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Tissue engineering of cultured skin substitutes

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Abstract

Skin replacement has been a challenging task for surgeons ever since the introduction of skin grafts by Reverdin in 1871. Recently, skin grafting has evolved from the initial autograft and allograft preparations to biosynthetic and tissue-engineered living skin replacements. This has been fostered by the dramatically improved survival rates of major burns where the availability of autologous normal skin for grafting has become one of the limiting factors. The ideal properties of a temporary and a permanent skin substitute have been well defined. Tissue-engineered skin replacements: cultured autologous keratinocyte grafts, cultured allogeneic keratinocyte grafts, autologous/allogeneic composites, acellular biological matrices, and cellular matrices including such biological substances as fibrin sealant and various types of collagen, hyaluronic acid etc. have opened new horizons to deal with such massive skin loss. In extensive burns it has been shown that skin substitution with cultured grafts can be a life-saving measure where few alternatives exist. Future research will aim to create skin substitutes with cultured epidermis that under appropriate circumstances may provide a wound cover that could be just as durable and esthetically acceptable as conventional split-thickness skin grafts. Genetic manipulation may in addition enhance the performance of such cultured skin substitutes. If cell science, molecular biology, genetic engineering, material science and clinical expertise join their efforts to develop optimized cell culture techniques and synthetic or biological matrices then further technical advances might well lead to the production of almost skin like new tissue-engineered human skin products resembling natural human skin.

Keywords: skin substitutes • keratinocyte culture • massive burns • tissue engineering • fibrin sealant • biological matrices • monolayers • collagen • hyaluronic acid • allografts

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Introduction

Skin is the body's largest organ. Among many complex functions - including the immune response - it protects against environmental influences. Acute or chronic loss of this barrier requires the elementary processes of tissue repair for any organism to survive. Within this process reepithelialization of the wounded surface is the primary destination of skin wound healing [132].

Ever since the introduction of skin grafts by Reverdin in 1871 the transplantation of the patient´s own skin from a healthy donor site either as a full thickness or as a split thickness skin graft has become the surgical "gold standard" to cover skin wounds. This is the reason why every tissue engineered skin substitute will have to be measured against the performance of autologous skin grafts.

The recognition that extensive wounds require a barrier protection to prevent infection and desiccation and cell guidance by dermal elements to maximize healing led to the evolution of biologic and synthetic dressings and skin substitutes [65]. In massive burns the mere extent of wound surfaces and the considerable loss of skin necessitated the invention of various temporary or permanent skin substitutes, simply because there are not enough skin resources of the patient´s own body that would allow for recovery. To achieve long-term recovery certain properties of both dermal and epidermal layers of skin are important to incorporate. The clinical use of cultured skin substitutes for wound closure has reduced the amount of donor skin required by more than 10 times compared with conventional skin grafts and has reduced the number of surgeries required to harvest donor skin while at the same time decreasing the time of recovery of severely burn-injured patients. The experiences made in burn wound treatment have propagated the use of cultured skin substitutes to treat chronic problem wounds that are difficult to heal. However, results have not been similar to those gained in burns. This application is therefore still under discussion and many technical advances are under investigation to optimize tissue engineered skin substitute performance in these indications [57, 59, 61, 63, 74, 103]. Besides the direct clinical impact skin cell culture research has significantly contributed to the basic understanding of skin regeneration and biology [71, 72, 90, 92, 107, 125].

Scientific and clinical basis of currently available skin substitutes by their fundamental clinical design properties

Wound closure requires a material that restores the epidermal barrier function and becomes integrated into the healing wound, whereas materials for wound cover basically rely on the ingrowth of granulation tissue for adhesion [76]. The latter ones are most suitable for superficial burns and contribute to an improved environment for epithelial regenaration [14, 84].

Conceptually, skin substitutes are either permanent or temporary; epidermal, dermal or composite; and biologic or alloplastic (synthetic). Biologic components are either autogenous, allogenic, or xenogeneic. Logically, research efforts of different groups are centered on several possible permutations of these traits, whereas practically most designs rely on a permanent or temporary engraftment of the material. Because of these various aspects different attempts have been made to classify the methods currently available with regard to their clinical application as well as to the basic scientific categorization. Researchers in the field have defined the common techniques according to their principal biological action in the patient:

A) temporary [material designed to be placed on a fresh wound (partial thickness) and left until healed]
B) semi-permanent [material remaining attached to the excised wound, and eventually replaced by autogeneous skin grafts]
C) permanent [incorporation of an epidermal analogue, dermal analogue, or both as a permanent replacement]

Historically many various approaches have been developed as an offspring from the clinical practice, especially in burn surgery. Some of these developments had been fostered by clinical needs and other methods have then been transferred back to the field of research. Terms such as biologicals, skin substitutes, dermal analogues and culture-derived tissues have been widely used to discern between the approaches (Table 1).

