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Keratinocyte Culture Techniques in Medical and Scientific Applications

Kuhn Stefanie, Radtke Christine,
Allmeling Christina, Vogt Peter M. and Reimers Kerstin

*Department of Plastic, Hand and Reconstructive Surgery, Medical School Hannover,
Germany*

1. Introduction

Human skin is a complex organ essentially organized in a thin non-vascular epidermis, a thick collagenous dermis, and subcutaneous fat tissue called hypodermis. The epidermis is subdivided into five layers termed: stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and stratum germinativum. Furthermore, skin appendages, like hair follicles and glands, are derived from the epidermis but project deep into the dermis (Priya et al, 2008). They fulfil specialized functions for body homeostasis and temperature control.

Cellular differentiation and growth follow precise temporal and spatial patterns. In the epidermis constant replacement of cells lost by desquamation in the outer layers by cells of the basal layers takes place (Atiyeh & Costagliola, 2007). The basal layers of the epidermis retain two cell populations with different proliferation capacity: stem cells with high potential for self-renewal have low level proliferation rates but retain their ability to generate daughter cells throughout life; other cells display high frequencies of cell divisions but finally are destined for terminal differentiation. These cells are called transit-amplifying (TA) cells. Epidermal stem cells have been found in the bulge region of hair follicles and in the epidermal bottom of the deep rete ridges (Lorenz et al, 2009). After wounding, keratinocyte stem cells contribute to wound closure by giving rise to daughter cells which migrate to the site of defect (Ito et al, 2005).

Human keratinocyte stem cells and TA cells can be differentiated by their panel of cell surface marker gene expression; while stem cell markers CD29 and CD49f are highly expressed in both cell types, the transferring receptor CD71 is expressed in higher amounts in the rapidly proliferating TA cells (Lorenz et al, 2009). Moreover, keratinocyte stem cells have the ability to grow clonogenically with the number of colony-forming units, obtained per mm² skin biopsies, varying between 94 and 3190. The growth potential of clonogenic keratinocytes can be differentiated by the morphological appearance of the colonies: colonies with significant growth potential (holoclones and meroclones) contain mostly small cells and have a regular round appearance, paraclones, however, contain large, terminally differentiated cells and appear rather irregular (Ronfard et al, 2000). Due to the restricted growth potential of terminally differentiated cells colony-forming units increase when kept under ideal culture conditions. Conditions supporting keratinocyte differentiation, however, decrease growth potential of the culture.

2.1 Cultured epithelial autografts

In the treatment of burn surgery, considerable technical progress in intensive care management, ventilation, resuscitation and nutrition, directed in specialized burn centres, greatly improved the survival rates of severely burned patients. Early tangential excision and quick skin grafting to limit inflammation and restore skin barrier function are applied at large wounds changing the repair process basically from wound healing to graft-take (Ehrlich, 2004). Beside the clear advantages of an early restoration of the outer barrier of the body thus preventing infection, fluid loss and catabolism, the practice of grafting minimizes granulation tissue to the thin space between skin graft and wound bed leading to scar reduction (Ehrlich, 2004). In the same time autologous material used for split thickness skin autografts is limited by massive tissue destruction and/or unsuited donor sites (e.g. hands and face). In order to replace autografts a number of approaches have been developed, essentially they are either based on the use of acellular matrices, transfer of cultured cells or a combination of both.

Since the 80ties of the last century the use of autologous keratinocytes has been reported by different burn centres and established as an alternative for severely burned patients with little donor skin (De Corte et al, 2011). Keratinocyte transplantation after burns and in chronic wound treatment is potentially useful in clinical practice to improve functional outcome in burn patients. After the establishment of keratinocyte growth on a mitotically inactivated and lethally irradiated feeder layer of murine embryonic fibroblasts (Rheinwald & Green, 1975) first clinical application of graftable epithelia (cultured epithelial autografts, CEA) for treatment of burns was reported by O'Connor et al. in 1981 (Atiyeh & Costagliola, 2007). However, general use of CEA in burn treatment is hampered by the extensive infrastructure needed for production of CEA as well as high costs. Technical problems include CEA susceptibility to infection due to a high vulnerability of cultured epithelial sheets to bacterial proteases and toxins. Take rates are variable and depend to a large extent on skilled preparation of the wound bed (Atiyeh & Costagliola, 2007). Histological analyses of the grafts indicate delay in rete ridge formation and basement membrane maturation leading to prolonged skin fragility after wound healing (Rennekampff et al, 1996, Hernon et al, 2006). Critical disadvantages like the loss of clonogenic cells by enzymatic separation of the epithelial sheets from the feeder layers and alteration of integrin expression patterns by high cellular density in the sheets still are in need of technical refinements (Ronfard et al, 2000, Poumay & Pittelkow, 1995). As an alternative to enzymatic treatment, for example use of Ca²⁺-chelating agents or temperature-responsive culture dishes have been proposed (Ebara et al, 2003, Inoue et al, 2006). Nevertheless, due to the capability of epidermal cells to extensive expansion, CEA is an important means for treatment of large skin defects without the possibility of conventional skin transfer. As an example a 3 cm² biopsy is sufficient to cover the body surface of an adult after a 5,000-10,000 fold expansion in a culture period of 3-4 weeks (Williamson et al, 1995).

