EPITHELIAL - MESENCHYMAL INTERACTIONS IN NORMAL AND NEOPLASTIC HUMAN ORAL MUCOSA

A STUDY ON *IN VITRO* ORGANOTYPIC MODELS

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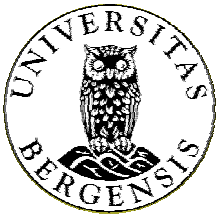

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ABBREVIATIONS

NHOM	- Normal Human Oral Mucosa
OSCC	- Oral Squamous Cell Carcinoma
NHOK	- Normal Human Oral Keratinocytes
HHOF	- Normal Human Oral Fibroblasts
DOK	- Dysplastic Oral Keratinocytes (DOK cell line)
OT	- Organotypic Cell Cultures
MEM	- Minimum Essential Medium Eagle
KSFM	- Keratinocyte Serum Free Medium
DMEM	- Dulbecco's Modified Eagle's Medium
PBS	- Phosphate-Buffered Saline
TBS	- Tris-Buffered Saline
FAD	- 3:1 Mixture of DMEM and Ham's F12 media
FCS	- Foetal Calf Serum
BPE	- Bovine Pituitary Extract
EGF	- Epidermal Growth Factor
KGF	- Keratinocyte Growth Factor
GM-CSF	- Granulocyte-Macrophage Colony-Stimulating Factor
TUNEL	- Terminal Deoxynucleotidyl Transferase Mediated Dutp Nick End Labeling
IHC	- Immunohistochemistry
CK	- Cytokeratin
Ab	- Antibody
PI	- Proliferation Index
AI	- Apoptotic Index
MMP	- Metalloproteinase
HGF	- Hepatocyte / Scattered Growth Factor

LIST OF PUBLICATIONS

The thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I. Costea D.E., Dimba E.A.O., Loro L.L., Vintermyr O.K., Johannessen A.C., The Phenotype of *In Vitro* Reconstituted Normal Human Oral Epithelium is Essentially Determined by Culture Medium. *J Oral Pathol & Med, in press.*

II. Costea D.E., Loro L.L., Dimba E.A.O., Vintermyr O.K., Johannessen A.C., Crucial Effects of Fibroblasts and Keratinocyte Growth Factor on Morphogenesis of Reconstituted Human Oral Epithelium. *J Invest Dermatol*, *121:1479-1486, 2003*.

III. Costea D.E., Johannessen A.C., Vintermyr O.K., Fibroblast Control on Epithelial Differentiation is Gradually Lost During *In Vitro* Tumour Progression. *Accepted to be published in Differentiation*.

IV. Costea D.E., Johannessen A.C., Vintermyr O.K., Species-Specific Fibroblasts Trigger Invasiveness of Early Neoplastic Oral Keratinocytes. *To be submitted to J Dent Res*.

SUMMARY

Epithelial-mesenchymal interactions are necessary for the development of oral epithelium during embryogenesis, but adult oral epithelium is also under the influence of mesenchymal tissue in both normal and neoplastic conditions. The aim of this study was to investigate the role of fibroblasts on morphogenesis of normal and neoplastic human oral epithelium. *In vitro* organotypic models of normal, early neoplastic and neoplastic human oral mucosa have been developed (Papers I and III). The cultured tissues obtained in the laboratory were assessed by morphometry, immunohistochemistry and the TUNEL method. Our data showed that fibroblasts had a crucial effect on cell growth and differentiation of the reconstituted normal human oral epithelium. Epithelial growth, but not differentiation could be restored by keratinocyte growth factor (KGF), one of the soluble factors synthesised by them. A novel finding was that fibroblasts were essential for restoring the normal pattern of cell death in reconstituted normal human oral epithelium (Paper II). The studies done on step-wise models of oral carcinogenesis showed that the role of fibroblasts on epithelial growth was maintained during *in vitro* tumour progression (Papers III and IV). In contrast, the tight control exerted by fibroblasts on epithelial differentiation and cell death of *in vitro* reconstituted normal human oral epithelium was gradually lost during *in vitro* neoplastic progression (Paper III). Furthermore, our results pointed to an important role for the keratinocyte-fibroblast cross talk and fibroblast-derived diffusible factors in triggering local invasiveness of early neoplastic oral keratinocytes. An interesting finding was the species-specificity of fibroblasts required for the invasive growth of early neoplastic keratinocytes to occur (Paper IV). This finding brings some concerns on the sensitivity of the *in vivo* xenotransplantation method as single test system for identifying putative malignant human neoplastic cells. In conclusion, this study brings *in vitro* evidence for the role of fibroblasts in coordinating the major biological processes of the suprajacent epithelium in both normal and neoplastic human oral mucosa.

Key words: oral, cancer, organotypic cell culture, fibroblast, keratinocyte, growth, differentiation, invasion.

INTRODUCTION

There is an increasing trend in cancer research towards investigations that aim a better understanding of the role that different components of the tumour stroma might play in the development of malignancies. The mesenchymal part of epithelial tumours has attracted interest during the recent years mainly due to the studies demonstrating that activated tumour stroma is a prerequisite for carcinoma invasion (Atula et al., 1997; Olumi et al., 1999; Mueller et al., 2001; De Wever et al., 2004). On the other hand, tumour-stroma interactions can only be understood on the basis of the interactions between normal epithelial and stromal cells (Fusenig, 1994; Tlsty and Hein, 2001). Therefore, these fundamental regulatory mechanisms have also become a central area of the tumour-stromal research field, which has as a long-term goal to identify and characterise tumour-specific epithelial-mesenchymal alterations as new targets for therapy.

The role of the stromal microenvironment in epithelial tumour progression is not yet understood. The lack of appropriate experimental models made the experimental study of these mechanisms in previous years difficult (Mueller and Fusenig, 2002). However, the development over the last two decades of the *in vitro* organotypic cell culture method that mimics the native architecture of the tissue of origin, with a network of different cell types spatially arranged as they are in the tissue of origin, has allowed pertinent studies on cell-to-cell interactions (Freshney, 2000; Mueller and Fusenig, 2002).

The work presented here took advantage of the use of such three-dimensional (3D) organotypic models in order to investigate the epithelial-mesenchymal (keratinocyte-fibroblast) interactions at different stages of keratinocyte transformation in human oral mucosa.

AIMS

The general aim of the present work was to study the role of fibroblasts on oral epithelial morphogenesis in normal human oral mucosa (NHOM) and during tumour progression, by use of the *in vitro* organotypic models.

SPECIFIC AIMS

1. To establish reproducible *in vitro* models of normal, early neoplastic and neoplastic human oral mucosa (Paper I and III).
2. To investigate the role of underlying fibroblasts and their products, including keratinocyte growth factor (KGF), on growth and differentiation of *in vitro* reconstituted normal human oral mucosa (Paper II).
3. To investigate the role of underlying fibroblasts on differentiation of oral epithelium during *in vitro* tumour progression (Paper III).
4. To investigate the role of underlying fibroblasts on *in vitro* growth and invasiveness of neoplastic keratinocytes, and to identify the fibroblast-related factors that trigger local invasion (Paper IV).

RESULTS AND DISCUSSION

1. METHODOLOGICAL CONSIDERATIONS

1.1. The choice of the experimental model

To study epithelial-mesenchymal interactions appropriate and functionally relevant experimental models are needed. The conventional cell culture models, in which cells grow two-dimensionally (2D) in monolayers, lack the interactions with the other cells normally found *in vivo* in their neighbourhood (Freshney, 2000). To some extent these interactions can be reproduced by growing different cell types in co-culture. However, for some cell types (*e.g.* fibroblasts) it is also essential for their optimal functionality to restore in culture the 3D structure of their environment (Grinnell, 2003). Thus, although easy and convenient to set up with good viability and reproducibility, the monolayers still have a major limitation in order to be used for studies on epithelial-mesenchymal interactions (Freshney, 2000). Organ cultures represent another *in vitro* model system with potential use for studying epithelial-mesenchymal interactions due to the fact that in these cultures the microenvironment of the epithelial tissue remains intact (Sacks, 1996). However, this system has its own problems, with difficulties in obtaining a good viability, and a sufficient number of parallel specimens necessary for a full experimental set up. On the other hand, *in vivo* studies on the functional role of the stromal compartment in normal tissues or neoplasms are often difficult to interpret due to the systemic influences that interfere with the local microenvironment (Mueller and Fusenig, 2002).

To circumvent these difficulties organotypic *in vitro* models have been elaborated. These models imply that the tissue of interest is reconstituted in the laboratory from isolated and purified cells in a 3D structure that follows the specific spatial arrangement and architecture of the native tissue. In the case of oral mucosa, this reconstitution implies at the most simple level that oral fibroblasts are embedded into a collagen type I gel (to reconstruct the connective tissue equivalent), on top of which are seeded the oral epithelial cells (to reconstruct the epithelial tissue equivalent) (Fig. 1). In this way, the *in*

vitro organotypic (OT) system allows the restoration of oral epithelium under the influence of the connective tissue environment.

The second great advantage of this model system is that each of the two compartments can be modified, thus complex study designs can be achieved. By changing, for example, the type of cells used in the epithelial compartment (primary normal, immortalised or neoplastic keratinocytes), multistage models of epithelial cancer can be constructed (Fusenig and Boukamp, 1998; Hansson et al., 2001). By using various types of fibroblasts, collagens, or combinations of different layers of collagen with or without fibroblasts, the mesenchymal compartment can be changed in such way that the role of its specific components on the development of the suprajacent epithelium can be assessed. One particular innovation in this way is the heterotypic model obtained by combining cells derived from different species in the same organotypic culture (Maas-Szabowski et al., 2001). The use of cells from different species, *e.g.* mouse fibroblasts in the connective tissue equivalent and human keratinocytes in the epithelial tissue compartment, facilitates the identification of the origin of compounds involved in epithelial tissue reconstitution, and thus the precise analysis of growth and differentiation regulatory mechanisms (Stark et al., 2004).

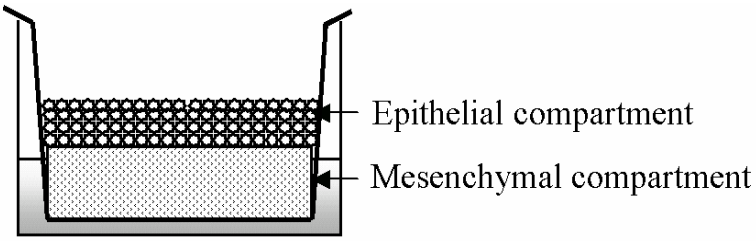

Fig. 1 Schematic illustration of the organotypic cell culture system. The keratinocytes grow air-exposed on a fibroblast-containing collagen gel. The matrix gel is in contact with the culture medium through a metal grid.

The organotypic cultures have also their limitations (Table 1). An alternative to overcome the time limitation is to xenograft the OTs in nude mice after 1-2 weeks of *in vitro* growth (Boukamp et al., 1990). The efforts to introduce immunocompetent cells into these types of cultures seem also to have some success, as recently reported (Lamarque et al., 2004).

However, there is a general consensus that the behaviour of keratinocytes in organotypic culture closely resembles the one of keratinocytes in native oral mucosa (Grafstrom, 2002). Moreover, the organotypic cell cultures are considered to date the most appropriate *in vitro* models for keratinocyte - fibroblast interaction studies (Freshney, 2000; Mueller and Fusenig, 2002).

Advantages of OT models
Allows epithelial-mesenchymal interactions
Offers a great flexibility for the study design as each of its compartments can be modified
Human material that poses less ethical considerations
Reproducible
Standardised (especially the serum free models)
Limitations of OT models
Restricted life span
Lack of vascular and immunocompetent compartment
Technically and financially demanding

Table 1. Advantages and limitations of the in vitro 3D organotypic models.

1.2. Optimisation of the method for growing organotypic cultures of NHOM

1.2.1. Type of medium

The “antagonism“ between epithelial and connective tissue cells in culture is so well known that it was even mentioned in one of Einstein’ autobiographic books citing the work of his friend Katzenstein (Schilpp, 1949; Fusenig and Worst, 1975). This “antagonism”, meaning that the cells have different inherent requirements for optimal growth and function in culture, has been of great importance when trying to assemble these cells together in organotypic cultures. Keratinocytes grow better in low Ca^{++} concentrations and differentiate in presence of serum and physiological levels of Ca^{++}, while fibroblasts require physiological levels of Ca^{++} and serum in order to proliferate and maintain their functionality in culture. In order to find the condition that ameliorates these inherent needs, tests were first conducted on primary normal human keratinocytes (NHOK) and fibroblasts (NHOF) on monolayers. From all media tested, only the FAD medium (mixture 1:3 of DMEM and Hams, F12 media) was able to reduce the dependency on serum for optimal fibroblastic growth, at the same time diminishing the negative impact that high Ca^{++} concentration had on the growth of keratinocytes (Paper

I). Further tests on organotypic cultures showed that the use of FAD medium was of a significant importance for optimal epithelial growth (Fig. 2) and differentiation (Fig. 3).