Since many of such attempts to apply tissue engineering principles together with clinical experience have led to the development of commercially available products it seems also valuable to summarize the diversity of provided products that

Table 1 Systematic classification of currently available biologic and synthetic skin substitutes

Biologicals (Naturally occurring tissues)
- Cutaneous allografts (?- irradiated, deep frozen, glcerolized) - Cutaneous xenografts - Amniotic membranes
Skin substitutes
- Synthetic bilaminates - Collagen based composites
Collagen based dermal analogs
Deepithelized allograft
Culture-derived tissue (see also Tab.2)
- Cultured autologous keratinocytes (sheet grafts, cell suspensions) - Bilayer human tissue - Polyglycolic or acid mesh - Fibroblast seeded dermal analogs - Collagen-glycosaminoglycan matrix - Epithelial seeded dermal analogs

have found their way into clinical applications up to now (Table 2).

Specific problems of the burn wound

The initial stimulus to develop cultured skin substitutes stem from the clinical need of large amounts of autologous skin graft to treat extensive burns. Modern treatment concepts in extensive burn surgery are based on the perception that early surgical removal of heat denaturated proteins and devitalized tissue from a wound [staged serial debridement between the second and tenth day after the injury = early necrectomy] turns the burn into an excisional (primary) wound which can heal faster than a secondary wound [1, 31, 35, 114].

Primary excision has reduced mortality, morbidity and later reconstructive measures by a factor of 50% when compared to results obtained by awaiting spontaneous separation of eschar with later grafting. Early excision and the early reconstitution of a functional skin as early as possible is considered to be crucial for the further course and for the patient´s survival [55, 62, 69, 102] and leads to better wound healing with improved functional results [4].

Today, autologous split thickness skin autografts still represent the "gold standard" to resurface large wounds. In massive burns the available skin donor sites for auto grafting may be very limited. This has fostered the development of alternative means such as skin substitutes. The increasing emphasis on rehabilitation and "quality of skin cover" has further accelerated this field. A skin substitute which has the properties of a dermis is the marker for gauging a permanent substitute.

Allogeneic or alloplastic skin substitute coverage as a temporary solution is necessary until definitive cover can be achieved [41, 55, 62, 101, 113, 123, 124, 127, 142, 153]. Allogenic skin grafts may be completely integrated into the healing wound initially and bridge the critical time gap in the early phase of burn treatment, but in the further course irrevocably undergo immunogenic rejection [5, 51, 55, 56, 62, 70, 79, 124, 140, 146, 153]. Theoretically the application of *in vitro* cultivated autologous skin substitutes is able to overcome this specific deficit of today´s burn treatment and reconstructive surgery.

Skin cell culture based developments

The possibility to rapidly multiply a large number of epithelial cells under culture conditions with intervals of cell-multiplication of less than 24h based on the breakthrough technique by Rheinwald and Green in 1975 that allowed keratinocytes to be successfully cultured and subcultured in clonal cell densities on a "feeder-layer" of lethally irradiated mouse fibroblasts [118] renders the chance to grow epidermis in the quantity of the complete body´s surface within three to four weeks out of a single small skin biopsy. Isolated colonies of epithelial cells expand into broad sheets of undifferentiated epithelial cells. These cul-

Table 2 Currently comercially available or marketed matrices and products for tissue engineered skin substitutes

Material	Brand Name	Manufacturer
Collagen gel + cult. Allog. HuK + allog. HuFi	Apligraf™ (earlier name: Graftskin™)	Organogenesis, Canton, MA
cult. Autol HuK	Epicell™	Genzyme Biosurgery, Cambridge, MA
PGA/PLA + ECMP DAHF	Transcyte™	Advanced Tissue LaJolla, CA
Collagen GAG-silicone foil	Integra™	Integra LifeScience, Plainsborough, NJ
Acellular dermis	AlloDerm™	Lifecell Corporation, Branchberg, NJ
HAM + cult. HuK	Laserskin™	Fidia Advanced Biopolymers, Padua, Italy
PGA/PLA + allog. HuFi	Dermagraft™	Advanced Tissue Sciences, LaJolla, CA
Collagen + allog HuFi + allog HuK	Orcel™	Ortec International, Inc., New York, NY
Fibrin sealant + cult. autol HuK	Bioseed ™	BioTissue Technologies, Freiburg, Germany
PEO/PBT + autol. HuFi + cult autol HuK	Polyactive ™	HC Implants
HAM + HuFi	Hyalograft 3D™	Fidia Advanced Biopolymers, Padua, Italy
Silicone + nylon mesh + collagen	Biobrane™	Dow Hickham/Bertek Pharmac., Sugar Land, Tx

ECMP = extracellular matrixproteins, DAHF= derived from allog. HuFi, GAG=glycosaminoglycan, PGA = polyglycolic acid (Dexon™), PLA = polylactic acid(Vicryl™), PEO = polyethylen oxide, PBT = polybutyliterephthalate, cult. = cultured;autol. = autologous, allog. = allogeneic, HuFi = human fibroblasts, HuK= human keratinocytes, HAM = microperforated Hyaluronic Acid Membrane (benzilic esters of hyaluronic acid =HYAFF-11®)

tures are then treated with trypsin and the cells are taken to secondary culture using the same techniques. Numerous and outstanding research efforts of various groups led to the formulation of perfectly defined commercially available media, which enable keratinocytes to be cultured without a feeder layer and serum free. The combination of the concepts of early staged burn debridement with temporary skin cover and the technique of keratinocyte multiplication in culture gave rise to the hope that each burn wound no matter how extensive it is may be covered within 3 to 4 weeks [24, 30, 45, 51–53, 55, 59, 61, 148].