The growth potential of epidermal cells led to the innovative approach to graft keratinocyte single cell suspensions in non-solid delivery devices e.g. fibrin sealants or lactate solutions (Cervelli et al, 2010, Horch et al, 1998) or microcarriers (Seland et al, 2011). Aerosol spraying techniques have been developed when 2005 the first commercial system Recell® was introduced into clinical practice (Gravante et al, 2007).

Full-thickness skin wounds treated with split thickness autografts, transplanted keratinocytes or CEA with little or no dermal parts tend to poor stability, excessive contracture and scarring. Therefore, many different dermal substitutes are already available

commercially, e.g. acellular human or animal dermis like human Alloderm® (Lifecell Corporation, Branchburg, NJ) and porcine Strattice™ (Lifecell Corporation, Branchburg, NJ). Other substitutes are biological derivatives, mostly collagen I, like Integra™ (Integra Life Science, Plainsboro, NJ) and Matriderm® (Dr. Suwelack Skin & Health Care, Billerbeck, Germany), or substitutes combining synthetic material with biological material like polyglactin Dermagraft® (Advanced BioHealing, LaJolla, CA) (van der Veen et al, 2010) or Biobrane® (Bertek Pharmaceuticals, Morgantown, WV). Biological constructs might be seeded with autologous or allogenic cells, e.g. Epicel® (Genzyme Tissue Repair) or Apligraf® (Organogenesis, Canton, MA). Interestingly, in contrast to allogenic keratinocytes which provoke strong rejection after restoration of immunocompetence, allogenic fibroblasts are tolerated by the host enabling preparation of fibroblast seeded dermal substitutes prior to use (Coulomb et al, 1998). Nevertheless, autologous cells are favoured by recent reports in terms of functional and aesthetic outcome (Atiyeh & Costagliola, 2007).

Recent progress in innovative approaches, e.g. tissue engineering of living skin equivalents as well as cell transplantation and gene therapies, led to an urgent demand of obtaining pure cell cultures in sufficient amounts and quality. A major obstacle to achieve this aim is the standardization of keratinocyte preparation and the maintenance of pure proliferative cultures. Another potential problem for clinical application of cultured human keratinocytes is the use of serum because of safety issues. Other companies offer recombinant growth factors and enzymes for animal-free production of cell therapeutics like trypsin-like dislodging enzyme (TrypLE, Gibco, Invitrogen) (De Corte et al, 2011).

2.2 Pharmaceutical equivalents

Living skin equivalents are not only needed in clinical applications but also in the vast field of experimental research, including the pharmacological and cosmetic industry as *in vivo* animal experiments should be replaced by *in vitro* experiments if appropriate. Therefore, alternatives urgently need to be developed. Keratinocytes have been cultured *in vitro* in a two-dimensional (2D) fashion for a long time, using feeder layers of fibroblasts. Now, the culture of keratinocytes is possible using a special keratinocyte medium without serum which is important for the future use of the cells in animal or human applications (see above). Nevertheless, an organotypic – that is three-dimensional (3D) – culture of skin cells is an important issue as it mimics the natural skin and presents better interaction of the different skin cells.

Skin equivalents have been produced and improved already for a long time, including primary cells or cell lines (Schoop et al, 1999, Marionnet et al, 2006). Now, numerous 3D different skin equivalents exist – either commercially available or in-house equivalents (see below). They may comprise different kinds of cells, materials and structures. Some only include the epidermis while others represent a complete skin containing also a dermis. Also, some use de-epidermized dermis with others being made of fibroblasts integrated into a collagen gel with keratinocytes seeded on top. Constantly, new 3D skin equivalents are being generated and tested for many different purposes.

2.2.1 Use of skin equivalents as alternatives to animal testing

Evaluation of cosmetic ingredients is mainly done by irritancy, toxicity and corrosive testing. Up to now the rabbit Draize test has been used for this purpose, consisting of determination of oedema / erythema after topical application of the test substance. But

apart from the animals suffering pain, the prediction of the irritancy potential is not always correct (Macfarlane et al, 2009). As the 3D skin equivalents are physiologically similar to natural skin they present an alternative to animal testing (Mertsching et al, 2008). As such, they can be used for the testing of drugs, cosmetics and other substances like e.g. sunscreens but also for irritancy and toxicity tests as well as for the study of wound healing, cancer research or infection biology. Additionally, skin equivalents offer the possibility to gain more knowledge about the mode of action of (skin) diseases and the according drugs (Ponec, 2002).

