development of the dermis, and finally cultivation of an epidermal layer on top. The self-assembly approach does not contain any exog-

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enous elements like scaffolds, but promotes fibroblasts to secret ECM components by the use of ascorbic acid in the culture medium. Embedded in their own ECM, keratinocytes can then be seeded above this conglomerate. Finally, complex reconstructed models are derived from co-cultivation of melanocytes and keratinocytes in order to produce pigmented skin followed by seeding endothelial cells from umbilical vessels with fibroblasts possibly resulting into a "pre-vascularised" skin equivalent. The reconstruction of the hypodermis ultimately completes the reconstruction of tissue engineered skin equivalents resulting in a reconstructed three-layer conglomerate of cultured skin [60, 64].

The herein presented efforts bear despite their drawbacks during the development process, and resultant clinical outcomes of keratinocyte culturing, a solid foundation for the future. The ultimate challenge however remains, bringing any of these efforts into clinical practice, while avoiding the failures of the past.

Conflict of interest

The authors declare no financial or personal conflict of interest.

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