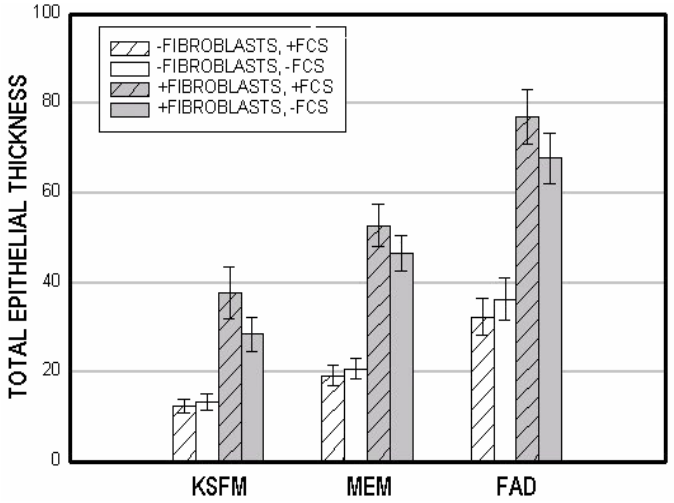

Fig.2 The effect of medium type and serum on total epithelial thickness of in vitro reconstituted human oral epithelium. *Human oral epithelium was cultured on top of either simple collagen gel or on collagen biomatrix containing fibroblasts in KSFM, MEM and FAD medium, in absence or presence of serum and BPE. Total epithelial thickness was measured by histomorphometry. Data represent the mean (µm) ± SEM of 5 different experiments.*

Since fibroblasts were also found to be crucial for keratinocyte growth and differentiation (Paper II) we ran a correlation analysis in order to identify the real importance of medium type for morphogenesis of reconstituted NHOM. The analysis showed that FAD medium indeed had a direct effect on epithelial cell proliferation independent on fibroblasts (Fig. 3 of Paper I). However, its effect on epithelial differentiation was mediated through the fibroblasts (Fig. 3).

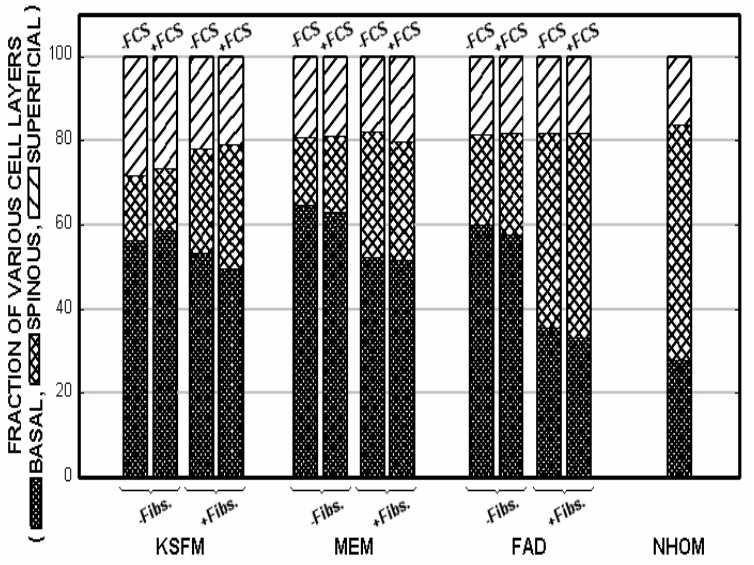

Fig. 3. The effect of medium and serum on the relative distribution of various cell layers in reconstituted human oral epithelium. *Reconstituted epithelium was grown in KSFM, MEM and FAD medium in absence or presence of serum and bovine pituitary extract (BPE), in absence or presence of fibroblasts in the collagen gel, for 10 days. The thickness of the basal, spinous, and superficial epithelial cell layers were analysed by histomorphometry. The mean values were determined from 5 separate experiments. Measurements of native oral epithelium (n=12) were included for comparison.*

Cytokeratin 13 (CK 13), a marker of differentiation for nonkeratinised oral epithelium (Presland and Dale, 2000), was used to assess the potential that different medium types have on epithelial differentiation of *in vitro* reconstituted human oral

epithelium. Few scattered positive cells were detected in the suprabasal cell layers of cultures grown in keratinocyte serum-free medium (KSFM) (Fig. 4). Cultures grown in minimum essential medium eagle (MEM) showed a strong staining, but localised only in the upper suprabasal cell layers (Fig. 4). Cultures grown in FAD medium showed a strong, uniform staining of all suprabasal cell layers (Fig. 4). Collagen IV, a major constituent of the basement membrane, could not be detected in KSFM grown cultures (Fig. 4). In MEM grown cultures, a strong positive staining for collagen IV was detected immediately underneath epithelium and around the neighbouring fibroblasts (Fig. 4). The staining was granular and not well confined to the epithelial-mesenchymal interface. Cultures grown in FAD showed a positive granular staining and a deposition of collagen IV confined towards the epithelial-mesenchymal interface (Fig. 4). Cell death, as visualised by the TUNEL method, was less frequent in the epithelium of FAD cultures (Fig. 4, Table 2).

		KSFM		*MEM*		*FAD*		*NHOM*
		+FCS +BPE	*-FCS -BPE*	*+FCS +BPE*	*-FCS -BPE*	*+FCS +BPE*	*-FCS -BPE*	
Total Epithelial Thickness	*-KGF*	38.8 ±5.7	27.5 ±3.8	52.9 ±4.6	46.8 ±6.0	77.3 ±6.6	67.9 ±6.6	
	+KGF	61.5 ±4.6	48.7 ±5.3	80.5 ±7.3	81.5 ±6.0	126.4 ±11.2	120.8 ±8.2	151.1 ±12.0
PI basal cell layer	*-KGF*	8.1 ±1.0	6.9 ±1.3	14.5 ±1.9	14.1 ±2.2	16.9 ±2.3	16.1 ±1.9	
	+KGF	13.3 ±1.3	12.4 ±1.1	22.1 ±2.9	21.2 ±1.8	25.3 ±3.2	24.3 ±1.2	23.8 ±1.5
PI suprabasal cell layers	*-KGF*	3.7 ±1.5	3.0 ± 1.8	4.8 ±2.7	4.6 ±0.4	1.1 ±0.3	0.4 ±0.2	
	+KGF	5.5 ±1.0	5.2 ±0.5	6.9 ±2.0	6.5 ±1.3	1.2 ±0.2	0.6 ±0.2	0.01 ±0.0
AI basal cell layer	*-KGF*	4.2 ±1.6	4.5 ±0.9	8.0 ±1.4	8.3 ±1.1	1.6 ±0.4	1.8 ±0.3	
	+KGF	2.6 ±1.5	3.7 ±0.4	7.6 ±2.0	7.5 ±1.3	1.5 ±0.2	1.1 ±0.4	0.3 ±0.1
AI suprabasal cell layers	*-KGF*	3.9 ±1.1	4.4 ±1.1	7.0 ±1.8	6.0 ±0.7	5.0 ±0.5	5.4 ±0.1	
	+KGF	4.6 ±1.3	4.7 ±0.9	7.1 ±1.4	7.0 ±0.9	7.2 ±0.3	7.8 ±0.4	8.1 ±0.6

Table 2. Histomorphometry, proliferation and apoptotic indexes of reconstituted human oral epithelium and native oral epithelium. *Oral epithelium was reconstituted on fibroblast-containing collagen matrices for 10 days in KSFM, MEM and FAD medium, in conventional (with serum and BPE) and defined conditions (serum free and without BPE), with or without addition of 10ng/ml KGF. Data represent mean ± SEM of 5 independent experiments and 12 native human oral mucosa specimens.*

In conclusion, these findings indicated that FAD medium was superior compared to other media tested for optimal growth and differentiation of NHOM in culture (Paper I). In this medium the cultures showed a defined proliferative basal region, a well-developed spinous cell layer and a clear terminally differentiated zone, comparable to native human buccal mucosa (Fig. 4).

1.2.2. Foetal calf serum (FCS) and bovine pituitary extract (BPE) supplementation

The use of defined culture conditions offers several advantages including less experimental variability and the possibility of identifying factors that directly regulate cell proliferation and differentiation (Freshney, 2000). Therefore, attempts have been done to establish organotypic cultures in defined conditions (Stark et al., 1999). Notably, the serum-free period of these "defined" cultures was limited only to the generation of the epithelium (Grafstrom, 2002). Fibroblasts were still derived in conditions with serum and the construction of matrix still involved the use of FCS. A similar approach was followed by our procedure for developing organotypic cultures of NHOM (Paper I). The keratinocytes were initially seeded in KSFM on top of a collagen type I matrix in which primary human fibroblasts have been previously embedded together with 1 vol. FCS (see Appendix). On day two of co-culture the culture medium was essentially shifted into serum-free FAD medium that was routinely used for the rest of the co-culture period. About 50% of the culture medium was replenished every second day with fresh serum-free medium. The original prepared collagen matrix, both in absence and presence of fibroblasts, contained about 9% serum. Serum from this compartment was expected to become essentially diluted with time of culture due to the procedure described above, but it is also likely that some remnants of serum have remained bound to this compartment during the whole period of cultivation. The growth of fibroblasts in the first day of co-culture with keratinocytes in KSFM medium could have benefited from this superfluous supplement of serum that remained in the matrix compartment. When the medium was switched into FAD medium on day two of co-culture, the dependency for serum on growth and survival of fibroblast was most probably less crucial, based also on our *in vitro* monolayer data (Paper I).

We (Papers I and II) and others (Stark et al., 1999) referred to the above described culture procedure as "defined" although there were present some remnants of serum due to the presumable leakage of serum from the matrix compartment initially assembled in presence of serum. Assuming that serum was freely diffusible in the collagen matrix and not retained (unspecifically bound) in the matrix, the amount of serum present in the culture media would be less than 0,08 % on day 10 in co-culture.

However, further refinements to develop strictly defined conditions for organotypic cultures while maintaining an optimal cell growth and differentiation are still needed.

The cultures grown in serum-free and BPE-devoid culture medium had impaired to a certain extent (not significantly) epithelial growth (Fig. 2, Table 2). However, this “defined” culture medium was able to support equally well the differentiation of the reconstituted human oral epithelium (Fig. 3). Moreover, the organotypic cultures grown in “defined” media were better organised, had a more delineated basement membrane deposition (Fig 4), and less fatty vacuoles as compared with FCS and BPE supplemented media (Paper I).

1.2.3. Growth factors

Keratinocyte growth factor (KGF) versus epidermal growth factor (EGF)

Although FAD supported an optimal differentiation of the reconstituted epithelium, its thickness was significantly lower than normally found in the suprapapillary region of the native buccal human mucosa (Table 2). KGF (10 ng/ml) significantly increased epithelial thickness and stimulated cell proliferation in all types of medium used in the study ($p < 0.05$, Table 2). Cell death rate (AI -Table 2), epithelial stratification (Fig. 4), and expression of various markers (Paper II) were not significantly changed by addition of KGF. Of note was the finding that keratinocytes derived from gingival mucosa were less responsive to the proliferative stimulative effects of KGF (Fig. 5). This suggests that keratinocytes from oral mucosa with different keratinisation patterns have different sensitivity KGF, and that this property might be part of their intrinsic regulatory mechanisms of growth.

Even though it was not a statistical significant effect ($p > 0.05$), EGF increased epithelial thickness from 60.6 ± 6.7µm to 76.9 ± 6.3µm when added alone (Fig. 6 Panel B). Addition of EGF in presence of KGF decreased the thickness of reconstituted epithelium in FAD medium from 137.6 ± 13.0µm to 123.2 ± 10.7µm, although not significantly ($p > 0.05$). This indicates that EGF and KGF had non-additive effects on oral epithelial growth (Fig 6), and that oral keratinocytes showed low sensitivity to EGF growth promoting effects.