2.2.2 Skin barrier function, irritancy and toxicity testing

The skin serves as a barrier and therefore protects us against exogenous harms like environmental hazards, UV-irradiation and pathogenic microbes, but also against endogenous water loss. For the effect of topically applied drugs or cosmetic products, the way of their penetration into the skin is of great importance (Ponec et al, 2001). Here, the stratum corneum serves as the main barrier in the skin and its composition and morphology are major factors in the permeation process of drugs into the skin. Ceramides and lipid composition of the epidermis are important key features with respect to the structure of the epidermis. In this context a model to predict the stratum corneum lipid organization has been developed (Bouwstra et al, 2001). Additionally, the barrier function and organisation of a skin equivalent composed of epidermis reconstructed on de-epidermized dermis (RE-DED) was tested (Ponec et al, 2001). The formation of the stratum corneum barrier in vitro was similar to that in vivo, including all major stratum corneum lipid classes and ceramide fractions, but displaying some differences in the organisation and composition of the fatty acids. Also, the architecture and lipid composition of commercially available skin equivalents (EpiDerm®, Episkin® and SkinEthic®) and the RE-DED skin model were compared (Ponec et al, 2002). The overall ultrastructural appearance of the epidermis in the skin models was similar to that of native skin. Nevertheless, some differences with respect to the differentiation of the keratinocytes and the exact composition of the fatty acids and ceramides were observed. The same skin equivalents were tested in spite of minor variations they were found suitable for the in vitro testing of permeation and percutaneous absorption of topical products like cosmetic ingredients since the reproducibility of such testing was given (Lotte et al, 2002).

On the basis of the aforementioned results, skin equivalents may serve as models for the prediction of human skin irritation, inflammation and toxicity of topically applied substances. Here, single-cell assays under submerged culture conditions, epidermal equivalents, skin equivalents and excised skin under air-liquid-interface culture conditions serve as different kinds of models with different advantages and drawbacks of each (Gibbs, 2009). Now, several commercially available skin equivalents (EpiDerm®, Episkin® and SkinEthic®) are validated as stand-alone test replacements for standard animal experiments (rabbit Draize test) (Macfarlane et al, 2009). But even fifteen years ago, skin models have already been tested for their use in the prediction of skin irritation and for the study of mechanisms of contact irritant dermatitis (Osborne & Perkins, 1994). Three commercially available test systems were analyzed comprising human epidermal keratinocyte cultures (Clonetics), and partially or fully cornified keratinocyte-fibroblast co-cultures (Skin2, Advanced Tissue Sciences and Testskin, Organogenesis). The results of the cell damage measurement in the keratinocyte cultures were not very satisfactory as it did not always correlate with human skin patch data. Different endpoints for the assessment of cytotoxicity

and inflammation in response to test formulations were successfully developed in Skin2 and Testskin and proved very useful (Osborne & Perkins, 1994).

Additionally to skin irritations, toxicological testing may be performed on skin equivalents, including phototoxicity, photoprotection and efficacy tests of cosmetic molecules (Damour et al, 1998). The StrataTest® skin model permits the testing of a broad range of test substances. It contains keratinocyte progenitors and forms a fully stratified tissue including epidermal and dermal components and a barrier function of the epidermis (Rasmussen et al, 2010). The skin was exposed to ozone, cigarette smoke or ultraviolet (UV) irradiation, resulting in the formation of reactive oxygen species (ROS). Antioxidants as well as sunscreens could successfully be tested with StrataTest®.

Since nanotechnology has developed very rapidly, humans are exposed to nanoparticles in many different situations. In contrast, the knowledge about their potential harmful effect is still under investigation. As the outer barrier of the body, the skin plays an important role in the nanoparticle penetration and they have been found in different depths and locations in the skin (Smijis & Bouwstra, 2010). The nanoparticles interact with the skin cells and may cause cytotoxicity or undesired immune responses, but the results are often contradicting.

2.2.3 Ultraviolet (UV) irradiation and DNA repair

UV irradiation is present all the time and causes mutations resulting in cancer if the DNA repair system is not functional in the skin cells. Melanin production by melanocytes and sunscreens protect the skin from damage, e.g. DNA changes and tumor formation, and skin equivalents containing a dermis as well as a stratified epidermis can be used to test the effect of sunscreens (Nelson & Gay, 1993). The release of pro-inflammatory mediators was also measured as a response to the UV irradiation.

Skin equivalents may also serve to analyze phenomena like skin photo aging and cancer development (Bernerd, 2005). The effects of different wavelengths of irradiation can be detected in the different layers of the skin. UV-B directly induces DNA lesions and therefore apoptosis in keratinocytes, while UV-A afflicts the dermis through the generation of ROS, fibroblast alterations and extracellular matrix modifications. DNA-repair deficient skin models can be generated by the use of cells (keratinocytes and fibroblasts) from patients with xeroderma pigmentosum. These patients suffer from a severe deficiency in the nucleotide excision repair of UV-induced DNA lesions (Bernerd et al, 2001). These skin equivalents display histological similarities to native skin of patients suffering xeroderma pigmentosum and thus may help to achieve a deeper understanding in the fields of photo aging, photo carcinogenesis and tissue therapy. The proliferation of keratinocytes increased while their differentiation was delayed and reduced. Epidermal invasions into the dermis could be shown as seen in native xeroderma pigmentosum tissue.