The further development of *in vitro* cultured skin substitutes since the first clinical applications can been characterized by three principially different ways of culture ideas:

Table 3 Summary of possible skin substitute techniques utilizing cultured human keratinocytes with regard to the various possible designs that are currently used or experimentally developed

I. Autologus cultured human keratinocytes
1. Autologous epidermal sheet transplants ("sheet grafts" = gold standard)
2. In-vitro cultured and constructed dermo-epidermal autologous transplants:
2.1. Keratinocytes on a collagen gel + fibroblasts
2.2. Keratinocyte sheets + Kollagen-Glycosaminoglycane-Membrane +Fibroblasts
2.3. Keratinocyte sheets on a layer of fibrin -gel
2.4. Keratinocyte sheets on cell free pig dermis
2.5. Keratinocyte sheets on cell free human dermis
2.6. Keratinocytes on bovine or equine collagen matrices
2.7. Keratinocyte sheets on micro-perforated hyaluronic acid membranes
2.8. Keratinocyte sheets on collagen + Chondroitin -6-sulfate with silicon membrane coverage (living skin equivalent)
3. Combination of allogenic dermis (in vivo) with epidermal sheets
4. non-confluent keratinocyte suspensions
as a spray suspended in saline solutions
as spray or clots suspended in a fibrin matrix
4.1. exclusively
4.2. in clinical combination with fresh or preserved allogenic skin
4.4. as non confluent keratinocyte monolayers on equine or bovine collagen matrices or on top of hyaluronic acid membranes
4.6. in combination with collagen coated nylon on silicone backing
4.7. dissociated keratinocytes without cell culture
4.8. outer root sheath cells (from plucked hair follicles) cultured or without culture
5. Three dimensional cell cluster cultures (spherocytes)
5.1. Cultured on microspheres as carrier systems (experimentally on: dextrane, collagen, hyaluronic acid)
5.2. Cell seeded microspheres + allografts/biomaterials
II. Allogeneic keratinocytes
Allogeneic Keratinocytes
6.1. Keratinocyte -sheets – (as a temporary wound cover)
6.2. Allogeneic keratinocyte suspensions (experimentally)
6.3. Syngenic-allogeneic keratinocytes
In-vitro constructed dermo-epidermal composites / analogues
6.4. Keratinocytes and Fibroblasts (collagen matrices)

- the growth of multilayered epithelial transplants (so called "sheet grafts") [13, 14, 37, 44, 46, 53, 57, 59, 61, 93, 110, 118, 120, 121, 141, 145];
- the construction of composite multi-layered dermal-epidermal analogues [13, 18, 20, 24, 25, 30, 37, 41–43, 48, 52, 53, 59, 61, 63, 78, 81, 84, 110, 130, 133, 135–137, 143, 144, 147, 149];
- and the concept of preconfluent cell grafting [13, 19, 23, 29, 39, 47, 49, 50, 58–63, 67, 68, 76, 82–85, 87, 100, 126, 134, 141, 143, 145].

A summary of the most well known techniques to produce and apply cultured skin substitutes and most of the currently available methods and research directions can be found in Table 3. Regarding these different approaches some implications for the further development and research can be deduced which are mainly based on clinical problems with cultured skin.

Conventional "sheet graft" (CEA) skin substitutes

Cultured sheets of human autologous epithelium (CEA = cultured epithelial autografts, so called "sheet grafts") offered a fascinating feasibility of

new grafting perspectives. However, there are ongoing controversies about the optimal indications and the pros and cons of this exciting technique [95, 101, 121]. Among the disadvantages especially the high costs have tempered a more widespread use. In a clinical trial conventional meshed autografts were found to be superior to CEA for containing hospital cost, diminishing length of hospital stay, and decreasing the number of readmissions for reconstruction of contractures. Our group tried to survey the costs of survival after the treatment of a patient with burns of 88% TBSA (total body surface area) using CEA. The direct costs were figured out to sum up to 425.000 Euro (currently 540.000 USD) for the successful primary care of this patient [54, 66]. Data from the literature revealed the cost for the successful treatment of 1% of TBSA with CEA in 1995 to account for 13.000 USD, although the reported "take" (engraftment) rates of CEA and the necessity to repeatedly graft the areas again can be extremely variable and may differ considerably [81, 151].

Wound infection, that is, clinically significant bacterial contamination, is probably one of the main causes of graft failure. Particularly in the first few days following grafting, the fragile non-cornified epithelium and the not yet fully developed dermoepidermal junction are much more susceptible to the damaging effects of bacterial infection than a meshed skin graft [68].

The earlier literature data of multicenter trials reported a high engraftment rate of cultured CEA, whereas later on others and especially single center experiences with a larger number of CEA grafted patients reported considerably lower take rates (between 15% and 65%) [24, 25, 30, 53, 81, 94, 98, 101, 116, 128, 129]. The true "take rate" derived from cumulative literature metaanalysis may therefore reach an average value of only 50% or less [40]. This is consistent with the original data of the pioneering works. The engraftment rate was initially reported to variy considerably between 0% and more than 80% take in adults and 50% take in children [25, 43].