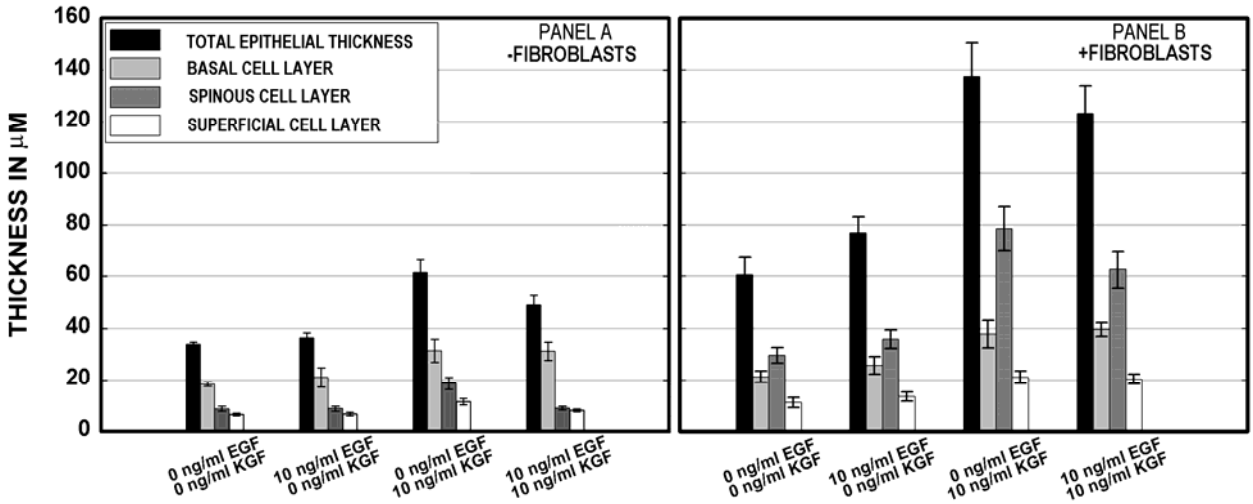

Fig. 6. Non-synergistic effects of EGF and KGF on morphogenesis of in vitro reconstituted human oral epithelium. *The cultures were grown for 10 days in FAD medium, in absence or presence of EGF (10 ng/ml) and KGF (10 ng/ml), in presence (Panel A) or absence (Panel B) of fibroblasts, as indicated in the figure. Total epithelial thickness, thickness of the basal, spinous and superficial cell layers were analysed by histomorphometry. The data represent the mean (μm) ± SEM from 3 different experiments run in duplicates.*

Granulocyte-macrophage colony-stimulating factor (GM-CSF)

As shown in Paper II, our data indicated an important role of fibroblasts not only for epithelial growth, but also for epithelial differentiation. In order to identify the fibroblast-related factor responsible for the differentiation effect of fibroblasts on epithelium, GM-CSF was tested alone or in various combinations with other growth factors and cytokines. GM-CSF has been reported previously to be the main responsible, together with KGF, for optimal differentiation and organisation of *in vitro* reconstituted skin (Szabowski et al., 2000). However, those tests were done on c-jun deficient mouse fibroblasts that did not produce KGF and GM-CSF, but could still secrete other factors important for epithelial differentiation. A better defined model for testing the role of various fibroblast-derived factors on epithelial differentiation would be a model devoid of any fibroblasts. We have run such tests including GM-CSF alone, in a range of 0.1 – 100 ng/ml, GM-CSF (0.1 – 100 ng/ml) in combination with KGF (0.1-10ng/ml), and GM-CSF (0.1 – 100 ng/ml) in combination with other growth factors and cytokines (EGF, TGFα, Il1, SCF, HGF in a range of 0.1-100ng/ml)(all from Sigma). None of the above mentioned conditions could restore an optimal differentiation of reconstituted NHOM (Fig 7). Further tests showed that addition of GM-CSF antibody (100ng/ml) (Sigma) did not

impair epithelial differentiation of reconstituted NHOM (Fig. 7). These findings suggest that the differentiation of reconstituted oral epithelium is not modulated by addition of GM-CSF (Fig 7).

1.2.4. Other additives

Insulin

Intracellular vacuoles could be detected on formalin fixed, paraffin embedded tissues of reconstituted NHOM, stained with haematoxilin-eosin (Paper I and II, Fig. 4). These vacuoles were found to be, at least in part, accumulations of glycogen or lipids (Paper I). The supra-physiological levels of insulin that were routinely used in the culture medium could have been a possible reason for the appearance of such vacuoles. In order to test the role of insulin for optimal growth and organisation of organotypic cultures of NHOM, we added insulin to the culture media in a concentration range of 0-5 µg/ml. The decrease of the insulin level bellow 5 µg/ml did not impair formation of intracellular vacuoles, but impaired formation of an orderly stratified and even epithelium (Fig. 8). Although insulin has been reported to influence the growth of oral keratinocytes in monolayers (Formanek et al., 1996), our results showed that the major effect of insulin on oral keratinocytes in organotypic cultures was to support an optimal epithelial architecture and not epithelial growth. These tests suggested further use of the supra-physiological levels of insulin for growing oral keratinocytes in organotypic cultures, as reported previously to be optimal for the growth of skin keratinocytes (Rheinwald and Green, 1975).

Three-iodo-thyronine and cholera toxin

Addition of three-iodo-thyronine (1.35 ng/ml) and cholera toxin (0.1 μg/ml or 8.33 μg/ml) did not significantly influence epithelial growth or differentiation. The only difference noticed was the presence, although not consistent, of epithelial protrusions into the collagen matrix in cultures supplemented with three-iodo-thyronine and cholera toxin (Fig. 9).

Lipid metabolits

Another potential reason for the presence of intracellular accumulation of tryglicerides in reconstituted NHOM (as shown by Red oil staining – Paper I) could have been an imbalanced metabolism of lipids, as previously suggested for OT cultures of skin (Ponec et al., 2000). The differences in the fatty acid content of reconstituted skin compared to the native tissue were ascribed to the cell culture conditions in which the growth medium was deficient in linoleic acid (Ponec et al., 2001). Addition of either linoleic acid-BSA (1mg/ml BSA, approx. 10 μg/ml linoleic acid) or pure linoleic acid (10 μg/ml) to the culture medium of reconstituted NHOM did not affect epithelial organisation or the presence of intracellular vacuoles. The persistence of an increased production of tryglicerides despite an optimal content of linoleic acid has also been reported for reconstituted skin (Vicanova et al., 1999). In our hands, linoleic acid-BSA was found to better support epithelial growth (129.1 ± 9.7μm) than simple bovine serum albumin (113.3 ± 8.8μm) (Fig. 10), although not statistically significant ($p > 0.05$).

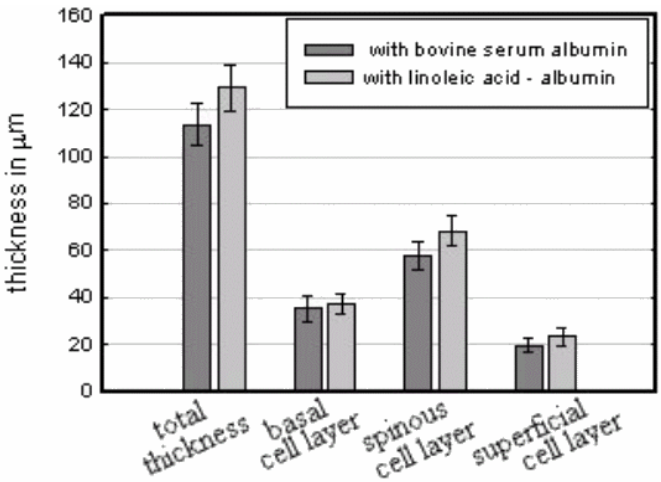

Fig. 10 The effect of linoleic acid-albumin on total epithelial thickness of in vitro reconstituted human oral epithelium. *NHOK were organotypically cultured on top of fibroblast-containing collagen biomatrix for 10 days in FAD medium supplemented with either BSA or linoleic acid-BSA. Total epithelial thickness was measured by histomorphometry. Data represent mean (μm) ± SEM of 3 different experiments.*

1.2.5. Longevity of OTs

OT cultures maintained a good histology up to 12 days. After 14 days of culture they already showed signs of matrix degradation (lysis) and increased desquamation and keratinisation of the epithelium (Fig. 5). KGF and FCS seemed to have an effect in maintaining the tissue architecture up to 14 days, especially in cells derived from buccal mucosa (Fig. 5).

1.3. Neoplastic models

1.3.1. Neoplastic cell lines

Spontaneously immortalised cells have been suggested as better models of step-wise tumourigenesis than virally immortalised cells, due to their stable phenotype for extended culture passages (Boukamp et al., 1997; Mueller et al., 2001). Such spontaneously immortalised cell lines isolated from human oral mucosa were sought to represent early and late stages of neoplastic transformation. Only one dysplastic oral keratinocytic cell line could be found commercially available, from the European Cell Culture Bank: human (caucasian) dysplastic oral keratinocyte (DOK) cell line, accession no. 94122104. This cell line was established from a tongue dysplasia that evolved after 11 years into a well differentiated oral squamous cell carcinoma (OSCC). It was reported not to form tumours in nude mouse, and it was considered to have a transformed, but not fully malignant phenotype (Chang et al., 1992). Later it was showed that it harbours p53 mutations (Burns et al., 1994). DOK cells were routinely grown on plastic surfaces without feeding layers, in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma) supplemented with 10% FCS, 20 μg/ml L-glutamine, and 5 μg/ml hydrocortisone. Addition of antibiotics and antimycotics in the culture medium impaired DOK cell growth. Therefore their addition into the DOK culture medium was avoided. The seeding density was also critical for propagating the DOK cells without feeding layers. A seeding density of at least 15 000 cells/cm^2 (splitting ratio 1:3) was needed for optimal platting and successful growth of subsequent cultures of DOK cells.

From many commercially available human neoplastic oral keratinocytic cell lines, we have chosen the PE/CA-PJ 15 cell line, accession no. 961211230, (Berndt et al., 1997). This cell line has been isolated from a well differentiated buccal SCC. It was reported to form tumours in nude mice with histology similar to the native carcinoma (Berndt et al., 2001). PE/CA-PJ 15 cells were routinely grown in Iscove's Modified Dulbecco's Medium (IMDM) (Sigma) supplemented with 10% FCS and 20 μg/ml L-glutamine.

In order to ensure experimental reproducibility, stokes of frozen cells were done immediately after their arrival from the ECACC. In this way, only DOK cells in passage 29 and PE/CA-PJ 15 cells in passage 9 were used for experimental purposes.

1.3.2. OT step-wise models of oral carcinogenesis

DOK and PE/CA-PJ 15 cells were grown organotypically following the same procedure as described for NHOK (see Appendix) in serum-free medium, but without the supplement of growth factors, antibiotics or antimycotics.

The organotypic cultures of DOK cells formed a disorganised epithelium with cellular atypia and hyperchromatic nuclei. DOK cells grew invasive into the fibroblast-containing biomatrix (Fig. 11). However, a certain degree of epithelial maturation, a polarised proliferation to the basal cell layer, and a positive staining for CK 13 in the spinous cell layer could be detected (Paper III). Therefore, the model derived from this cell line was considered more representative for early neoplastic than for dysplastic human oral mucosa.

The PE/CA-PJ 15 cell line formed an epithelium with no polarisation, cellular atypia and hyperchromatic nuclei. These cells invaded in a front–like manner into the fibroblast-containing biomatrix (Fig.11). This model was considered representative for neoplastic human oral mucosa.

The epithelial origin of these cell lines was verified by immunostaining for a pancytokeratin tissue marker (Fig.11).

2. EPITHELIAL - MESENCHYMAL INTERACTIONS

The aim of this review section was to bring together and discuss the information available from published reports on epithelial-mesenchymal interactions in both normal and neoplastic human oral mucosa. The mesenchyme or stroma is remarkably complex and has a number of different cellular components. Therefore the focus was restricted to the keratinocyte - fibroblast interactions. Reports from experimental work on human derived tissues (mainly oral mucosa, but also skin when data on oral mucosa were missing) would be discussed, but some descriptive studies on human archival material were also included. Only few studies from other research fields were included, when needed, for the discussion purposes.