To achieve a deeper understanding of the mechanisms present in skin, the cellular responses of basal keratinocytes to UV irradiation can be analyzed with the help of skin equivalents comprising a dermis and a differentiated epidermis. The basal keratinocytes can be divided into two subgroups, one of which expressing high levels of $\beta 1$ integrin, the other expressing low levels of $\beta 1$ integrin. Both populations react differently to UV irradiation with respect to their proliferation and thus representing different subgroups of keratinocytes (Hendrix et al, 1998).

UV irradiation effects are of course dependent on the melanocytes and their pigmentation present in skin. A skin equivalent model was developed by seeding keratinocytes and melanocytes onto a de-epidermized dermis (DED) resulting into a reconstructed epidermis

and a physiologic distribution of dendritic melanocytes in the basal layer showing melanosome transfer to keratinocytes (Bessou et al, 1995). UV-B irradiation led to an increase of melanocyte numbers and stimulation of pigmentation as well as an increase of melanosome transfer. In an analogous model, melanin was detected in the melanocytes and the neighbouring keratinocytes. UV-B irradiation resulted in an increase of pigmentation, quantity and dendricity of melanocytes, while the quantity and distribution of melanin appeared to be unchanged at the light microscopic level (Todd et al, 1993).

2.2.4 Cancer models

Cancer can be induced by carcinogenic substances. Therefore, skin equivalents like EpiDerm™ (MatTek Co., MA), which have already been used to study cytotoxicity and irritant potential of cosmetic ingredients and consists of a reconstituted human epidermis, was also employed to evaluate the genotoxicity of carcinogenic agents (topical application of benzo[a]pyrene or UV-A / UV-B irradiation and psoralen-ultraviolet A radiation) (Zhao et al, 1999). The resulting expression of c-fos, p53 and other substances after the treatment of the skin equivalent was similar to that in native skin receiving the same treatment. Thus, this model is a convenient and cost-effective alternative to animal testing for the assessment of genotoxicity and the study of the mode of action of mutagens and carcinogens.

Another great concern with respect to cancer is the invasion and metastatic potential of the cancer cells. Four human melanoma cell lines with different metastatic potentials were analyzed in a skin model containing keratinocytes and melanoma cell lines (Dekker et al, 2000). The cells were either cultured submerged or at the air-liquid-interface (epidermal and dermal part present). In the interface cultures the cancer cells formed tumour cell nests in the epidermis, similar to the situation in vivo. Two of the four cell lines were found to invade into the dermal compartment. Here, a correlation was found between the expression pattern of certain adhesion molecules and the ability for invasive growth. The invasion potential was also analyzed in case of the squamous cell carcinoma (SCC) of the oral cavity (Kataoka et al, 2010) as a model for cancer invasion and metastatic activity. Here, the metastasis involves the destruction of the basement membrane and the invasion into the submucosal tissue. Two SCC (human tongue) cell lines and normal epithelial cells from gingiva as a control were cultured on AlloDerm®, a skin equivalent consisting of allogeneic acellular dermis. One cancer cell line invaded through the basement membrane into the dermis and thus represents an adequate in vitro model for SCC.

Cancer may be caused by human papilloma viruses (HPV). The molecular mechanisms were analyzed using a skin equivalent model with primary adult human epidermal keratinocytes, which were transduced with retroviruses expressing HPV genes (Akgul et al, 2005). They were seeded onto a de-epidermized dermis, previously repopulated with primary dermal fibroblasts. The expression of the HPV E7 gene led to an enhanced terminal differentiation and hyperproliferation of the keratinocytes. E7 uncouples differentiation and proliferation in vivo, such that the differentiating keratinocytes remain in a DNA replication competent stage. Also, E7 caused the keratinocytes to migrate through the basement membrane and thus invade the dermis. An over expression of several metalloproteinases (MMPs) was detected. Thus, the model proved very useful for the study of HPV genes in vitro.

2.2.5 Wound healing and angiogenesis

Wound healing still proves difficult in many situations. Therefore, models for wound repair need to be established and validated giving the opportunity to test new wound healing

therapies as well as to optimize dosages. Skin equivalents consisting of DED seeded with either keratinocytes or fibroblasts or both were developed. A full-thickness wound being inserted into the skin model decreased in the course of time, thus representing a wound model with the ability to heal (Xie et al, 2010). The growth factor VNGF was added to the wounds and resulted in an increased and earlier wound closure. Also, the wound model proved useful in the field of mechanistic studies and the assessment of a synthetic biomimetic gel.

The re-epithelialization of a wound is dependent on many factors and another wound model was used to determine the role of fibroblasts in this process and in the formation of the dermal-epidermal junction (DEJ) as well as the analysis of the differential protein expression during the re-epithelialization process (El Ghalbzouri et al, 2004). The fibroblasts facilitate wound closure and affect the deposition of several basement membrane components. Both, keratinocyte growth factor (KGF) and epidermal growth factor (EGF) accelerated the re-epithelialization in full-thickness but not in superficial wounds. The presence of laminin 5 and type IV and VII collagen did not seem to be required for keratinocyte migration.