Cultured human epithelial "sheet grafts" usually consist of 3 to 5 cell layers and are very delicate to handle. Secondary devices are necessary to manipulate them [43]. Hitherto unsolved problems are the lack of adherence and the tendency to form blisters even months after the engraftment when exposed to shearing forces, thus restraining CEA grafted areas from mechanical stress.

Several attempts have been made to explain the reasons for the uncertain take rates of sheet grafts. One of the commonly believed theories relates this phenomenon to the abnormal structure of the anchoring fibrils under culture conditions and due to the enzymytic treatment of sheets when detached from the culture flasks before the grafting process [18, 21, 24, 62, 106, 135–137]. Until now only parts of the body have been successfully covered with sheet grafts in large burn wounds. When resurfacing third degree burns or chronic wounds the lack of dermis remains a clinical challenge. The development of dermal analogues or substitutes or combinations of keratinocytes and dermal substitutes or matrix cells such as fibroblasts [5, 8, 34, 63, 81, 96, 101, 133, 143, 147] aims towards this problem.

Clinically, the expectations from the laboratory findings or animal experiments with regard to the clinical performance of cultured keratinocytes skin substitutes have not been fulfilled by their true performance yet. Therefore alternatively the temporary coverage of debrided wounds had been propagated. It relies on the engraftment of at least parts of the allograft dermis that remain after the immunogenic rejection process or after surgical removal of the allogenic epidermis [64]. In burn patients, clinical experience has shown that early excision and covering with allograft that temporarily engrafts may keep the wound bed clean and well- vascularized and enhance the likelihood of sheet graft take [55, 56, 62, 64, 86, 96, 121, 152]. Allogenic temporary skin coverage may serve as a biological and infection preventing *in-vivo*-culture environment after surgical debridement and consequent autografting until the allogenic epidermis is rejected. There is however a major problem of availability. Xenografts (tissue from another species) are more accessible than allografts. However, xenografts cannot be permanently re-vascularized from the wound so the tissue breaks down and sloughs off the wound [60].

In burn wounds human allografts allow for a stable temporary wound cover until further skin harvesting of the already used donor sites is possible. The autologous split skin is then subsequently mechanically expanded by different

