27.6 Optimizing the Mucosa Graft: Developing Gingival Keratinocyte—Fibroblast Construct

From the clinical application it is known that there is considerable wound shrinkage, which may be due to lack of differentiation in the graft. The environment of epithelial cells may influence their differentiation as, for example, restoration of differentiation was described for keratinocytes in vitro, e.g., when using an air-liquid culture technique [2] or when combining keratinocytes with a submucosal layer of fibroblasts [55]. Further, perfusion culture systems help to maintain or promote a high level of cell differentiation in epithelial cells in vitro [1, 17 31]. However, whether such an effect on cell differentiation is also observed on human oral keratinocytes has only been poorly studied. Therefore, two approaches were made:

1) Gingival keratinocytes were cultured in a perfusion culture system.
2) Gingival keratinocytes were grown in as co-cultures together with gingival fibroblasts.

For perfusion cultures, primary gingival keratinocytes cultures were trypsinized and seeded in a concentration of 200,000 cells on polycarbonate membranes (Corning-Costar, Bodenheim, Germany; diameter 1.3 cm, 3 µm pore size), mounted in sterile carrier rings (Minucells and Minutissue, Bad Abbach, Germany). These secondary cultures were kept in Keratinocyte-SFM (Gibco, Eggenstein, Germany) and DF-Medium (see above) in a ratio of 1:1 and additives 2.5% (v/v) inactivated fetal calf serum, 1% (v/v) penicillin/streptomycin, and 7% (v/v) HEPES buffer as:

1) *Standard cultures*, cell-seeded membranes mounted on tissue carriers in six-well plates, were left in the cell incubator for 16 days (Heraeus Instruments, Osterode, Germany) at 37°C, 95% air and 5% CO_2 humidity atmosphere, medium being changed every third day.
2) *Perfusion cultures*, cell-seeded membranes mounted on tissue carriers, were transferred into a perfusion culture container (Minucells and Minutissue, Bad Abbach, Germany) after 48 h of cell adherence. Cells were perfused for another 14 days continuously with KD-Medium at a rate of 0.7 ml/h (IPC-N8 peristaltic pump, Ismatec, Zürich, Switzerland).

Cultures were processed for light microscopy by embedding the specimens in methyl methacrylate (Technovit 8100, Kulzer, Wehr, Germany) at 4°C after fixation in 3% buffered paraformaldehyde and dehydration. Semithin sections were cut and prepared for histology or immunohistochemistry. Antibodies against cytokeratins 1, 2, 10, 11; 5, 6; etc. were applied by indirect immunoincubation using the avidin-biotin technique. Slides were examined using a BX-61 apparatus (Olympus, Japan) and photographs were taken by using a digital camera in combination with analysis software (Soft Imaging Systems, Münster, Germany).

Morphology after perfusion culture showed continuous dense cell growth with a mean of 3.4 cell layers (Fig. 27.5) and a standard deviation of 0.4 cell layers only. After standard culture the continuous cell layers were interrupted by areas with little growth,

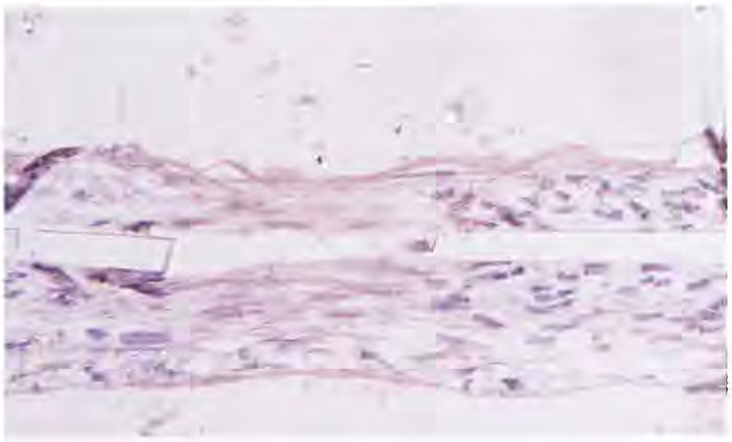

Fig. 27.5 Perfusion culture of gingival keratinocytes. Gingival keratinocytes cultured on polycarbonate membrane (PCM) under perfusion conditions show an increase in cell layers. (×300)

and histologically there was an average of 2.4 layers and a standard deviation of one layer. The increase in cell layers had been reported previously [36] for perfusion cultures of a human oral mucosal keratinocyte cell line.

After perfusion, cells attached to the polycarbonate membrane had more a cuboid shape with round nuclei, whereas the cells forming the top layers were flat without a smooth surface. After standard culture the cells and their nuclei showed flat shapes and the cytoplasms contained numerous vacuoles. The fewer vacuoles in the cytoplasm of the perfusion cultures may be signs of a less impaired metabolism of the fatty acids of keratinocytes [52], as vacuoles are interpreted as the physiological answer to cellular damage [11]; so in this respect, the cells in perfusion culture seem to suffer less cellular stress.

With respect to the expression of cytokeratins as markers of differentiation, for CK 13, CK 14, and CK 1, 2, 10, 11 differences were found. CK 13, a marker for suprabasal cells in nonkeratinizing epithelia, was very strongly expressed in all cell layers during the adherence phase and under standard culture conditions. After perfusion culture the CK 13 expression was limited to a few cells only at the basal aspect of the epithelium.

After standard culture anti CK 14 reacted mainly with the cells close to the membrane. After perfusion culture, CK 14 was only seen in cells that were close to the pores or filling the pores.

After perfusion culture CK 1, 2, 10, 11, markers of terminal differentiation of cornified epithelium, showed a positive staining reaction in the cytoplasm of all cells within the whole epithelium. After standard culture fewer cells were binding to the antibody. Only cells in close relation to the carrier membrane were expressing these cytokeratins.

Consequently, the culture conditions influence the differentiation pathway of the oral mucosa cells. Perfusion culture enhances the expression of the terminal differentiation markers CK 1, 2, 10, 11, indicating a differentiation as gingival keratinocytes [33], whereas after standard culture cells support differentiation as alveolar mucosa cells expressing CK 13 [41]. Hence, these morphological and cell biological changes clearly indicate a higher differentiation of oral keratinocytes cultured under perfusion conditions.

27.7 Gingival Keratinocyte—Gingival Fibroblast Co-cultures

To create mucosa looking like gingival epithelium after transplantation, the tissue engineered mucosa graft needs a fibrous connective tissue. Beside perfusion culture conditions, the importance of the submucosa connective tissue/fibroblast component has been demonstrated in vivo in transplantation studies as well as for in vitro investigations [7, 16, 42, 55]. Therefore, gingival biopsies were separated in epithelial cells and in fibroblasts. Primary gingival epithelial cultures were established using the explant technique and for fibroblast cultures the single cell suspension technique.

Different approaches were made to create complex keratinocyte fibroblast constructs, namely: (1) sandwich constructs, consisting of a fibroblast and a keratinocyte in two different biomaterials put on top of each other and (2) composite constructs, consisting of keratinocytes and fibroblasts in one biomaterial.

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