A more mechanistic study concentrated on the cellular response to injury and the angiogenesis of wound healing (Herman & Leung, 2009). The three-dimensional skin equivalent model comprises several cell types found in normal human skin or chronic wounds. A microvascular component within the dermis-like extracellular matrix mimics the microvasculature of native skin and therefore is used to analyze angiogenesis in response to injury.

2.2.6 *Candida albicans* infections and bacterial contaminations of wounds

Candida albicans is able to colonize the surfaces of certain mucous membranes in humans, e.g. causing cutaneous candidiasis. A skin equivalent model is used to estimate the viability of *C. albicans* based on the quantitative detection of its actin mRNA (Okeke et al, 2001). As after the application of the antimycotic amorolfine the viability of *C. albicans* proved to be reduced, this technique may be useful to evaluate the therapeutic efficacies of antifungal drugs in the treatment of candidiasis. Also, the cytokine expression patterns in cutaneous candidosis were investigated (Schaller et al, 2002). Expression of pro-inflammatory cytokines was induced which is important for the recruitment of neutrophils, macrophages and lymphocytes in vivo and the induction of a Th1-type cytokine response.

Infections are feared of in the therapy of wounds and burns since they often lead to complications in the healing process or the rejections of skin substitutes but also resulting in sepsis and death in case of severe infections. Different kinds of skin substitutes (skin equivalent, dermal equivalent, xenograft, control saline gauze) were tested for their effect on wound closure (Fiala et al, 1993). Full-thickness wounds were inserted into rabbits and subsequently inoculated with *Staphylococcus aureus*. Wounds dressed with skin equivalents or dermal substitutes had lower bacterial counts than wounds treated with xenografts. The grafts took well in case of the skin equivalents and dermal equivalents and both are as such more effective as biological dressing materials than xenografts. Furthermore, skin equivalents have been used to analyze the biofilm development of clinical relevant bacterial strains (*Pseudomonas aeruginosa*, *Staphylococcus aureus*) on wounds (Charles et al, 2009). Those bacteria may also account for the chronicity of wounds. A skin equivalent wound model was developed on the basis of the commercially available Apligraf® / Graftskin, which comprises primary keratinocytes, forming a stratified epidermis, and primary fibroblasts

forming a dermis. Full-thickness wounds were inserted and inoculated with the above mentioned bacteria. Biofilm formation is dependent on the inoculation time as well as the bacterial strain, but occurred within some hours. As such, this model is useful for the study of biofilm growth, prevention and eradication.

2.2.7 Psoriasis and (de)pigmentation diseases

Psoriasis is an inflammatory disease of the skin, which is characterized by hyperproliferation of keratinocytes. TNF-alpha is a pro-inflammatory cytokine and as such implicated as a key cytokine in the pathogenicity of psoriasis. Therefore, its effect on keratinocytes from healthy and psoriatic skin was analyzed (Fransson, 2000). The keratinocytes were combined with fibroblasts from either healthy or psoriatic skin and as such formed a skin equivalent. Nevertheless, no effect of TNF-alpha on proliferation or differentiation of healthy or psoriatic keratinocytes could be found. Further, wound healing in a psoriatic skin may be different from that in normal skin and can be tested in a skin equivalent model (Konstantinova et al, 1998). The nerve system seems to be connected with the psoriasis disease and thus the expression of nerve growth factor was analyzed. While the keratinocytes invaginated into the dermis, it was found under the basal membrane zone, suggesting an influence on the migration of nerves into the regenerated tissue. Another model for psoriasis as well as graft versus host disease (GVHD) is a (mouse) model containing (healthy and psoriatic) keratinocytes, fibroblasts and activated NK cells (Kalish et al, 2009). The isolated, resuspended cells were placed into the chambers in skin of immunodeficient mice, forming skin after several weeks. NK cells were then added for the production of a psoriatic mouse model. Analogously, when T cells were injected, a GVHD model was achieved.

A major disadvantage of the current burn therapies with skin substitutes is the lack of pigmentation, perspiratory glands or hair follicles. Also, pigmentation disorders like hyperpigmentation in café-au-lait macules are the subject of research (Okazaki et al, 2005). A skin equivalent for the research on this disease was developed. It comprises fibroblasts, keratinocytes and melanocytes from café-au-lait macules resulting in a much higher amount of pigment than found in normal skin. Especially the fibroblasts seem to play a major role in this effect. Another approach is the construction of a skin equivalent containing melanocytes to achieve a skin equivalent with normal pigmentation using keratinocytes and melanocytes isolated from hair follicles (Liu et al, 2011). The skin equivalent was able to successfully repair skin defects in nude mice. A further group centred on the questions by which factors the melanocytes are influenced in their proliferation and melanogenesis (Hedley et al, 2002). The basement membrane was found to be necessary for the positional orientation of the melanocytes, i.e. without the basement membrane proteins melanocytes moved to the upper keratinocyte layers. The melanocytes pigmented spontaneously. The addition of fibroblasts led to a decrease of spontaneous pigmentation and neither α -melanocyte-stimulating-hormone nor cholera toxin was followed by pigmentation.