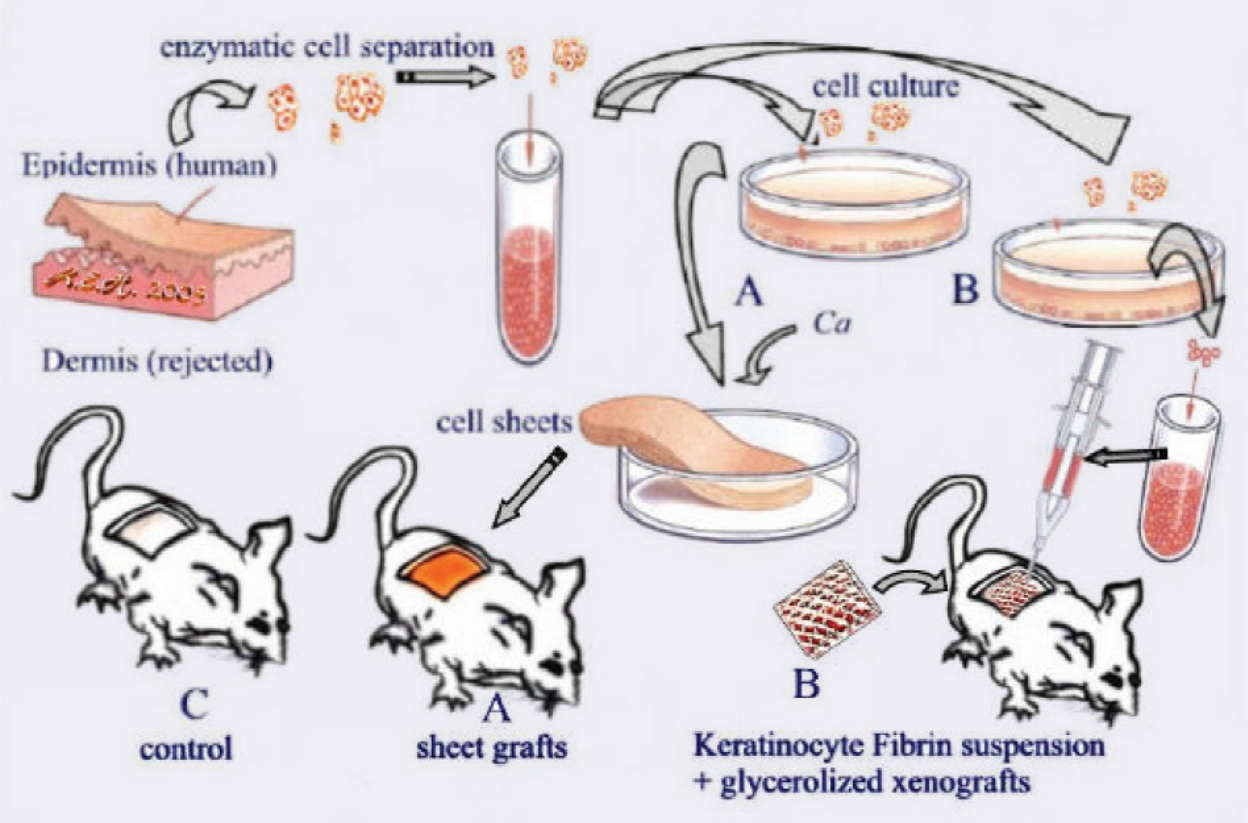

Fig. 1 Experimental setup of testing the performance of tissue engineered skin substitutes in full thickness nude mouse skin wounds: here comparison of cultured and stratified epithelial sheet grafts after calcium addition (A) versus subconfluently cultured non stratified keratinocyte single cell suspensions in fibrin sealant (B) with xenogenic meshed allograft skin as an overlay versus control wounds (C); model mimicking the clinical reality in large burn wounds.

means. Thus - even with limited donor sites - extensive burn areas may sequentially be covered with a definite result [4]. Among other reasons the limited resources in various health systems over the world have recently once again favoured the further development of these conventional techniques to circumvent cultured skin substitutes whenever possible. This has led to the revival of a conventional surgical technique called "microskin grafting" [55, 56, 62, 86, 96, 121, 152].

Cell suspensions

A techique of "epithelial cell seeding" had been published by von Mangoldt as early as in 1895 to treat chronic wounds and wound cavities with surprisingly good clinical results [91]. In his original description he harvested epithelial cells or cell clusters by scraping off superficial epithelium from a patient´s forearm with a surgical blade "until fibrin was exudated from the wound". This mixture was then applied to wounds. He claimed reduced donor site morbidity and a more regular aspect of the resurfaced wounds when compared to the method of Reverdin, that was the common method at this time. One of his key observations was the fact that single cells or cell clusters would better attach to the wound bed than conventional pieces of skin.

This technique was further modified by Pels-Leusden at the beginning of the 19th century, who mixed the epithelial cell-serum/blood suspension and injected it into the wound bed of chronic wounds with a syringe. However, because others feared the formation of epithelial

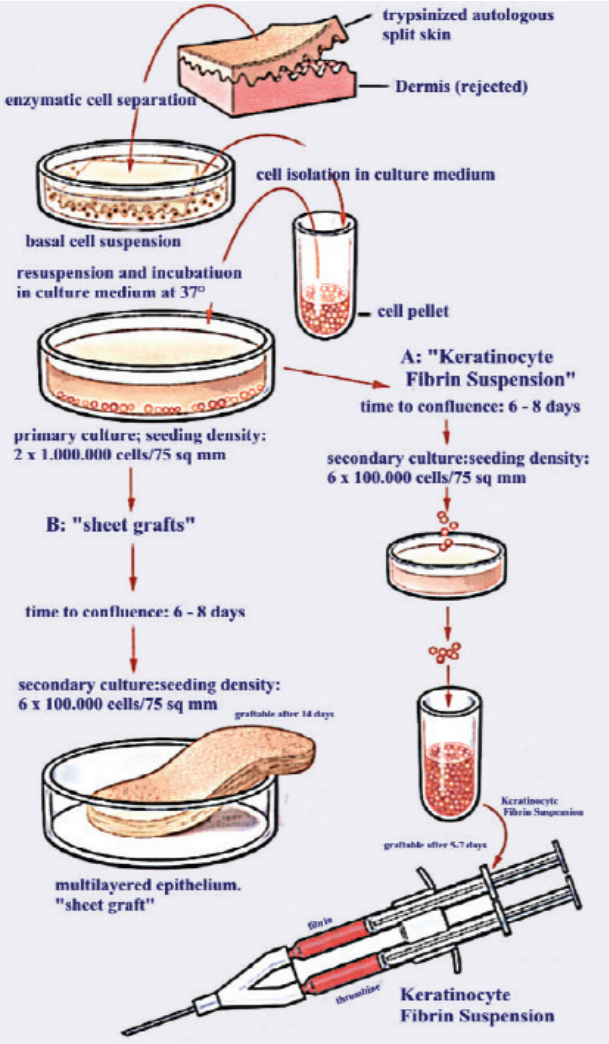

Fig. 2 Comparison between conventional grafting of differentiated multiplayer "sheet grafts" (B) of cultured epithelium versus grafting of subconfluently cultured single cell keratinocyte suspensions in fibrin sealant biological carriers (A) saving around one week of time from bench to bedside.

cell cysts beneath the closed skin wounds his method was not widely adopted [112].

Further attempts to transplant epidermal cell-suspensions in saline solutions, and without a binding matrix such as fibrin, were published in 1952, but did not yield consistent clinical results [17]. To obviate the problem of keratinocyte attachment Hunyadi *et al.* reported upon the successful transplantation of non-cultured keratinocytes gained by trypsinization from biopsies and suspended in a fibrin matrix to heal chronic venous leg ulcers. In his control group trypsinized keratinocytes suspensions without fibrin sealant did not lead to reepithelialization [59]. Fibrin, a naturally occuring substrate, is believed to play a key role in wound healing. Positive results with the use of fibrin sealant to fix skin grafts on burns and other wounds have been published [6, 10, 59, 110]. Cell culture studies have shown that the relative percentage of holoclones, meroclones, and paraclones of basal kerationcytes is maintained when keratinocytes are cultivated on fibrin, proving that fibrin does not induce clonal conversion and consequent loss of epidermal stem cells; the clonogenic ability, growth rate, and long-term proliferative potential are not affected by the new culture system; when fibrin-cultured autografts bearing stem cells are applied on massive full-thickness burns, the "take" of keratinocytes is high, reproducible, and permanent; and fibrin allows a significant reduction of the cost of cultured autografts and eliminates problems related to their handling and transportation [110].

