The Viennese culture method: cultured human epithelium obtained on a dermal matrix based on fibroblast containing fibrin glue gels

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Abstract

The aim of this study was to develop a new keratinocyte culture system on a dermal equivalent suitable for skin wound closure. Our dermal matrix is based on a fibrin glue gel containing live human fibroblast (from human foreskin). Keratinocytes obtained from primary culture according to the Rheinwald and Green method, were seeded on to the gel. In all cases, the keratinocytes plated on the dermal equivalent grew to confluence and stratified epithelium was obtained. After 10 days an irregular multilayer could be observed. The cells showed active interaction with the fibrin support, presenting as cell formations projecting into the matrix.

After 15 days a regular epithelial sheet consisting of three to four layers of cells was formed. A limiting membrane demarcating the keratinocytes from the fibrin matrix was discernible. Squamous differentiation similar to Strata reticulare and corneum found in vivo could be observed. Nuclei of basal cells were regularly spaced from each other and the chromatin was of homogeneous appearance without prominent nucleoli. The last time point (20 days) showed signs of disintegration of the epithelial sheet. A basement membrane-like structure could not be seen any more. Detachment of the basal cells was associated with subepithelial vacuoles. Basal cells contained irregular nuclei. Therefore, we conclude that 15 days of culture were optimal for the generation of a keratinocyte layers with signs of differentiation; this new culture system could be an important step forward in covering severely burned patients due to a number of advantages, as for example a large expansion factor, the shortening of the optimal culture time to 15 days, the usage of commercially available fibrin glue gels and the versatile manipulation of composite cultures.

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1. Introduction

Since the development of the method for growing epithelial sheets with the support of lethally irradiated 3T3 cells in submerged culture conditions in 1975 [1], cultured epithelium has been used as grafting material in different clinical situations such as the treatment of burn wounds [2], chronic skin ulcers [3] and oral mucosal defects [4]. This method has gained particular attention in the treatment of seriously burned patients, since it is impossible to obtain, in a short period of time, an epithelium surface, large enough to cover the needs of a patient whose skin is largely damaged [5]. Rapid and effective burn wound closure is one of the most important aspects in the treatment of burn patients, because the patient is in a sub-septic condition until all skin defects are closed.

The sheet grafts used clinically consist of cultured epidermal cells attached to gauze with surgical clips. They are usually available three to five weeks after initial biopsy. The
grafts consist of three to five cell layers [6]. The surgical handling with secondary devise is delicate [7,8]. Moreover, it has been found out that keratinocyte grafts still have persistent problems: blistering and a lack of stability. These drawbacks have limited their clinical application and provided further stimulus to the development of keratinocyte culture methods on surfaces that mimic the human dermis (dermal equivalent). Several dermal equivalents of diverse composition have been reported [9–11]. In addition to the dermal bed that is often damaged by the burn, dermal substitutes increase the possibility of graft take [12]. However, the keratinocyte expansion factor found under these conditions (i.e. the total surface of keratinocyte culture that can be obtained in a given period) is lower than that obtained by the traditional culture in the presence of 3T3 cells [13]. So far, this restriction and the elevated cost of materials precluded the use of dermal equivalents for seriously burned patients, who require a large skin surface within a short period. Data from several laboratories indicated the possibility of growing fibroblasts and endothelial cells on/in fibrin gels [14–16]. Moreover, autologous keratinocytes in fibrin glue suspension have been employed in the treatment of burned patients [6,17–19] and acellular fibrin gels have been used as biological support for keratinocyte cultures [20]. Based on these reports, we investigated the possibility that fully commercially available fibrin gels enriched with human fibroblasts could act as a dermal substitute for keratinocyte culture. Bearing in mind the possible use of this type of culture for the treatment of seriously burned patients, we have evaluated our system in regard to architecture, expansion rate and handling of the composite culture for grafting purposes.