2.3 Gene therapeutic approaches

Transfection of keratinocytes is a promising tool for gene therapy either for temporary production of growth-stimulating factors in wounds (Vogt et al, 1994) or as a bioreactor for systemic expression of genes (Meng et al, 2002). Modification of healing processes by ectopic expression of stimulating polypeptides like cytokines or growth factors has several advantages over direct application of these factors, which are expensive and are restricted in their efficiency by high turn-over rates under systemic conditions.

Transduced keratinocytes are easily accessed, monitored and even removed if necessary, which is all favourable for establishment of gene therapeutic protocols; furthermore, epidermal cells are able to produce and secrete efficient amounts of ectopic protein. Secreted polypeptides have been shown to cross the epidermal-dermal barrier enabling systemic delivery of target proteins (Fenjves et al, 1994). Choice of genetic tools depends on the specific requirements of the therapeutic approach and considerations of safety and reliability. Correction of genetic diseases depends on permanent expression of the target gene e.g. on transgene integration into the host genome - ideally in cells with self-renewal capacity, like stem cells - or on extrachromosomal replication of an episome. Transient gene expression might be adequate for influencing temporal conditions as might occur in wound healing processes. Viral and non-viral gene delivery systems meet these demands to different extents and depend on different conditions for successful gene transfer. While non-viral methods and adenoviruses are essentially transient, viral vectors like retroviruses and lentiviruses might integrate into the host genome.

Most published techniques were performed with viral vectors in order to reach high transfection efficiency (Braun-Falco et al, 1999, Garlick et al, 1991). Keratinocytes have been transfected with several types of viral vectors including adenovirus, adeno-associated virus, retrovirus and lentivirus. Cells were expanded on mouse fibroblast feeder layers and kept under serum to ensure keratinocyte viability and proliferation. Thus, high exposure to virus particles and sufficient transfection rates can be achieved (Aasen & Belmonte, 2010).

These approaches contain several disadvantages, however. Viral vectors bear the risk of uncontrolled immune reactions and provide only limited genomic space for additional genetic material. Many studies focus on the use of retroviruses, e.g. as a corrective transgene for junctional epidermis bullosa; nevertheless, inactivation of gene expression by the host immune response is a serious drawback of this method (Kikuchi et al, 2008). In contrast to retroviruses and lentiviruses adenoviruses do not integrate into the host genome and thus are less prone to arbitrary and possibly harmful activation of host genes. They are able to infect non-dividing cells but are of limited use in gene correction due to their transient expression. Keratinocytes and fibroblasts are transduced by adenoviruses with less efficiency than other cell types due to their low levels of Coxsackie-adenovirus receptor and probably their negative charge of membrane glycoproteins (Kikuchi et al, 2008). As high viral load is needed strong immune response has been observed after treatment with adenovirus (Raper et al, 2003).

Non-viral methods have been developed as an alternative. Keratinocytes have been demonstrated to take up naked plasmid DNA *in vivo* (Hengge et al, 1995) but this has so far not been shown for an *ex vivo* setting. Transfection has also been mediated by calcium phosphate precipitation, DEAE-dextran and polybrene treatment followed by a dimethylsulfoxide shock (Jiang et al, 1991). Liposome-mediated transfer yielded about one third of transfected cells (Li et al, 2001). Electroporation can be used *in vitro* and *in vivo* (Kikuchi et al, 2008) while nucleofection is an interesting tool for broad transfection of keratinocyte populations because the protocol enables the transferred DNA to directly enter the nucleus. Thus, gene transfer to quiescent and terminally differentiated cells is possible which is important in keratinocyte cultures (Distler et al, 2005). High rates of gene transfer are necessary when functional assays are employed to investigate the success of gene transfer in contrast to the use of reporter genes.

In order to establish a protocol for the enrichment of positive cells we propose magnetic cell separation of transfected cells as described by us previously (Radtke et al, 2009). This

enrichment technique has been used for different types of cells intended for gene therapeutic purposes (Kube & Vockerodt, 2001). Essentially, cells were seeded on 10 cm uncoated plastic dishes and grown to 60% confluence. Transfection with pEGFP-C3 using 18 μ l Fugene6 (Roche Applied Sciences) complexed with 6 μ g DNA was performed 24 hrs after seeding. Transfection reagent was mixed with culture medium and incubated at room temperature for 5 minutes, followed by a 15 minutes incubation period with added DNA to allow for formation of transfection complexes. Importantly, the transfection reagent should not be allowed to attach to the used plastic ware and transfection complexes should be added to the cells in a strict dropwise manner. Fugene6 has been used efficiently in many gene therapeutic contexts (Arnold et al, 2006, Elmadbouh et al, 2004, Young et al, 2002) and has been proposed for generation of induced pluripotent stem cells (Aasen & Belmonte, 2010). It has been used successfully by us and others for keratinocyte transfection (Aasen & Belmonte, 2010, Distler et al, 2005, Radtke et al, 2009). The majority of cells maintained attached and viable. It has been reported that keratinocyte transfection can result in termination of proliferation due to cell-cycle arrest (Jensen et al, 2000). In our approach cells continued to proliferate and grew into a monolayer.