Our group demonstrated [67] in clinical trials that extensive burned areas up to 88% TBSA can be successfully covered with a cultured keratinocyte-fibrin-sealant-suspension in 6 patients and 14 transplantations using a commercially available two component fibrin sealant [15, 54, 57, 59, 61, 82, 134]. The KFS (=Keratinocyte-Fibrin-Suspension) was available for grafting after only 10 days since no epidermal differentiation was needed for the single cell suspensions, while the CEA were available after 3 weeks only (Fig. 1) [58]. Our initial attempts of KFS grafting without meshed allograft overlays in third degree burns did lead to reepithelialization within one week when applied to long lasting and non-spontaneously healing wounds, but failed to show mechanical stability as has been reported from CEA grafted areas.

These findings led to the combination of a preliminary wound bed preparation with allograft skin followed by subsequent KFS transplantation together with meshed split thickness allograft skin as an overlay. This biological overlay helps to secure all grafted cells additionally and to protect them from desiccation during the healing process (Fig. 2). While the allografts in this combination healed initially with signs of revascularization (like autologous skin grafts) a slight and progressive immunogenic rejection period was then noted after 12 to 14 days [69]. This was sequentially fol-

lowed by stable wound coverage within two more weeks, after the epithelial parts of the allografts had been rejected. It seems notable that stable wound closure was achieved even over stress prone areas like knees and elbow joint regions without signs of mechanical instability as was seen after simple epithelial grafting without allografts [15, 54, 57, 59, 61, 134].

Allogenic keratinocytes as a source of readily available grafts of cultured epithelium, which are of special interest for large burns, have been fostered by others [38, 80]. In extensive burns superficial wounds are thought to be immediately covered or third degree wounds may be covered with a biological skin substitute until enough autologous grafts are available. Skin donor sites are described to heal faster and thus repeated harvest of thin skin grafts may be facilitated. However, the duration of persistance of such allogenic cells or substitutes in the wound and the timely course of rejection is unclear and has been controversely discussed [24, 30, 45, 51–53, 55, 61–63, 94, 95, 97, 98, 105, 121, 123, 148].

One more way of securing keratinocyte cell suspensions in culture medium after delivery to a wound bed has been published recently utilizing a commercially available bilayer skin substitute in an animal model. It remains to be seen to what extent human keratinocytes might behave in a similar fashion in a clinical situation [76].

Membrane cell delivery systems

A cell membrane delivery system is a means of support, chiefly mechanical, for cultured keratinocytes or other cells while in culture and in the early post-transplantation period [89]. Several such systems have been introduced, based on biological or alloplastic carrier materials, but are not widely adopted in clinical practice yet [49, 50].

Cultured cells and alloplastic or mixed synthetic-biological carriers

The combination of cultured autologous keratinocytes with various alloplastic dermal regeneration templates has been studied by various authors [76, 150]. Yannas and coworkers reported in 1989 that trypsinized whole-skin-cell suspensions (uncultured keratinocytes and fibroblasts) centrifuged into a collagen-glycosoaminoglycan (C-GAG) matrix and grafted onto guinea pigs were able to facilitate the regeneration of a healthy epidermis [150]. Clinically the centrifugation of cells into the membrane and the combination of cell-seeding techniques together with commercially available off-the-shelf products with a number of regulatory questions has hindered a more widespread use of these methods when compared to techniques that rely only on the material from individual laboratories.

One of these materials that has been propagated for reconstructive and burn surgery for many years [76] consists of a bilayer membrane product with a a well-characterised so called "dermal portion" that consists of a porous lattice of fibers of a cross-linked bovine collagen and glycosaminoglycan (GAG) that is supposed to be replaced by new collagen synthesized by fibroblasts that grow in from on full-thickness wounds [104, 99] and a so called "epidermal" cover of synthetic polysiloxane polymer (silicone). Once the collagen layer has been integrated into the wound bed, the silicone layer either may start to separate spontaneously or has to be peeled away and replaced with an ultra-thin split-thickness skin graft (SSG) of approximately 0.1 mm. Cultured autologous keratinocyte sheets have been proposed as an alternative form of definitive wound cover for this maneuver, but results have not been consistent yet [76]. In experiments from our group cultured keratinocytes seeded directly into such a bilayer-matrix performed well in the laboratory, but did not succeed in mice full thickness wounds or in the clinical setting, whereas others found a reformation of epithelium in full thickness mice wounds [20, 75, 76, 131].