2. Material and methods

2.1. Primary keratinocyte culture

To obtain primary keratinocytes, normal human keratinocytes derived from adult skin donors or from burn patients, where a small particle of skin is harvested within first the 3 days (at the first operation), were isolated following previously described methods [5]. The skin should be harvested as soon as possible in order to avoid hospital acquired bacterial infection. The skin particle is incubated in trypsin-EDTA for 18 h at 4 °C in order to obtain individual cells. Keratinocytes were cultured on the fibrin glue/fibroblasts gels according to Rheinwald and Green [1]. The medium was changed every third day. After 10, 15 and 20 days the fibroblast/fibrin glue/keratinocytes sheet was peeled off the culture flask, without the use of enzymes.

2.2. Preparation of fibrin and fibroblasts gels

Human fibroblasts were isolated from human adult full thickness skin by enzymatic digestion, and cultured in DMEM containing 10% FCS. Approximately $4 \times 10^6$ human fibroblasts were isolated and incubated in a 115 cm$^2$ large culture flask. Twenty four hours later, a fibroblast containing fibrin glue (FFG) was layered on top of the human fibroblasts.

The FFG was prepared as follows: approximately $4 \times 10^6$ human fibroblasts were mixed together with the “thrombinpart” (thrombin 5IE/ml) of a two components glue (Tissucol, Baxter, Austria). This mixed “thrombin-cell part” was added to the “fibrinpart” on the floor of the culture flask, which was seeded with human fibroblasts (Figs. 1 and 2).

After solidification of the glue another layer with approximately $2 \times 10^6$ human fibroblasts were seeded onto the FFG. The incubation of this new matrix was done at 37 °C with wet atmosphere and CO$_2$-concentration of 5% up to keratinocyte cultivation.

2.3. Secondary culture of keratinocytes on gel of fibrin and fibroblasts

The primary keratinocytes, prepared as described above, were seeded onto the fibrin fibroblast gel (Fig. 3).

Keratinocyte growth was followed by using an inverted microscope. After 10, 15 and 20 days of culture, the gels...
were manually detached from the culture flask (without the use of enzymes) (Figs. 4 and 5).

2.4. Histology and immunostainings

Fibrin-supported keratinocyte sheets were fixed in neutral buffered formalin overnight at room temperature and embedded in paraffin. Two micrometer sections were processed for standard hematoxylin and eosin staining. Immunohistochemical studies with monoclonal antibodies against type IV collagen (Dako), laminin (Sigma). Moreover, electron microscopy was performed in order to evaluate the ultrastructure of our sheets.

Morphological analysis and microphotography were performed on 6 samples per time point using the Olympus VANOX AHBT3 microscope and the Olympus DP50 CCD digital camera, respectively.

3. Results

3.1. Fibrin/fibroblasts gels

Within 10 days of culture a confluent epithelium multilayered was achieved. In all cultures, it was possible to peel off the fibroblast/fibrin glue/keratinocytes sheets from the culture flask without the use of enzymes.

3.2. Morphological and differentiation features of keratinocytes growing on fibrin fibroblasts gels

At time point one (10 days) an irregular keratinocyte multilayer could be observed. A delicate extracellular
structure was deposited between cells and matrix. Mitoses were regular and frequent. Nuclei were located centrally and nucleoli easily visible. The cells showed active interaction with the fibrin support, presenting as cell formations projecting into the matrix (Fig. 6).

After two weeks a regular epithelial sheet consisting of three to four layers of cells had formed. A limiting membrane demarcating the keratinocytes from the fibrin matrix was discernible. Squamous differentiation with flattening out of cells similar to Strata reticulare and corneum found in vivo could be observed. Nuclei of basal cells were regularly spaced from each other and the chromatin was of homogeneous appearance without prominent nucleoli. Mitoses were rare (Fig. 6).