The ectopic expression of the truncated mouse H-2Kk molecule on the cellular surface was used for magnetic labelling via specific antibodies coupled to paramagnetically labelled beads. To this end cells were transfected with pEGFP-C3 (Clontech) and pMACS Kk (Miltenyi). After 24 hours cells were trypsinized until they appeared rounded. The reaction was stopped with 100 μ l FCS and the cells were scraped off the dish. Antibodies were added (80 μ l anti Kk) and the cell-antibody mixture was incubated at room temperature for 15 min. The volume was adjusted to 2 ml with phosphate buffered saline / ethylenediaminetetraacetic acid. The magnetically labelled cells were separated by MACS as previously described (Wei et al., 2001). Keratinocytes transfected with Fugene6/DNA complexes yielded 35% positive cells under optimized conditions without induction of apoptosis despite growing the cells under serum-free conditions (Radtke et al, 2009).

2.4 Keratinocyte culture and isolation

Keratinocytes can either be maintained under feeder layer-dependent conditions as essentially described by a method developed by Rheinwald and Green or under defined conditions in serum-free, media with a low calcium concentration as proposed by Boyce and Ham in 1983 (Rheinwald & Green, 1975, Boyce & Ham, 1983). Both methods have certain advantages and disadvantages and the users have to decide about their culture strategy based on the specific demands of their application.

For example, in cultures kept in conditions without feeder cells contamination with other cells, growing rapidly under feeder-layer conditions, is suppressed; nevertheless replication of keratinocytes is limited (Krueger et al, 1994, RoCHAT et al, 1994). In contrast, serum-containing culture medium significantly increases the amount of undesired cells (e.g. fibroblasts and melanocytes) decreasing the amount of attaching keratinocytes at the same time.

Other advantages of serum- and feeder-based techniques include higher resistance to apoptosis, e.g. after adenoviral infection. It has also to be taken into concern that it is possible to switch to serum-free culture conditions at any time point while changing from serum-free medium to serum-based conditions is not recommended (Aasen & Belmonte, 2010). Nevertheless, cell size enlargement has been observed by several authors (Inoue et al, 2006, van Rossum et al, 2004) when serum-containing media were used in contrast to serum-

free cultures. Lorenz et al. explain this by stating that keratinocyte stem cell characteristics are better preserved in serum-free media (Lorenz et al, 2009). This is in accordance with the fact that the number of clonogenic cells is increased under appropriate culture conditions. As keratinocytes usually proliferate in low-calcium (0.15 mM CaCl₂) and differentiate in high-calcium medium (Daniels et al, 1995) it is of importance to avoid any long-term exposure to high levels of calcium. Compared to some cell types such as fibroblasts, keratinocytes require more care and avoiding apoptosis in low density cultures, and differentiation and senescence when reaching confluence is difficult.

As most commercial products available for keratinocyte culture are optimized for human cells, cultures with cells of non-human organisms are especially difficult to obtain. A method for long-term culture of newborn C57/BL6 mouse epidermal keratinocytes by using highly supplemented rodent fibroblast conditioned medium was described by Hager et al. (Hager et al., 1999). Adult mouse keratinocyte subcultures were established without using fetal bovine serum (FBS), feeder layers, fibroblast conditioned medium (FCM) or bovine pituitary extract (Yano & Okochi, 2005). A detailed description of isolation and culture of keratinocytes from newborn and adult mice was published by Lichti et al. (Lichti et al, 2008). Rodent monocellular protocols are complex, time consuming and the reproducibility of the cell isolation is difficult, but as genetically modified mice are a valuable preclinical tool for understanding pathologies based on genetic disorders, protocols should be optimized and refined as well. Three dimensional skin models with keratinocytes from wildtype or mutant mice might thus profit by the recent progress in protocol modification (Lichti et al, 2008).

We established a simplified method for isolation and maintenance of proliferating human keratinocytes as described before (Radtke et al, 2009). Following this protocol a monoculture of human keratinocytes could be established without the necessity of co-cultures with fibroblasts. From our experience several factors played a role in the preparation of pure, proliferating keratinocyte cultures: 1) donor skin is best from young donors and 2) there are different regions of the body where the probability of harvesting healthy attaching keratinocytes is higher. We suggested that the best skin for preparation of keratinocyte cultures should be taken from anatomical regions without previous mechanical irritation, less cornification and regions without hair to diminish infection rates; while we did not recommend using scar tissue or skin containing striae.

2.4.1 Methods for cell culturing of human keratinocytes

Full thickness human skin has to be cut into small pieces and incubated in a dispase II solution overnight at 37°C. The epidermis should be removed from the dermis with fine forceps and centrifuged at 220g for 5 minutes. The pellet should be resuspended in a 0.1% trypsin/0.02% EDTA solution and incubated at room temperature for 15 minutes. Activity can be stopped by trypsin inhibitor followed by centrifugation. The cells should be resuspended in Waymouth medium and filtered through a 100 µm mesh. Dissociated cells can be seeded on uncoated tissue culture flasks. After 48 hours, cell debris and non-adherent cells should be removed and the culture medium changed to a serum-free keratinocyte growth medium. The plated cells typically reach 70-80% confluence after 5-7 days.