Another system based on preconfluent grafting of monolayers of human kerationcytes on synthetic polymeric membranes or on polyurethane would allow for early coverage of excised burn wounds within only a few days of receiving the biopsy. Since only propagation of actively dividing cells and no further differentiation is needed, the time until grafting can be significantly shortened. This is of major importance in large burns,

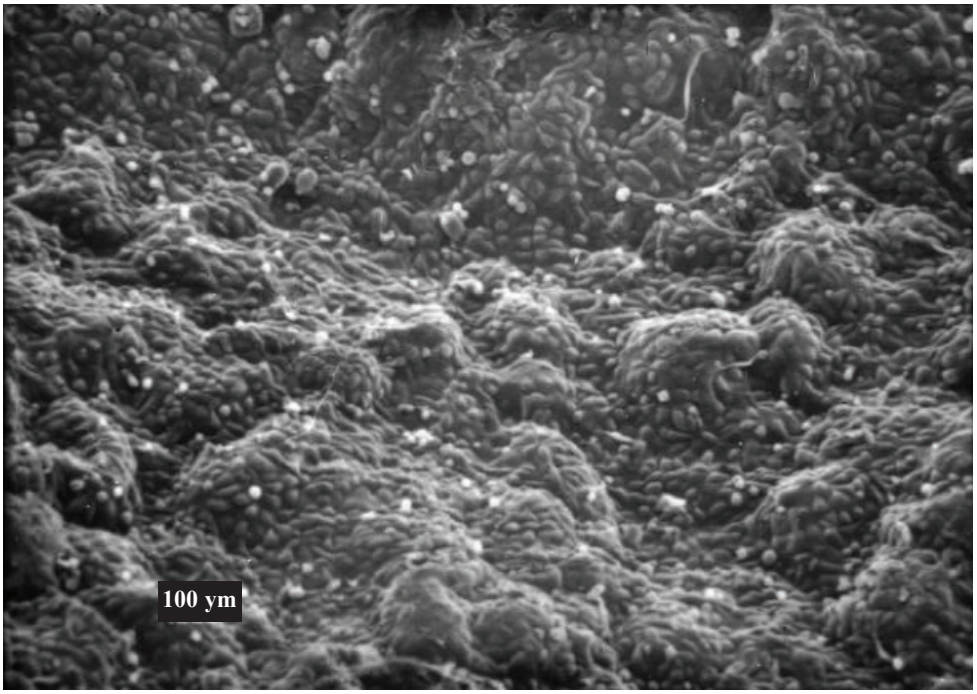

Fig. 3 Secondary culture of confluent human keratinocytes on equine collagen type I carrier in a non stratified monolayer; Environmental Scanning Electron Microscopy; bar = 100ym

because these patients are prone to progressive infections until the wounds are closed. When such films are inverted onto the wound the keratinocytes are supposed to migrate to the recipient wound bed and to form an epithelium [49, 50]. Serum free culture is recommended for this type of cell transfer to maintain a single layer of keratinocytes with basal characteristics.

Cultured cells and biological carriers

As mentioned previously the time between the patient biopsy and grafting is of vital importance for the survival of patients with major burns in opposition to chronic problem wounds where a time delay does not matter. If preconfluent monolayer grafts would be used and no differentiation period would be necessary, a significant reduction of the required time could be achieved. Theoretically, only the proliferating basal cells are responsible for the initial reformation of an epithelium. Cells in transition/differentiation or those which have lost the basal characteristics are probably not contributing to the resurfacing of wounds. From our previous studies [61, 82] and clinical trials [74] we delineated the concept of early preconfluent monolayer grafting of cultured epithelia. This does significantly shorten the time between skin substitute production in the laboratory and (re)-transplantation of cultured epidermal cells back into their more natural wound healing environment. Clinically these techniques may be combined with different surgical approaches, such as allografting together with simultaneously delivered cultured human keratinocytes [13, 57, 59, 61–63, 69, 82, 87] (Fig. 3).

One of the unsolved problems is the constant and reliable delivery of cultured cells to the recipient wound bed. Using fibrin sealant as a biological cell carrier, some groups now have introduced spray techniques [39]. This enables the dispersion and distribution of cultured cells to a maximum surface compared to our initial traditional approach without spray systems. The fact that cultured keratinocytes do survive this procedure has recently been shown in vitro by our group [63] and experimentally as well as clinically by others. Another way to optimize keratinocyte growth and delivery is the method of Ronfard and coworkers who cultured keratinocytes on a stabilized fibrin sealant in the gel phase [120]. After sufficient multiplication the whole fibrin-keratinocyte graft can be mechanically removed from the culture systems and be transplanted to the recipient. By this elegant technique a reliable and simple delivery of keratinocytes is enabled, as was shown

experimentally and clinically [63]. This approach was developed for burn victims and has not been used widely in clinical situations by others.

The enzymatic detachment from the culture dishes - necessary in the delivery of CEA sheets - is potentially harmful to the cultured cells and has been accused of being the main reason for the lack of adherence of sheets to the wound [24, 26]. Attempts to mount cultured epithelia on dermal matrices have been a possible way to facilitate handling, to avoid enzymatic treatment before grafting and at the same time to deliver a dermal analogue. Various templates have been used. One of the most common materials has been collagen in combination with glycosaminoglycane (C-GAG), with or without a cover of a gas permeable silastic membrane, that serves as a barrier to fluid loss [105]. The question whether dermal fibroblasts seeded into such composites are necessary or not has not been definitively answered up to now [33, 105, 115, 139, 146).