2.1. Epithelial-mesenchymal interactions in normal human oral mucosa

As early as three decades ago experimental work clearly demonstrated that the interactions between epithelium and mesenchymal tissue play an important role not only for epithelial morphogenesis during embryonic development, but also for differentiation and maintenance of adult oral epithelium (for review see Mackenzie, 1994). These studies, which recombined whole sheets of epithelia with pieces of connective tissue stroma, were rather difficult to interpret since they included many different cell types and various unknown factors that could have influenced the outcome. The use of the more defined organotypic cell culture system could overcome some of these problems, and allow a more controlled study of specific interactions between the major stromal cells (fibroblasts) and keratinocytes (Fusenig, 1994). Such studies have pointed to an important role for fibroblasts on morphogenesis of *in vitro* reconstituted oral epithelium (Paper II) (Igarashi et al., 2003). Cultured keratinocytes showed full differentiation only if grown on top of a connective tissue equivalent that contained fibroblasts. Collagen matrix alone could support to some extent keratinocyte attachment and proliferation, but not an optimal differentiation. Therefore, the fibroblasts could be considered not only necessary, but also sufficient for optimal oral epithelial differentiation. These findings corroborate well with reports from *in vitro* reconstituted skin (El Ghalbzouri et al., 2002;

El-Ghalbzouri et al., 2002). An interesting finding of our work was the observation that the underlying fibroblasts could control the cell death programme of reconstituted oral epithelium by decreasing spontaneous cell death in the basal cell layer and promoting it in the superficial cell layers (Paper II). This suggested for the first time, by our knowledge, an important role for fibroblasts in directing the pattern of cell death in suprajacent epithelium.

Fibroblasts are not a homogeneous population, and they differ in morphology and function between tissue types, and even within the oral mucosa (Giannopoulou and Cimasoni, 1996). Thus, one might suspect that different types of fibroblasts could induce different patterns of differentiation and keratin expression in oral keratinocytes. As expected, some studies on organotypic cultures could show that new cytokeratins are induced in oral keratinocytes by different types of fibroblasts (Kautsky et al., 1995; Okazaki et al., 2003), although the original cytokeratin pattern of the epithelium persisted. A more extensive study with regards to the expression of epithelial differentiation markers showed that sheets of epithelium from skin, buccal and palatal mucosa grown on top of skin fibroblast-containing gels maintained their tissue-specific differentiation markers (Gibbs and Ponec, 2000). Later it was shown that except for alveolar fibroblasts, the type of fibroblast had little influence on keratinocyte differentiation (Chinnathambi et al., 2003). Interestingly, that latter study showed also that some tissue specific differentiation markers could be induced by the underlying oral fibroblasts in skin keratinocytes, but not by skin fibroblasts in oral keratinocytes. Taken together, all these studies done on organotypic cell cultures confirmed the suggestions drawn from earlier recombination studies that, at least in part, the phenotype of different epithelia is regulated by intrinsic properties of the keratinocytes (Billingham and Silvers, 1967; Mackenzie and Hill, 1981; Schweizer et al., 1984).

To explain the influence that the connective tissue exert on epithelial cells, two hypotheses have been proposed: 1) the connective tissue provides a physical substrate for attachment and orientation of basal keratinocytes; and 2) the connective tissue cells synthesise diffusible proteins that influence both growth and differentiation of epithelial cells (Melbye and Karasek, 1973). In support of the first hypothesis are the reports

which showed that the growth of a fully differentiated oral epithelium can be achieved in the absence of fibroblasts if the simple collagen type I gels are coated with collagen IV and laminin (Kim et al., 2001). From these results the authors suggested that these two major basement membrane proteins play an important role in the process of differentiation of oral keratinocytes. That the basement membrane is essential for epithelial organisation and differentiation has also been suggested by studies on *in vitro* reconstituted skin (Andriani et al., 2003), although the de-epidermised dermis used in that study could have contained residual growth factors that might have influenced the outcome of the experiments.

However, some studies could show that there is no need for a direct contact between skin keratinocytes and the connective tissue for a full epithelial differentiation (Boukamp et al., 1990). Studies from *in vitro* organotypic skin cultures confirmed that the effect of the connective tissue on epithelial morphogenesis was solely due to the diffusible factors synthesised by fibroblasts (El Ghalbzouri and Ponec, 2004). The growth and differentiation of skin keratinocytes was entirely restored by diffusible factors synthesised by fibroblasts when co-cultured with keratinocytes, but not by diffusible factors synthesised by fibroblasts grown in monolayers. This suggests not only that the fibroblasts influence the suprajacent keratinocytes, but also that the keratinocytes influence the underlying fibroblasts, implying that there is a continuous cross-talk between these two types of cells. In reconstituted skin this cross-talk seems to involve a double paracrine regulatory pathway: interleukin-1 synthesised by keratinocytes stimulates fibroblasts proliferation and synthesis of keratinocyte growth factor (KGF), which in turn acts on keratinocytes, stimulating their proliferation and synthesis of interleukin-1 (Maas-Szabowski et al., 2001). Reports from the latter group suggests that KGF and GM-CSF are the main fibroblast-derived mediators of skin keratinocyte proliferation and differentiation (Szabowski et al., 2000). What about the growth factors controlling the growth and differentiation of oral epithelium? It has been suggested that oral mucosa is different from skin with regards to the mechanisms of control of epithelial growth and differentiation (Dabelsteen et al., 1997; Sanale et al., 2002). The first experimental proof for this suggestion has come from the finding that oral fibroblasts

were more potent in producing KGF than their dermal counterparts (Gron et al., 2002; Okazaki et al., 2002). Recently, it was shown in organotypic models of NHOM that KGF could promote oral epithelial growth and proliferation, but unlike in the above described skin models KGF did not affect oral epithelial differentiation (Paper II). Unpublished observations (section 1.2.3. of the present study) suggested that neither GM-CSF, nor the combination of KGF and GM-CSF were responsible for the fibroblast-induced differentiation of oral epithelium. The results from our laboratory suggest that the oral fibroblasts possess additional mechanisms to KGF and GM-CSF synthesis that modulate differentiation of reconstituted human oral epithelium. This suggestion has also been recently proposed for dermal fibroblasts (El Ghalbzouri and Ponec, 2004). The latter study used reconstituted skin on simple collagen gels as test system. The previous study that reported an important role for KGF and GM-CSF on epithelial differentiation had used c-jun deficient mouse fibroblast-containing collagen matrices (Szabowski et al., 2000). Such fibroblasts, although deficient in KGF and GM-CSF synthesis, might have secreted other factors that could interact synergically with KGF or GM-CSF. This might explain why the dramatic effect reported for KGF and GM-CSF on epithelial differentiation in that particular test system was difficult to reproduce by use of other test systems. In favour of this rationale is a very recent report from that group which works on c-jun deficient fibroblasts. They seem to have identified a new fibroblast-derived diffusible factor (named Keratinocyte Commitment Factor) of importance for skin keratinocyte differentiation (Knebel et al., 2004).

In conclusion, it seems now generally accepted that the underlying fibroblasts play an essential role for organization, growth and differentiation of adult oral epithelium. Important factors in influencing the growth of oral keratinocytes have been identified, but the factors involved in oral epithelial differentiation are still to be unveiled.

2.2. Epithelial-mesenchymal interactions in neoplastic human oral mucosa

The efforts done in order to understand the pathobiology of the OSCC have been primarily focused on genetic alterations during the development of OSCC (Todd et al., 1997; Williams, 2000). Although the genetic and epigenetic alterations in the neoplastic cells occurring during the development of OSCC have been identified, the specific

factors that induce the transition from a benign non-invasive to a malignant invasive phenotype are still not known (Scully et al., 2000). However, invasion occurs within a tumour-host microecology, where stroma and tumour cells exchange enzymes and cytokines that modify the local microenvironment (Liotta and Kohn, 2001). Therefore, an alternative view emerged suggesting a potential involvement of the stromal tissue in tumour progression (Lewis et al., 2004). Recent advances in the biology and biochemistry of other epithelial cancers have also led to similar views on tumour stroma and fibroblasts as an important contributors to epithelial tumour development (Tlsty and Hein, 2001; Mueller and Fusenig, 2002; De Wever and Mareel, 2003; Radisky and Bissell, 2004).

2.2.1. Findings from descriptive studies on human archival material suggesting a role for fibroblasts in oral carcinogenesis.

For long it has been observed that the connective tissue undergoes a number of changes in the vicinity of oral carcinomas, most frequently described as a proteolytic degradation of the extracellular matrix (ECM) (Jones and Coyle, 1969). Initially it was thought that cancer cells are the ones that must break down their local ECM environment, *i.e.* basement membrane (McKinney and Singh, 1977). This concept is supported by several studies that showed an increase in the content of metalloproteinases (MMPs) and their activation in the tumour cells of OSCCs (Kurahara et al., 1999; Yorioka et al., 2002). An important role was attributed to MMPs 2 and 9 for basement membrane degradation. MMP-9 was proven to be highly expressed by malignant keratinocytes located at the tumour/stroma interface (Yoshizaki et al., 1997). However, several studies have showed that the MMP-2 present in carcinomas may not be derived from the tumour cells, but from the tumour stroma (Charous et al., 1997; Sutinen et al., 1998). In these studies, immunoreactivity for MMP-2 could be found in the peripheral cell layer of neoplastic islands, but MMP-2 mRNA expression was confined to fibroblasts with no signal detected in the epithelial tumour cells. This suggested that tumour cells are capable of utilising MMPs produced by stromal cells and indicated an active role for stroma in tumour invasion (Thomas et al., 1999).

Many studies have reported a decrease in the expression of the basement membrane components (*e.g.* laminin, collagen IV, decorin, heparan-sulphate proteoglycan) along the tumour-stroma borderline (Harada et al., 1994; Kosmehl et al., 1999; Haas et al., 2000; Berndt et al., 2001; Patel et al., 2002). However, these studies also showed that concomitant with the focal loss of laminin 5 and collagen IV, there was an increased expression of fibronectin, tenascin and laminin gamma 2 isoform in the associated tumour stroma. As a consequence, these components resulting from the degradation of the basement membrane were suggested to play a role for tumour cell invasion (Haas et al., 2000). With regards to the type of cells involved in this process, several studies have showen that carcinoma cells could directly produce the ECM components of tumour stroma, namely Tn-C_L split variant of tenascin (Hindermann et al., 1999), and foetal chains (alpha2 and beta2) of laminin (Kosmehl et al., 1999), but cells within the tumour stroma were also found to synthesise Tn-C_L (Hindermann et al., 1999).

The fibroblasts from tumour stroma seem therefore to be actively involved in the process of OSCC invasion. These activated stromal fibroblasts were first suspected to be reverted to a foetal phenotype, or to acquire a particular phenotype that occurs during wound healing (Dvorak, 1986). The active fibroblast phenotype that occurs during wound healing was identified with the myofibroblast phenotype expressing α-SMA (Gabbiani, 1981). Such phenotype has later been identified in the tumour stromal tissue of breast (Ohtani and Sasano, 1980), colon (Martin et al., 1996), and prostate carcinomas (Tuxhorn et al., 2002), as well as in the salivary gland tumours (Prasad et al., 1999). For these type of tumours it is now widely accepted that the myofibroblast phenotype is a part of the stromal changes that occur with cancer development (De Wever and Mareel, 2002; Desmouliere et al., 2003). Of note is that in these tissues, unlike oral mucosa, scattered myofibroblasts are already present in normal conditions (Willis, 1967). However, recently the myofibroblast phenotype has also been demonstrated in the stroma of oral squamous carcinoma *in vivo*, particularly at the invasive front (Barth et al., 2004; Lewis et al., 2004).

It has been initially speculated that changes in the gene expression of tumour fibroblasts could occur as a consequence of factors released by the tumour cells or by

the local inflammatory response, or possibly even as a result of the increased tensile forces generated by the expanding tumour mass (Gray et al., 1992). Later on, based on TGF- β1 over-expression observed in breast and prostate carcinoma cells, it has been proposed that the reactive stroma is induced by cytokines produced by carcinoma cells (Ronnov-Jessen et al., 1996; Rowley, 1998). For OSCC the reports are still controversial. Some studies have shown an over-expression of subtypes 1 and 2 of TGF-β protein in OSCC cells, and this was found to be associated with their severity, including pathological grading, clinical stages and neck lymph node metastasis (Jin et al., 2001; Tang et al., 2004). In contrast, other reports showed a decrease in TGF-β1 expression in OSCC cells and no correlation with clinico-pathological parameters (Logullo et al., 2003).