Cells remain in a proliferative state by subculturing in serum-free medium every time before reaching confluence. For passage keratinocytes should be washed with PBS and slightly trypsinized with trypsin (0.05%/0.02% EDTA) at room temperature until the cells start to retract. The trypsinization has to be stopped after 3 to 5 minutes with trypsin neutralizer. Cells should be detached off the ground mechanically and immediately centrifuged at 220g

for 5 minutes. After splitting to uncoated cell culture dishes, cells usually adhere the same day and start to divide after 24 to 48 hours. This mild procedure allows passaging vital and proliferative cells for at least 5 passages in serum-free medium (Promocell, Heidelberg, Germany) without the need of a fibroblast feeder layer (Radtke et al, 2009). Expansion of plastic-adherent keratinocytes is achieved without signs of senescence and with doubling times of 5-7 days. This protocol may facilitate the clinical application of keratinocyte based therapies.

We cannot define the exact mechanism for attachment of keratinocytes of different species, but this is one of the key factors in culturing keratinocytes. The attachment of human keratinocytes in our cultures without large contamination of other cell types such as fibroblasts or melanocytes, which have normally a greater proliferation rate and would overgrow the culture in a couple of days, is a clear advantage of this method. The exact mechanism of keratinocyte attachment is not known and various coating matrices (e.g. laminin, poly-L-lysine) do not lead to significant differences. In conclusion, we were able to establish efficient protocols for the handling of keratinocytes for experimental and clinical purposes, including isolation, cultivation, storage and gene therapeutic manipulations (Radtke et al, 2009).

3. Conclusion

Keratinocytes are a useful tool in cellular transplantation studies to improve functional outcome in burn patients and chronic wounds. Although the best clinical outcome might be achieved by transplantation of full-thickness skin grafts, limitations in donor tissue availability - especially in case of large burn wounds - leads to the necessity to use split-thickness grafts as commonly preferred skin replacements. Alternatively, transplantation of autologous keratinocytes - either precultivated to generate confluent keratinocyte sheets or intraoperatively prepared and transplanted in suspension - are used to fulfil special therapeutical requirements such as treatment of faces or when split-thickness grafts of sufficient amount or quality are difficult to obtain.

Beside the urgent need for therapeutical use keratinocyte cultures are an important issue for a number of biological applications. *Ex vivo* cultivation of skin and primary keratinocytes potentially integrated into different skin equivalents are recommendable model systems to investigate cellular behaviour of epithelial cells, skin diseases and pharmacological kinetics. Culture and subsequent transfection of keratinocytes might also be a promising tool for gene therapeutic approaches either for temporary production of growth-stimulating factors in wounds or as a biological vector for systemic expression of genes.

However, pure keratinocyte cultures are difficult to establish and to maintain for long-term culture. Cultivation in serum-containing media and on a mouse fibroblast feeder layer is not recommended prior to transplantation for safety reasons. We established a protocol for cultivation of keratinocytes under defined, serum-free conditions. From our experience, several factors may play a role in the preparation of a pure, proliferating keratinocyte culture: 1) donor skin is best from young donors and 2) there are different regions of the body with higher probability of harvesting healthy attaching keratinocytes. We suggest that the best skin for preparation and establishment of a pure keratinocyte culture without the need of a fibroblast feeder layer should be taken from anatomical regions without previous mechanical irritation, less cornification and regions without hair, the latter diminishing the infection rate. Skin originating from scar tissue or skin which contains striae is not recommended.

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5. References

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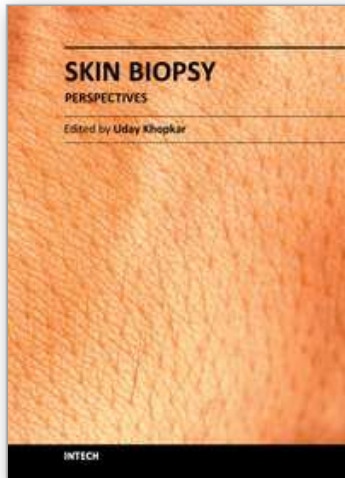
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Skin Biopsy - Perspectives is a comprehensive compilation of articles that relate to the technique and applications of skin biopsy in diagnosing skin diseases. While there have been numerous treatises to date on the interpretation or description of skin biopsy findings in various skin diseases, books dedicated entirely to perfecting the technique of skin biopsy have been few and far between. This book is an attempt to bridge this gap. Though the emphasis of this book is on use of this technique in skin diseases in humans, a few articles on skin biopsy in animals have been included to acquaint the reader to the interrelationship of various scientific disciplines. All aspects of the procedure of skin biopsy have been adequately dealt with so as to improve biopsy outcomes for patients, which is the ultimate goal of this work.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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