Due to a possible barrier function of the matrix material towards nutrients necessary for keratinocyte survival on top of such composites, questions of survival of such grafts in wounds remained open and the clinical long term success has yet to be demonstrated (Fig. 3a, 3b).

To avoid such obvious barrier functions of biological or synthetic carriers we transplanted composite grafts with the keratinocyte layer in an "upside-down" direction towards the wound bed, so that the collagen component serves as a carrier and as a biological dressing on top [61]. Further on we used subconfluent monolayers of cultured human keratinocytes instead of multilayered sheet grafts. Cells were cultured to monolayers in serum-free media to ensure maintenance of their basal cell characteristics during the culture period. Collagen, as an integral part of the wound healing process, is an obvious material to be used in dermal substitutes and has been applied in various forms to resurface full thickness wounds, as a cell-carrier material or dermal template. The classical way of trying to develop a nature-resembling two layered skin substitute in the culture laboratory is appealing, but until now has not performed sufficiently well in patients. This has contributed to the diffusion barrier that is created by the dermal analogue that blocks sufficient nutrition of the keratinocytes on top. These keratinocytes are thereby lost during the engraftment process before revascularization of the underlying dermal replacement has taken place.

Our experimental data with this "upside-down" grafting technique in full thickness nude mice wounds reveal the feasibility of this new approach [63]. In contrast to well known standard composite grafts there is no time required until revascularization is established and nourishment is reestablished so that a high number of transplanted cells can survive in the natural wound environment, similar to buried chip skin grafting [56]. Until now it is not known if parts of the biological carrier are integrated into the newly reconstituted skin. This method combines the *in vitro* expansion of graftable cells with advantages of the transplantation of actively proliferating cell populations on an appropriate biological carrier. Clinically it may become one more valuable tool to treat burns or chronic wounds.

Future perspectives

The concept of organ-specific regeneration has been challenged by discoveries that multipotent cells can be isolated from many tissues of the body [22, 27, 28, 32, 36, 71–73, 107]. Loosely referred to as stem cells, these cells in the adult body resemble pluripotent cell populations, such as derived from the early embryo, which may contribute to virtually any type of tissue under appropriate experimental or biological conditions [122]. While embryonic stem cells originate from undetermined early organisms without any history of differentiation, it is far less understood how a population of "reserve stem cells" retain their possibility in our adult bodies [122]. The potential of multipotent adult stem cells may well become a key to skin reconstitution once technical problems have been solved to influence the lineage of such cells and to guide their growth and differentiation [2, 9, 11, 12, 88]. Basal keratinocytes are the source of epithelial regeneration and have therefore been claimed to behave like adult stem cells [110]. Adult stem cells must be clonogenic and self-renewing during the lifetime of an organism through asymmetric division, with at least one daughter cell remaining pluripotent to main-

tain a stem-cell lineage, and the other daughter cell free to mature into a specialized and differentiated cell type [122]. DeLuca and coworkers tried to characterize the potential stem cell-like behaviour of basal keratinocytes into holoclones and meroclones [119], proposing that the proliferative compartment of stratified squamous epithelia consists of stem and transient amplifying (TA) keratinocytes. These authors were also [7, 8, 57, 87] able to demonstrate that epithelial cells from the corneal limbus have the capacity to reform a corneal epithelium with translucency after a culture period and retransplantation into the recipient eyes of patients [111]. They described that conjunctival keratinocytes with high proliferative capacity give rise to goblet cells at least twice in their life and, more importantly, at rather precise times of their life history, namely at 45–50 cell doublings and at ~15 cell doublings before senescence. Thus, the decision of conjunctival keratinocytes to differentiate into goblet cells appears to be dependent upon an intrinsic "cell doubling clock". These data open new perspectives in the surgical treatment of severe defects of the anterior ocular surface with autologous cultured conjunctival epithelium [108, 109, 117].

Gene therapy in combination with keratinocyte culture techniques lends itself as an obvious tool to enhance performance of cultured skin substitutes, to accelerate cell growth during the *in vitro* and *in vivo* process and to address difficult to heal wounds [3, 8, 57], [7, 8, 16, 57, 87, 138].

At the moment scientific principles and practical approaches to replace skin temporarily or permanently are advancing at a rapid rate. Although research in this area as well as the clinical application of cultured human skin substitutes is an expensive modality, we definitely need further progress to optimize skin substitute performance by tissue engineering procedures. Improved cosmesis and the ultimate regaining of lost skin functions including sensitivity, elasticity, normal physiological sweat gland and dermal appendage function, as well as normal pigmentation with invisible scars are the goals of future endeavors in this field. Research in this field may lead to improvements in skin reconstitution while at the same time overcoming current limits of donor sites and donor site morbidity in afflicted patients.

Both through basic and clinical research there will be major improvements in the understanding and ability to effectively deal with the problems of wound healing and to replace a truly functional skin with dermal appendages. But perhaps the major improvement will come through the ability to replace worn out, defective or damaged body parts through technologies that resemble regeneration. The concepts of Tissue Engineering applied to dermal replacement following burn injury or dealing with chronic wounds may well overcome many of todays limits in skin substitution [77].

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