The process of trans-activation of fibroblasts into myofibroblasts is associated with increased secretion of growth factors, chemokines, cytokines, as well as extracellular matrix proteins and proteases (Tomasek et al., 2002; De Wever and Mareel, 2003;). It seems likely that these factors produced by myofibroblasts cooperate in influencing the invasive behaviour of transformed epithelium, as shown by studies done on breast, prostate and colon carcinomas. Whether this is the case in OSCC remains to be seen. However, one should keep in mind that these mechanisms have been described for cancers having an extensive desmoplastic stromal reaction, a feature not commonly found in OSCC (Breuninger et al., 1997).

2.2.2. Experimental findings that indicate a role for fibroblasts in tumour growth and invasiveness of OSCC.

Several studies have shown an effect of normal gingival fibroblasts and their conditioned medium on promoting invasiveness of OSCC cells (Matsumoto et al., 1989; Sugiura et al., 1996). Own unpublished observations suggested that fibroblasts and fibroblast-derived diffusible factors were important not only in enhancing invasiveness of neoplastic oral keratinocytes with an already invasive phenotype like OSCC derived keratinocytes, but also for triggering invasiveness of neoplastic keratinocytes with a less transformed phenotype (Paper III). Of note is that our findings differ from previous

reports (Matsumoto et al., 1989; Sugiura et al., 1996) in that not the conditioned medium from pure fibroblast monocultures, but the conditioned medium from parallel organotypic cultures of DOK on top of human oral fibroblast-containing matrices was able to trigger invasiveness of DOK cells on simple collagen gels. Our data indicate therefore that transformed oral keratinocytes have the ability to affect the spectrum of fibroblast-secreted soluble factors, and point to the interplay between transformed keratinocytes and stromal fibroblasts as a key factor for tumour invasiveness (Paper IV).

HGF was pointed as the major fibroblast-derived factor that triggered migration of OSCC cells through the initial recruiting of integrins, cytoskeletal proteins, and p125FAK into focal adhesions (Matsumoto et al., 1994). Although another chemotactic factor of importance for enhancing OSCC cell migration has been discovered afterwards (Sugiura et al., 1996), later studies confirmed that the promoting effect that oral fibroblasts had on the invasive growth and migration of OSCC cells was due to the secretion of HGF (Uchida et al., 2001; Lewis et al., 2004). The latter study could show that the HGF-dependent increased migration of OSCC cells was a consequence of the fibroblast transdifferentiation into myofibroblasts under the influence of TGF-beta1 synthesised by OSCC cells. In this way it was demonstrated for the first time the existence of a double paracrine mechanism between SCC cells and fibroblasts (Lewis et al., 2004).

The concept of fibroblast-derived HGF-stimulated invasion of OSCC cells is widely accepted (Hasina et al., 1999). However, there are still discrepancies between the observed effects of HGF on OSCC cells (Ohnishi and Daikuhara, 2003). The reason for those discrepancies is not clear at present, but it might be related to the fact that different cell lines have different sensitivity to the HGF stimulation, and that different types of experimental models have been used to assess cellular invasion. The majority of these studies have used the transwell migration test or the 2D migration assay on basement membrane coated plastic surfaces. Recently it has been suggested that the results from 2D monolayer models can not be directly extrapolated to the *in vivo* situation, since the mechanisms involved in *in vivo* cell invasiveness are much more complex than the ones emerged from 2D monolayer experiments (De Wever et al., 2004). Our own results from a 3D collagen type I invasion assay showed that HGF did not have a significant effect on

oral neoplastic cell invasion, although the cells used in the study expressed c-met, the receptor for HGF (Paper IV). These findings could be taken as argument for the concept that neoplastic cells need more than just an increased motility in order to invade the collagen matrix. Although HGF increases cell motility through integrin and cytoskeletal reorganisation (Matsumoto et al., 1994), this might not be enough for a cell to degrade and invade a collagen matrix, as suggested by studies that have shown that HGF could not stimulate synthesis of MMPs in OSCC cells (Uchida et al., 2001). However, the importance of HGF for invasiveness of oral tumour cells needs more investigations, since there are contradictory studies on the effects that HGF might have on OSCC cells (Hanzawa et al., 2000).

In our hands, the invasion triggered by fibroblast-derived diffusible factors represented only 40 % of the invasion seen in the organotypic cultures with direct contact between the oral neoplastic keratinocytes and underlying fibroblasts (Paper IV). This suggests that apart from the diffusible factors synthesised by underlying fibroblasts, the direct contact between transformed keratinocytes and stromal fibroblasts might also have a role in keratinocyte invasion, but in a more supportive than primary inductive way. An important role for the direct contact between neoplastic keratinocytes and underlying fibroblasts in promoting cell invasiveness has also been suggested in the case of larynx carcinoma derived cells (Atula et al., 1997). In that study it has been suggested that the type and origin of the fibroblasts were important for neoplastic keratinocyte invasion. The origin of fibroblast was also suggested to be important in promoting OSCC invasion (Berndt et al., 1998), tumour derived fibroblasts being the ones that induced the most aggressive behaviour of OSCC cells.

With regards to the origin of fibroblasts, *in vivo* studies have shown that the organ-specificity of fibroblasts was important in promoting carcinoma cell invasion (Fabra et al., 1992; Hsieh et al., 1993; Kawai et al., 2000). Our own unpublished studies (Paper IV) showed that the species-specificity of underlying fibroblasts was also required for local invasion of early neoplastic oral keratinocytes. This issue is of importance since the formation of invasive tumours or metastasis after *in vivo* xenotransplantation on immune-deprived animals is still the only generally accepted sign of malignancy for a transformed

cell line, and the test is widely used in cancer research (Freshney, 2000). From this point of view, the species-specificity observed in our studies brings some concern for the use of heterologous animal models as reliable (sensitive) test systems for putative malignant cell properties.

The species-specificity described above could be related to the differences in cytokine and collagenase production between mouse and human species (DeVore et al., 1980; Mestas and Hughes, 2004). That both fibroblast diffusible factors and extracellular matrix components are important for cell invasion has also been suggested, although on a model of colon carcinoma (De Wever et al., 2004). In that study a combination of fibroblast derived growth factors (HGF) and ECM protein (tensacin TC) have been shown to be necessary and sufficient to promote carcinoma cell invasion in the collagen gels in the absence of fibroblasts, while HGF or TC alone could not promote tumour invasiveness. Both unspliced tenascin-C (TN-Cl) and ED-B+ fibronectin, synthesised by tumour fibroblasts, have been suggested to play a role in OSCC cell invasion (Ramos et al., 1997; Berndt et al., 2001;).

2.2.3. Fibroblasts and control of differentiation in oral keratinocytes during tumour progression

Clinical and experimental evidence indicate that differentiation and malignancy are, to some extent, inversely correlated parameters (Fusenig et al., 1995). However, experimental studies have demonstrated that complete or even substantial loss in overall epithelial differentiation is not a prerequisite for malignant growth of cancer cells (Boukamp et al., 1988). Progressive dedifferentiation that is often associated with advanced tumour stages, is also found in immortalised cells that are, however, nontumorigenic (Chang, 1986). Based on these observations it has been suggested that differentiation and carcinogenesis could be controlled separately and not causally linked (Fusenig et al., 1995), although this concept is not generally accepted (Guarino et al., 2004).

In relation to stromal cell involvement in the process of differentiation during tumour progression very little is known at present, and most of the knowledge has

emerged from skin models. A loss of the fibroblast control on epithelial differentiation is considered to occur during epithelial tumour development (Fusenig et al., 1995). In *in vitro* organotypic skin models, early neoplastic keratinocytes (virally immortalized cells) have been shown to be still under the differentiation control of the underlying fibroblasts, similar to normal skin keratinocytes (Kaur and Carter, 1992; Tsunenaga et al., 1994). Later on, *in vivo* studies showed that benign clones of transformed skin keratinocytes are still able to differentiate under stromal influence, while the malignant clones do not respond to stromal differentiation signals (Tomakidi et al., 2003). This would also fit with our own results from *in vitro* experiments on organotypic models of normal, early neoplastic and neoplastic oral mucosa, which suggest that the control of fibroblasts on epithelial differentiation is gradually lost with tumour progression (Paper III). Notably, in our hands the underlying fibroblasts determined a higher degree of differentiation in early neoplastic oral keratinocytes (DOK cells), concomitantly promoting their local invasion. This result might support the suggestions that differentiation and malignant growth are not causally linked (Fusenig et al., 1995).

2.3. Concluding remarks

There is now increasing experimental support for the view that far from being a physical barrier, the stromal fibroblasts actively participate in the invasive process of OSCC. Although good candidates emerged from *in vitro* monolayer studies, the specific fibroblast-derived factors involved in the *in vivo* process of invasion are yet to be identified. It is of hope that the findings from the field of epithelial-mesenchymal interactions will make significant advances in controlling oral cancer through the manipulation of tumor cell-host microenvironment interactions. Current reports from other types of cancer suggest that the approach of targeting genetically stable components of a tumour's microenvironment may provide significant new advances in therapeutic intervention (Zhu et al., 2002). The 3D organotypic cell culture models may now offer the possibility of using genetically modified cells. They might help to develop new strategies for the treatment and management of oral cancer.

CONCLUSIONS

Based on the findings of this study, the following conclusions could be drawn:

- The culture conditions were essential for developing reproducible *in vitro* models of NHOM (Paper I).
- Epithelial growth, differentiation, and cell death program of reconstructed NHOM were under the control of underlying fibroblasts (Paper II).
- KGF, a paracrine growth factor synthesised by fibroblasts, was important for oral epithelial growth but not for oral epithelial differentiation (Paper II).
- Major steps of oral carcinogenesis could be reproduced *in vitro* (Paper III).
- The role of fibroblasts on epithelial cell growth was maintained during *in vitro* tumour progression (Paper III).
- The tight control exerted by fibroblasts on epithelial differentiation of reconstituted NHOM was gradually lost during *in vitro* tumour progression (Paper III).
- Diffusible factors synthesised by species-specific fibroblasts in the presence of transformed keratinocytes were the key factor in triggering invasiveness of early neopalstic oral keratinocytes (Paper IV).
- Isotypic *in vitro* 3D organotypic models could serve as additional test system for malignancy to the *in vivo* classical xenograft test of human derived neoplastic cells (Paper IV).

FUTURE PERSPECTIVES

Further studies should investigate the potential role that the local microenvironment has on progression of early oral neoplasia (non-invasive) into invasive oral neoplasia. The following lines ought to be further investigated (Fig. 12):

1. Identification of the specific mechanisms by which underlying stroma enhances invasiveness of early neoplastic oral keratinocytes:

- elucidating the importance that stromal activation (fibroblast transition to a myofibroblast phenotype) has for progression of early oral neoplasia .
- investigating whether the myofibroblast phenotype can be reverted to the fibroblastic state, and whether this could disrupt their pro-invasive capacity.
- studying the effects of basement membrane components on invasiveness of early neoplastic oral keratinocytes.
- investigating the role of metallo-proteinases (MMPs) produced by tumour associated fibroblasts on invasion of early oral neoplasia.

2. Investigation of the mechanisms of cell-to-cell communication between early oral neoplastic keratinocytes and neighbouring normal oral keratinocytes, and evaluate their importance on lateral spreading and tumour invasion:

- investigating the behaviour of early oral neoplastic keratinocytes in *in vitro* “confrontation” models with normal oral keratinocytes.
- assessing cell behaviour and invasion of oral neoplastic keratinocytes after removal of cell-to-cell adhesion suppression exerted through E-cadherin by normal neighbouring keratinocytes.

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APPENDIX

CELL CULTURE PROCEDURES

1. TISSUE BIOPSIES

Before starting the laboratory work, consent for using human biopsies has been sought and obtained from the Ethical Committee of the University of Bergen. An information leaflet was drafted together with a consent form to be signed by the patients that would decide voluntary to join the project.

Samples of normal human oral mucosa (NHOM) were obtained from the superfluous oral tissue after wisdom tooth extraction. Only healthy patients were included in the study. To ensure that the collection procedure would be reproducible carried out, the oral surgeons were asked to fulfil the following criteria: to select for sample collection only healthy patients with no signs of local inflammation; to avoid taking samples if heavy bleeding occurs during surgery; to harvest buccal and not gingival tissue; to harvest samples of at least 0.5cm^2; to keep the samples in the collection medium at 4°C.