The last time point (20 days) showed signs of disintegration of the epithelial sheet. A basement membrane-like structure could not be detected any more. Detachment of the basal cells was associated with subepithelial vacuoles. Basal cells contained irregular nuclei. In the upper layers cell–cell junctions reminiscent of the Stratum spinocellulare could be noted, although intercellular vacuolization was associated with this step of differentiation as well. Importantly, apoptotic bodies were intermingled in suprabasal cell layers (Fig. 6).

4. Discussion

At the moment, several technologies are under active development as aids in cutaneous wound repair. However, many of them deal with a single aspect of the healing process that does not necessarily serve as primary source of new tissue. The biochemical and cellular composition of the composite graft described in this paper closely resembles that of skin during the first steps of wound healing. In fact, during the healing of a skin wound, the defect is temporally plugged with the formation of a fibrin clot. This clot is later infiltrated by inflammatory cells, fibroblasts and granulation tissue [21]. Thus, a high efficacy for wound closure can be expected, since an already accomplished step of healing is provided. We think that fibrin glue is an extremely useful tool in surgery, because fibrin glue is not only usable for graft adherence and take, but also as a template for cellular migration. Moreover, it is a useful delivery system for cultured keratinocytes, fibroblasts and growth factors. These postulations are in accordance to Currie et al. [19], who have analyzed the positive and negative role of fibrin glue in skin grafts and tissue engineered skin replacements.

One of the great advantages of our new culture system is the use of widely available commercial fibrin gels, which guarantee constant quality and reproducible sheet grafts.

In spite of the fact, that the use of a commercially available fibrin gel as a matrix for cultured epithelium has already been published by Ronfard et al. [16], our culture method differs significantly: e.g. our matrix is not only based on fibrin glue, it also contains different layers of human fibroblasts and we think that these fibroblasts play an important role as an accelerator and modifier. Moreover our matrix is at least twice as thick as the above mentioned matrix, and we think that the thickness of the matrix is very important for the architecture of the sheet graft and thereby of its stability.

Positive staining for two basal membrane proteins, type IV collagen and laminin were found in confluent cultures. The expression of these membrane proteins seems to rely on the presence of both keratinocytes and human fibroblasts in the culture. The presence of basal membrane like structures could favor the grafting success.

The culture of keratinocytes on gels of fibrin and fibroblasts is easy to handle. It can be performed in a closed culture flask with a lower risk of contamination than in a petri dish. Since the gel is transparent, the growth of the keratinocyte colonies can be followed with the inverted microscope. However, the greatest handling difference between this culture and “traditional” cultures is the fact, that these presented sheets can be manually detached from the flask. By using this method of separation, the continuity of the layer of the keratinocytes is preserved. Thus, no surface area is lost, which may occur when the sheets of isolated epithelium are processed with dispase [7]. This has been suggested by Harris et al., postulating that the use of enzymes reduced the adhesion potential of cells. Therefore, Currie et al. have recommended the use of keratinocytes cell sprays [6] and they mentioned the factor cultivation time as a big advantage of their method. Currie et al. postulated that culture time, which was needed to culture sufficient sheets is about 5 week,. By contrast, our culture system (short culture time → 15 days, no enzyme separation) has the advantages of sheet grafts without the above mentioned disadvantages.

In our view one of the major advantages using sheet grafts is the fact, that a homogenous distribution of cells covering the defect can be guaranteed. Moreover, in the case of our culture method the presence of keratinocytes and basal membrane like structures could favor grafting success. We think, that these grafts are capable of generating a normal epidermis for many years and favor the regeneration of the skin.

Based on our observations and data, we recommend a culture time of 15 days in order to generate keratinocyte sheets with signs of differentiation;

In conclusion, the culture of keratinocytes on gels of fibrin containing living fibroblasts offers the following advantages, when compared with other previously reported methods: (1) high expansion factor, (2) short culture time, (3) easy monitoring of keratinocyte growth, handling and delivery for grafting; (4) elimination of enzymatic treatment and (5) application of epithelial and mesenchymal cells in a single operative procedure. Therefore, we believe that our culture system can contribute to improving the strategy for closing burn wounds more quickly and adequately.
References