Collection and transportation of the biopsy samples were done in Minimum Essential Medium Eagle (MEM) (Sigma, St. Louis, M.O., USA) supplemented with 200 U/ml penicillin, 200 μg/ml streptomycin, 0.5 μg/ml amphotericin B, and 6 μg/ml fluconazole (all from Sigma). Specimens were transported and kept at 4°C until processing. They were further processed within 4h after excision.

2. ISOLATION OF PRIMARY NORMAL HUMAN ORAL CELLS

For isolation of primary normal human oral keratinocytes (NHOK) and fibroblasts (NHOF) we have adapted a protocol that combines mechanical and enzymatic techniques for disaggregating the tissue and obtaining single cell suspensions (Oda and Watson, 1990).

2.1. Epithelial-connective tissue segregation

To separate the epithelium from the connective tissue enzymatic digestion at 4°C with dispase (GibcoBRL, Grand Island, N.Y., USA), a protease isolated from cultures of Bacillus Polymyxa was used. Dispase has been proven to remove the lamina densa by selective action on type IV collagen and fibronectin, but to preserve the anchoring fibrils and the epithelial cells (Stenn et al., 1989). Therefore it is considered an effective, but gentle agent for separating the intact epithelium from the connective tissue. This strategy was chosen in order to protect the keratinocytes from the basal cell layer. The tissue biopsy was first washed twice in MEM with antibiotics and antimycotic for 5 min. Special attention was given to remove all traces of blood and the fat tissue. The main part of the connective tissue was also removed in order to isolate mainly papillary fibroblasts, since deep connective tissue and the connective tissue associated with epithelium have been shown to be different in their ability to support epithelial growth (Mackenzie and Fusenig, 1983; Mackenzie, 1994). The tissue was afterwards cut in pieces of approx. 0.25 cm^2 and removed with tweezers into a 15 ml centrifuge tube (Nunc, Naperville, I.L., USA) with 2.2 mil. Units/ml dispase solution (20 mg dispase I in 7.5 ml of MEM). The tissue was let overnight at 4°C. The second day, the epithelium and connective tissue were mechanically separated with tweezers and collected in two separate 35 cm diameter dishes (Nunc).

2.2. Isolation of NHOK

A combination of warm (37°C) trypsin and mechanical treatment was used. The epithelial strips were cut in small pieces (appox. 0.1 cm^2 or even smaller) in the dish they were collected, in a minimal amount of MEM medium (approx. 1 ml). The pieces of the epithelium were collected with a pipette that previously had the walls humidified with medium (to avoid the pieces of the epithelium to get stuck on the pipette walls), and discharged in a 15 ml centrifuge tube with 2 ml of pre-warmed (37°C) trypsin-EDTA 10x

(GibcoBRL). The tissues were incubated for 7 min at 37°C. The specimen was then carefully mixed with a Pasteur glass pipette for 2-3 min, then the trypsin was inactivated with 1 vol. FCS (Sigma), and the sample was further mixed for another 4 min. Special attention was given to:

- the tip of the pipette: oblique relative to the sample tube in order to avoid the damage of the cells;
- the force of pipetting: strong enough to detach the cells, but gentle enough not to damage them.

The above presented procedure gave, in our hands, the most reproducible results regarding cell viability and attachment, and was based on a series of tests that included:

- cutting the epithelial tissue in: absence of any medium, MEM, or trypsin-EDTA;
- incubation with: trypsin-EDTA 10x, or trypsin-EDTA 1x;
- time of incubation: 5, 10, or 15 min;
- pipetting: without neutralisation, with neutralisation with FCS from the beginning of the procedure, with neutralisation with MEM supplemented with 10% FCS from the beginning of the procedure;
- time of pipetting: for 5, 7, or 10 min.

After enzymatic digestion and mechanical dispersion of cells (pipetting), the cell suspension obtained was centrifuged for 7 min at 1000 rpm. The supernatant was discarded, and the cell pellet was collected and resuspended in 4 ml of KSFM (GibcoBRL) supplemented with 1 ng/ml EGF human recombinant (Gibco BRL), 25 μg/ml bovine pituitary extract (BPE – GibcoBRL), 200 U/ml penicillin (GibcoBRL), 200 μg/ml Streptomycin (GibcoBRL), 0.5 μg/ml amphotericin B (GibcoBRL), 6 μg/ml fluconazole (as Diflucan from Pfizer, Amboise, France), 20 μg/ml L-glutamine (GibcoBRL). The cells were plated at approx. $1x10^6$ cells per 25 cm^2 cell culture flask (Nunc), and incubated at 37°C and 5% CO2 in a humid environment. The cells were allowed to attach for 48 hours, after which 2 ml of the medium were gently replenished and the floating tissue debrids were removed.

2.3. Isolation of NHOF

A combination of warm (37°C) collagenase and mechanical treatment was used. The pieces of connective tissue that remained after detachment of the epithelium were minced, and afterwards transferred into a 15 ml tube with 1 ml pre-warmed (37°C) 2x collagenase solution obtained by mixing 2mg collagenase (Sigma) with 1 ml PBS (Sigma). Approximately 1 ml of MEM medium was carried over along with the pieces of tissue, resulting in a final collagenase solution of 1 mg/ml (1x). After 30 min incubation at 37°C, the mixture was vigorously pipetted for 5 min with a glass Pasteur pipette. After inactivation of collagenase with 1 vol. MEM supplemented with 10% FCS, the cell suspension was centrifuged at 1000 rpm for 7 min. The cell pellet was resuspended in 4 ml of MEM supplemented with 10% FCS, 200 U/ml penicillin, 200 μg/ml Streptomycin, 0.5 μg/ml amphotericin B, 6 μg/ml fluconazole, 20 μg/ml L-glutamin, and plated at approx. $1x10^6$ cells per 25 cm^2 cell culture flasks in a humidified chamber supplemented with 5% CO_2 at 37^0C. The cells were allowed to attach for 48 hours after which 2 ml of the medium was gently replenished and the floating tissue debrids were removed.

2.4. Propagation of NHOK and NHOF in monolayers

For further propagation the cells were trypsinised for 5 min at 37°C with 2 ml of pre-warmed (37°C) 0.05% trypsin-0.53 mM EDTA (GibcoBRL) for NHOK, and 0.5% trypsin-5.3 mM EDTA (GibcoBRL) for NHOF (NOHF were prewashed with 2 ml of pre-warmed at 37°C PBS). During trypsinisation the cells were regularly checked by shaking the flask to prevent the damage of the cells due to excessive trypsinisation. The trypsinised cells were then pooled in 1 vol. FCS and spun at 1000 rpm for 7 min. The supernatant was discarded and the cells split in a ratio of 1:4 and seeded in new 25 cm^2 cell culture flasks. In general, the media used for propagation of NHOK (KSFM) and NHOF (MEM) had a 50% reduced concentration of antibiotics as compared with the medium used for the isolation of cells. Four ml (out of 5 ml) of the respective culture medium was replenished every third day. By keeping one ml of the previous medium (that contained endogenous growth factors) we aimed to ensure favourable growth conditions ("endogenous conditioning").

2.5. Sample to sample variation

Some sample to sample variation occurs when dealing with primary cells (Freshney, 2000). We have also noticed a sample to sample variation when isolating primary oral keratinocytes. Approx. 10% of the samples did not plate from the beginning. In general this was mainly related to a small sample size. At this step, another 5-10% of samples were lost due to occasional contamination. In some (approx. 20%) of the samples that plated initially, the keratinocytes started to detach after 4-6 days, and did not show signs of further growth. On average, 60-70% of samples gave rise to primary cultures of oral keratinocytes. Further growth of primary keratinocytes from isolation until near confluence (80-90%) took approx. 2-3 weeks (range 12 - 30 days). Subsequent passages (2-4) showed a higher growth rate, the confluence being reached after approx. 6 days (range 4-9 days) of culture at the split ratio of 1:4. From the initial primary cultures of keratinocytes, approx. 20-25% of samples grew at a slower rate and presented an

altered cell morphology. Those cells were found to be less optimal for OT cultivation and therefore omitted from further studies. The omission criteria were as follows: (1) >25 days until they reach confluence after the first plating, (2) >9 days until confluence in subsequent cell passages with a split ratio of 1:4, (3) predominnace of large, flattened cells, that formed colonies with an appearance suggestive for meroclones or paraclones (Barrandon and Green, 1987). The samples that showed initially good attachment and extensive growth in subculture presented high numbers of colonies composed of small compact cells, with an elongated morphology (ovoid) rather than cuboid, suggestive for holoclones (Barrandon and Green, 1987). The latter sample to sample variation could have been related to differences in the population "stemness" of the isolated keratinocytes.

3. CONSTRUCTION OF ORGANOTYPIC CULTURES (OT)

For organotypic cultures, only NHOK and NHOF in passages 2-3 were used. NHOK and NHOF monolayer cultures had to be closely followed and synchronised, in order to use them for the construction of OTs just before they reached 90 % confluence. The change of the medium was avoided the day before OT construction.

3.1. Preparation of the connective tissue equivalent

3.1.1. Simple collagen matrix

The collagen matrix was made by mixing on ice 7 vol. (3.40 mg/ml) of rat tail collagen type I (Collaborative Biomedical, Bedford, M.A., USA), 1 vol. Dulbecco's Modified Eagle's Medium 10x (Sigma), 2 vol. reconstitution buffer (RB) pH 8.15 (2.2 g $NaHCO_3$, 0.6 g NaOH, 4.766 g HEPES in 100 ml dH_2O), and 1 vol. FCS. The pH was adjusted by use of the reconstitution buffer to a final value of 7.2. Attention was given to avoid air-bubble inclusion in the collagen matrix or polymerisation of the matrix on the pipette walls by using pre-chilled pipettes and not mixing big quantities of matrix at one time. Seven hundred μl of the collagen matrix were layered into the inner well of the organ culture dishes (Becton Dickinson, Franklin Lakes, N.J., USA). As container for the matrix we have also tried 3.5 cm dishes (Nunc), or 24 wells dishes (Nunc). The latter ones proved not to be so easy to manipulate at the lifting step. The matrix was allowed to polymerise for 1h in a humidified chamber at 37^0C, and then equilibrated with 1 ml of routine fibroblast culture medium.

3.1.2. Fibroblast-containing collagen biomatrices

NHOF were trypsinised as described above and resuspended in FCS at a concentration of approx. $10x10^6$ cells/ml. For construction of the biomatrix, 1 vol. of the NHOF suspension in FCS was added to 10 vol. of the mixture of 7 vol. collagen : 1 vol. DMEM 10x : 2 vol. RB, to a final concentration of $0.5x10^6$ fibroblasts/ml collagen matrix. Less than $0.1x10^6$ fibroblasts/ml were found insufficient to support an optimal epithelial growth and differentiation, while more than $10x10^6$ fibroblasts/ml were found to lyses and degrade the collagen matrix in less than 10 days. Of importance was to add the fibroblasts only after pH neutralisation (pH of 7.2-7.4) of the mixture. Seven hundred μl of the biomatrix were layered into the inner well of the organ culture dishes. The matrix was allowed to polymerise for 1h in a humidified chamber at 37^0C and then equilibrated with 1 ml of routine fibroblast culture medium ("equilibration medium").

3.1.3. Collagen IV-coated matrices

The matrices to be coated were allowed to polymerise for 20 min in a humidified chamber at 37^0C. Collagen IV solution was prepared on ice as indicated by the producer, by adding 1.666 ml of acetic acid 0.25% to the lyophilised powder (Sigma). The solution was neutralised 3:1 vol. with RB, and 18 μl of the neutralised collagen IV solution was gently poured on top of each of the polymerised matrix. Usually, one drop was first dropped at one of the poles of the cultures, and than the dish was tilted and rotated till the collagen IV solution touched the other pole of the matrix. A second drop was then dropped at the second pole and the dish was again tilted and rotated in the opposite direction in order to cover the remaining half part with collagen IV. The culture was let for 1 h in the incubator to allow the polymerisation of the layer of collagen IV, and thereafter 1 ml of the equilibration medium was added on top. However, the matrices coated with collagen IV did not support a better morphogenesis of reconstituted NHOM, therefore this strategy was not further used in the routine protocol of growing OTs.

3.1.4. Sandwich models

The principle behind the construction of sandwich models was to develop a model system in which the keratinocytes could be under the influence of the diffusible factors released by fibroblasts but without having a direct contact with them. Therefore the collagen matrix was composed of two different layers. For construction of the bottom layer, 500 μl of fibroblast-containing biomatrix were poured into the inner well of an organ culture dish. The fibroblast-containing biomatrix for the sandwich models was constructed as described at section 3.1.2. The only difference was that it contained a higher number of NHOF per ml ($0.7x10^6$ fibroblasts/ml), in order for the 500 μl fibroblast-containing biomatrix to provide the same number of fibroblast in the final sandwich co-culture as the "standard" model with 700 μl of $0.5x10^6$ fibroblasts/ml biomatrix. This bottom layer was let for 20 min to polymerise in the incubator. The intermediate layer of the sandwich model (or the upper layer of the collagen matrix) usually consisted of a collagen type I matrix without fibroblasts. 200 μl of simple collagen matrix were constructed as described in section 3.1.1. and poured afterwards on top of the bottom layer. The matrix was let for one more hour to polymerise in the incubator. One ml of the equilibration medium was then added on top of the second layer.

This protocol (bottom layer of 500 μl $0.7x10^6$ fibroblasts/ml, upper layer of 200 μl collagen type I matrix) was found to provide a sufficient diffusion of nutrients to the suprajacent epithelium while restricting the migration of fibroblasts to the epithelial cell layers. Different separation volumes in a range of 100-700 μl have been first tested.

3.2. Seeding of keratinocytes, lifting and maintenance of the cultures

Twenty-four hours after matrix construction, the equilibration medium was removed and 1 ml of keratinocyte routine culture medium (KSFM) with $0.5x10^6$ keratinocytes/ml was layered on top of each collagen matrix. The cultures were detached from the dish walls after additional 24 h, at a time point when the keratinocytes had not yet reached confluence. After detachment, half of the culture medium was replenished with serum free organotypic (FAD) medium: 3 vol. Dulbecco's Modified Eagle's Medium (Sigma) / 1 vol. Ham's F12 (Sigma), supplemented with 0.4 μg/ml hydrocortisone (Sigma), 5 μg/ml insulin (Novo Nordisk, Bagsværd, Denmark), 20 μg/ml transferrin (Sigma), 50 μg/ml L-ascorbic acid (Sigma), 1 mg/ml linoleic acid-albumin (Sigma), 200 U/ml penicillin, 200 μg/ml streptomycin, 0.5 μg/ml amphotericin B, 6 μg/ml fluconazole, 20 μg/ml L-glutamine. Two days later, the cultures were layered on curved metal grids that were covered with a thin filter paper soaked in organotypic culture medium. The cultures were lifted to the liquid-air interface and grown for 7 more days. We found of importance to follow the strategy of "endogenous conditioning" also for OT cultures. Therefore only half of the culture medium (2.5 ml) was changed every second day during the time of culture.

3.3. Harvesting of OTs

On day 10 of co-culture the organotypic cultures were harvested. To avoid the damage of the cultured tissues, they were collected together with the filter paper on which they laid during the culture time, and transferred onto a plane plastic surface. Any further manipulation was done only by touching the filter paper. Each tissue was divided in 2 equal parts with a sharp scalpel. One part was fixed in 4% buffered formalin pH 7.15 and embedded in paraffin. The other one was snap frozen in isopentane pre-chilled in liquid nitrogen. For an optimal orientation of the epithelium in the frozen samples, the tissue was first placed on a small plastic surface, covered with a drop of Tissue-Tek O.C.T. compound (Sakura Fintek, Zoeterwoude, The Netherlands), and frozen on that plastic surface. The frozen tissue became then harder, and therefore easier to manipulate. Afterwards it was taken with tweezers from the plastic surface, oriented perpendicular on a cold bolck support (that the tissue would stick on), and frozen again after complete covering with Tissue-Tek O.C.T.

Due to the fact that the artificial tissues were very thin (1-3 mm) and fragile, the routine protocol for fixation had to be adapted to the cultured tissues by excluding steering and decreasing the time of dehydration as follows: 4% buffered formalin pH 7.15 overnight; alcohol 70° 1h; alcohol 96° 1h; alcohol 100° 1h; xylen 1h; warm paraffin, 3h in the oven at 56°C. Special attention was given to orientate properly the tissues when embedded in paraffin.

4. IMMUNOHISTOCHEMSITRY

4.1. Attachment of OT tissue to the glass slides

Some tissues detached from the routine glass slides during the IHC procedure. Therefore, various types of slides have been tested:

- routine Menzel glasses (WWR, Braunshweig, Germany)
- Superfrost Plus Menzel glasses (WWR)
- ChemMate Capillary Gap Microscope glasses (DAKO)
- Silane (3-triethoxysilyl-propylamin) coated glasses (APES slides), a home made variant obtained by washing the routine Menzel glasses with silane for 5 min.

The best results were obtained using Silane coated glasses.

4.2. Pretreatment

For antigen retrieval in formalin fixed paraffin embedded tissue sections, the following pretreatments, either alone or in various combinations were tested:

- microwave treatment in 10mM citrate buffer pH 3.0 or pH 6.0
- microwave treatment in TRIS /EDTA buffer
- protease enzyme digestion (20 μg/ml Proteinase K – DAKO, in TBS buffer)

Microwave treatment at 750 W for 9 min followed by 15 min at 500 W in 10 mM citrate buffer (pH 6.0 for cytoplasmic/extracellular staining, pH 3.0 for nuclear staining) gave the best results. However, for collagen IV and laminin staining, protease treatment for 10 min at room temperature after microwaving was also necessary.

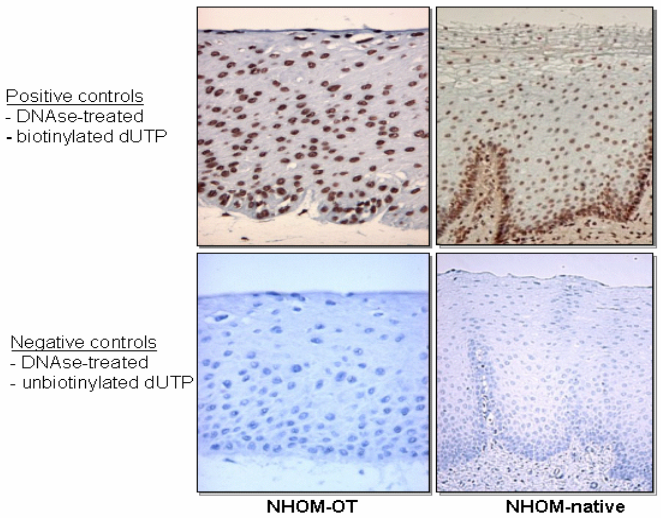
5. THE TUNEL METHOD

5.1. Pretreatment

The best results were obtained when a combination of microwave (850 W for 5 min, followed by 500 W for 5 min in citrate buffer 10mM, pH 3.0) and protease K treatment (15 min at 37°C) was used.

5.2. Positive and negative controls

For positive controls specimens were treated with 0.5 mg/ml DNase (Roche Diagnostics GmbH, Mannheim, Germany) in TBS buffer for 15 min at 37°C prior to incubation with bovine serum albumin. The specificity of the TUNEL reaction was tested by substituting the biotinylated dUTP in the TUNEL labelling mixture with unbiotinylated dUTP (Roche) in excess (negative controls).



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7. TROUBLE-SHOOTING GUIDLINES FOR GROWING OTs OF NHOM

No.	*Procedure*	*Pitfall*	*Trouble-shooting*
1.	Sample collection	Low viability / poor quality of cells	▪ Cut away the bleeding and destroyed pieces of the biopsy; ▪ Cut away the deep connective tissue; ▪ Process only samples bigger than 0.5 cm^2.
		Contamination	▪ Keep the transportation time less than 4h; ▪ Double the concentration of the usual AB/AM supplement; ▪ Use Fluoconazol 6 μg/ml in the transport media;
2.	Isolation of cells	Low viability of cells	▪ Check the integrity of the Pasteur pipettes' tips; ▪ Keep the pipette oblique to the tubes' walls; ▪ Do not use much force for pipetting.
		Low number of cells	▪ Use pre-warmed trypsin to efficiently detach the cells; ▪ Thorough pipetting.
3.	Propagation of cells	Poor quality of cells	▪ Replanish only ¾ of culture medium; ▪ Do not let the cells to reach more than 80-90% confluence.
		Contamination	▪ Use AB/AM supplements in the culture medium; ▪ Use EasyFlasks (Nunc); ▪ Use Biocidal (Wak-Chemie Medical GmBH, Bad Soden, Germany) in the water basin of the incubator and to spray the incubator.
4.	Construction of OTs	Low viability of cells	▪ Carefully check the Ph of the matrix; ▪ Avoid air bubble inclusion.
		Heterogeneous matrix	▪ Avoid premature polymerisation on the pipettes' walls; ▪ Mix well, sometimes using Pasteur pipettes; ▪ Do not prepare big quantities of matrix at one time.
5.	Lifting of OTs	Damage of the tissue	▪ Lay the matrix directly into the inner well of the organ culture dishes; ▪ Use teeth tweezers to clip the tissue.
6.	Maintenance of OTs	Poor epithelial growth	▪ Use FAD medium; ▪ Add KGF instead of EGF to the culture medium.
		Poor epithelial differentiation	▪ Use FAD medium; ▪ Do not use EGF; ▪ Keep the cultures dry.
		Lysis of the matrix and NHOF overgrowth	▪ Do not use FCS; ▪ Use the indicated amount/no. of fibs (as low as possible).
7.	Tissue harvesting	Epithelial-mesenchymal detachment	▪ Harvest the tissue with the filter paper from the culture grid; ▪ Manipulate the tissue only by catching the lens paper with tweezers; ▪ Always use new scalpels to cut the tissue in half.
		Poor orientation	▪ First freeze the tissue on a plastic surface.
		Tissue damage	▪ Do not let the tissues to be processed together with the routine biopsies.
8.	IHC on OT tissue	Detachment of tissue from the glass	▪ Use APES slides

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Paper I

The Phenotype of *In Vitro* Reconstituted Normal Human Oral Epithelium is Essentially Determined by Culture Medium

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The phenotype of *in vitro* reconstituted normal human oral epithelium is essentially determined by culture medium

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OBJECTIVE: To evaluate the role of various culture media and serum supplement on growth of oral cells in monolayer, and on morphogenesis of *in vitro* reconstituted normal human oral epithelium.
METHODS: Primary keratinocytes and fibroblasts were isolated from normal human buccal mucosa. The monolayers were assessed by growth curve analysis and morphology. The organotypic cultures were evaluated by morphometry, immunohistochemistry, and TUNEL.
RESULTS: FAD medium was able to support fibroblast growth in defined conditions, and to diminish the negative effect of physiological Ca concentration on keratinocytes in monolayers. Medium type had a profound influence on morphogenesis of *in vitro* reconstituted human oral epithelium. FAD medium was superior to other types of medium tested in supporting both epithelial growth and differentiation. Defined conditions supported epithelial morphogenesis equally well as serum-containing medium.
CONCLUSIONS: This study points to an essential role of medium composition for optimized growth and differentiation of primary organotypic cultures.
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Keywords: differentiation, keratinocyte, oral mucosa, organotypic cell culture

Introduction

Artificial tissues of human origin have become important tools for innovative research (1). As regards to reconstruction of human oral mucosa, various *in vitro* models have been developed (2–10), mainly by adoption of the methods and culture medium from skin models. Recent reports have suggested the importance of mesenchymal equivalent (7, 11), or culture medium (3, 7) for *in vitro* engineering of oral epithelium. However, the specific effect of these various types of medium on growth of oral cells in monolayers, or on morphogenesis of *in vitro* reconstituted oral epithelium has not been yet addressed. This study aims to evaluate the potential of various culture media (KSFM, MEM and FAD), and of conventional [supplemented with serum and bovine pituitary extract (BPE)] vs. defined conditions to support *in vitro* oral epithelial growth and differentiation. This methodologically oriented study provides clues to the importance of medium composition for an optimal morphogenesis of reconstituted normal oral epithelium in organotypic culture.

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Materials and methods

Tissue material

Eleven samples of normal human oral mucosa (NHOM) were obtained from the superfluous buccal tissue after wisdom tooth extraction, and were used to isolate the primary cells. The Ethics Committee of the University of Bergen approved the study, which included only clinically healthy donors after informed consent.

Culture of primary cells in monolayers

Primary human oral keratinocytes (NHOK) and fibroblasts (NHOF) were isolated from NHOM as previously described (4). NHOK were grown on plastic surfaces (Nunc, Naperville, IL, USA) without feeding layers, in serum free KSFM (GibcoBRL, Grand Island, NY, USA) supplemented with 1 ng/ml human recombinant epidermal growth factor (GibcoBRL), 25 μg/ml BPE (GibcoBRL), 20 μg/ml L-glutamine (GibcoBRL), 100 U/ml penicillin (GibcoBRL), 100 μg/ml streptomycin (GibcoBRL), 0.25 μg/ml amphotericin B (GibcoBRL), 6 μg/ml fluconazole (Pfizer, Amboise, France). NHOF were grown in minimum essential medium eagle (MEM) (Sigma, St Louis, MO, USA) supplemented with 10% fetal calf serum (FCS) (Sigma), 20 μg/ml L-glutamine, 100 U/ml penicillin, 100 μg/ml

streptomycin, 0.25 μg/ml amphotericin B, 6 μg/ml fluconazole. Cells in second or third passage were seeded in 24-well dishes at a cell density of 3×10^4 cells/well. After a period of 24 h, to allow the cells to plate, the routine medium was changed to media of various compositions to be tested: KSFM, MEM, or FAD, a 3:1 mixture of Dulbecco's modified eagle's medium (Sigma) and Ham's F12 (Sigma), supplemented or not with FCS (0, 1, 5 and 10%). KSFM was tested at two different Ca^{++} concentrations: 0.09 and 1.8 mM.

Culture of organotypic human oral mucosa
Second passage of primary NHOK and NHOF were used for preparation of organotypic cultures. The organotypic co-cultures were obtained by growing NHOK on top of a reconstituted collagen I biomatrix supplemented or not with NHOF, as previously described (Costea *et al*, 2003). The cultures were grown for 10 days in the various culture media tested: KSFM, MEM and FAD, in presence or absence of FCS (1% when used) and BPE (25 μg/ml when used). The basic supplements included in all the media tested were: 10 ng/ml epidermal growth factor (GibcoBRL), 0.4 μg/ml hydrocortisone (Sigma), 5 μg/ml insulin (Novo Nordisk, Bagsværd, Denmark), 20 μg/ml transferrin (Sigma), 50 μg/ml L-ascorbic acid (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, 6 μg/ml fluconazole, 20 μg/ml L-glutamine.

Histochemical staining
Periodic acid Schiff (PAS) staining was used for identification of glycogen on 15 μm formalin fixed, paraffin embedded sections. Parallel sections were pre-treated or left untreated with diastase (Sigma) for 25 min before staining with Schiff's reagent (Sigma) for 5 min as previously described (12). The presence of glycogen was demonstrated by loss of PAS positive staining on diastase pre-treated sections. Sections from vaginal mucosa and NHOM were used as positive controls. Fifteen-micrometer sections of fresh frozen tissue were used for identification of lipids with red oil solution (Sigma) for 10 min at 37°C, as described (13). Frozen sections of normal skin with subcutaneous fat tissue served as positive controls, and formalin fixed, paraffin embedded sections of subcutaneous fat tissue were used as negative controls.

Immunohistochemical staining
Five micrometer formalin fixed, paraffin embedded sections were processed as previously described (11). The primary antibodies and the titrations used in this study were as follows: Ki-67 (IgG 1), MIB-1clone, 1:50 (DAKO, Glostrup, Denmark); CK13 (IgG 1), KS-1A3 clone, 1:400 (Novocastra Laboratories Ltd, Newcastle, UK); collagen IV, CIV221 clone, 1:25 (DAKO).

TUNEL method
Cell death was detected by TUNEL method (terminal deoxynucleotidyl transferase-mediated dUTP *in situ* nick end-labeling) as previously described (14).

Evaluation of samples and statistical analysis
For tests run on monolayers growth curves were obtained after plotting the normalized values of cell density (cells/cm^2) against time using Sigma Plot program (SPSS Science Software GmbH, Erkrath, Germany). The number of population doublings (PDs) and population doubling time (PDT) were calculated as previously indicated (15). The morphometrical analysis of the artificial tissues was done as previously described (11). Statistical analysis was performed using the SPSS program version 11.0 (SPSS Inc. Chicago, IL, USA), Wilcoxon *U*-test with the level of significance set at 5%.

Results

FAD supports the growth of NHOF in defined conditions and diminishes the negative effects of physiological calcium on NHOK
During the logarithmic growth phase primary NHOK grown serum free in KSFM low Ca^{++} had a PDT of 27.6 ± 2.1 h (Fig. 1a). PDT increased to 34.5 ± 4.3 h ($P = 0.21$) in KSFM 1.8 mM Ca^{++}, 102.4 ± 8.7 h ($P < 0.05$) in MEM, and 28.8 ± 3.6 h in FAD ($P = 0.4$), showing that at physiologic Ca^{++} concentration FAD medium was superior even to KSFM in supporting epithelial growth (Fig. 1c,g). Addition of serum decreased keratinocyte proliferation in all media tested ($P < 0.05$), apart of FAD supplemented with 1% FCS (Fig. 1g).

Primary NHOF entered the log phase of growth after 1 day of culture (Fig. 1f) when cultured in MEM (1.8 mM Ca^{++}) supplemented with 10% FCS. The lag phase was significantly prolonged in KSFM with low Ca^{++}. The highest cell proliferation was reached in MEM supplemented with 5 and 10% FCS (4.2 ± 0.5 PDs, PDT 26.0 ± 3.1 h). NHOF cell growth was not significantly different among serum supplemented media tested in this study ($P > 0.05$) (Fig. 1b,d,f,h). The fibroblast proliferation was strongly impaired in serum deprived conditions apart of serum free FAD (Fig. 1h). The bipolar shape of fibroblasts and formation of characteristic parallel arrays and whorls was maintained in all serum supplemented conditions, but also in serum free FAD. Low Ca^{++} (KSFM) and serum free KSFM and MEM induced thin cytoplasmic extentions and accumulation of cytoplasmic vacuoles.

The growth of in vitro reconstituted human oral epithelium is modulated by the culture medium
Epithelial thickness showed significant differences between cultures grown in KSFM, MEM and FAD ($P < 0.05$; Table 1; Fig. 2), the thinnest ones being found in KSFM cultures. MEM cultures formed on average a thicker epithelium, but with variation in thickness along the tissue culture. The thicker epithelium of FAD cultures showed less variability along the tissue culture. Cell proliferation rates followed a similar trend, PI in the basal cell layer of KSFM cultures being significantly lower ($P < 0.05$) than the PIs observed in MEM or FAD cultures (Table 1). In all types of medium, the frequency of Ki-67 positive cells detected in

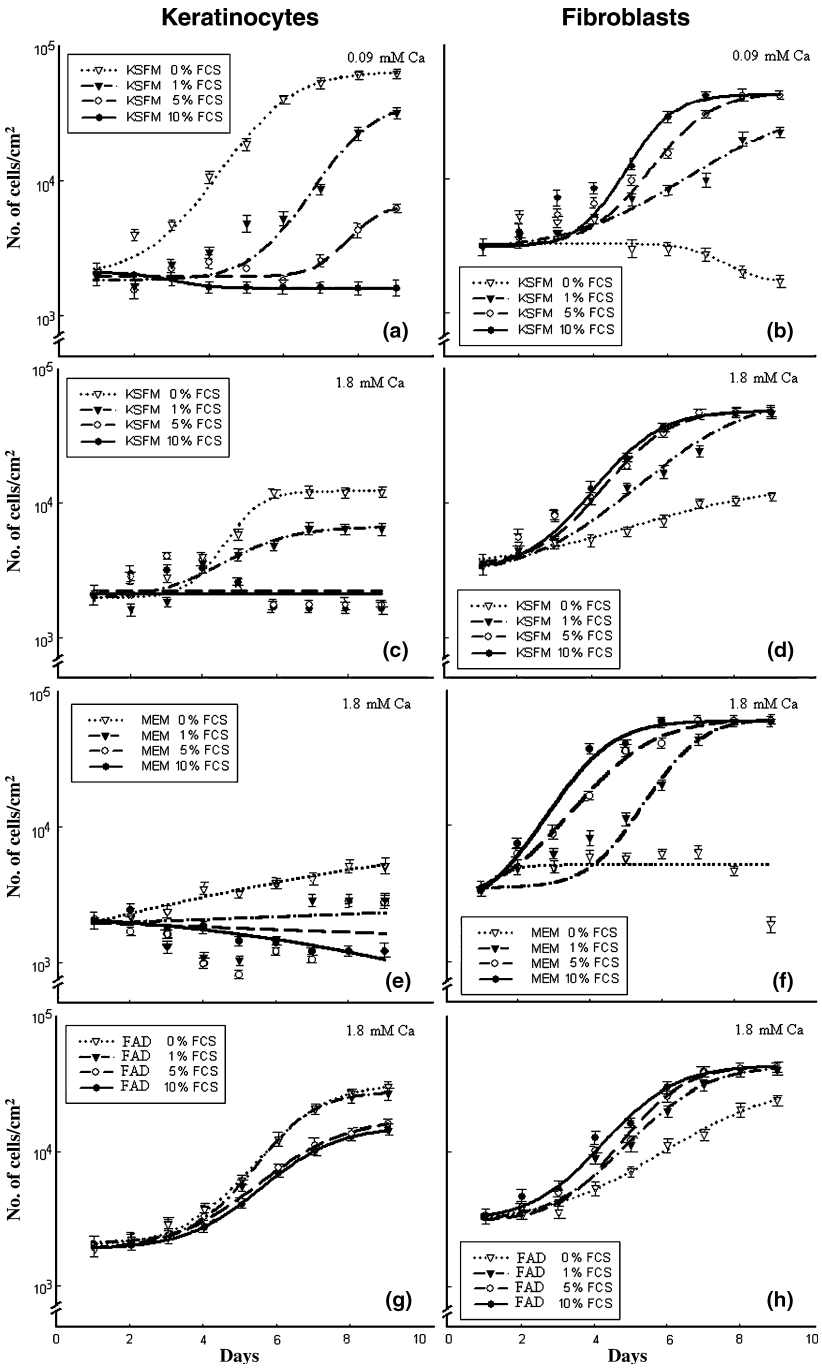

Figure 1 The influence of medium type, FCS and Ca^{++} concentration on growth of oral keratinocytes and fibroblasts in monolayer cultures. 3×10^4 cells/well were seeded in 24-well dishes. The effect of different media (KSFM, MEM and FAD), two different Ca^{++} concentrations (0.09 and 1.8 mM), and various concentrations of FCS (0, 1, 5 and 10%) were tested on primary NHOK and NHOF. The cells were daily counted for 10 days. Growth curves were obtained after plotting cell density (cells/cm^2) against time using Sigma Plot program. The data represent mean ± SEM from three separate batches of primary cells. The experiments were run in duplicates.

the suprabasal cell layers was significantly lower than in the basal cell layer ($P < 0.000$; Table 1).

We showed previously that the fibroblasts have a marked effect on growth of reconstituted human oral epithelium (11). Therefore, we wanted to investigate whether the above differences between reconstituted epithelium grown in various media could be attributed to the effect of these media on the growth and functional status of the underlying fibroblasts. To test the direct effect of media on growth of reconstituted epithelium, we grew NHOK on top of simple collagen gels. In this culture type, KSFM did not support the growth of epithelium (total thickness 13.3 ± 1.9 μm). The epithelium showed poor cell-to-cell adhesion and cell-to-matrix adhesion. MEM and FAD cultures showed a better epithelial growth, with a total epithelial thickness

Table 1 Histomorphometry, proliferation and apoptotic indexes of reconstituted human oral epithelium. Oral epithelium was reconstituted on a fibroblast containing collagen biomatrix for 10 days in KSFM, MEM and FAD medium, in conventional (with serum and BPE) and defined conditions (serum free and without BPE). Data represent mean ± SEM of five independent experiments

	KSFM		*MEM*		*FAD*	
	+FCS +BPE	*−FCS −BPE*	*+FCS +BPE*	*−FCS −BPE*	*+FCS +BPE*	*−FCS −BPE*
Epithelial thickness (μm)	38.8 ± 5.7	27.5 ± 3.8	52.9 ± 4.6	46.8 ± 6.0	77.3 ± 6.6	67.9 ± 6.6
PI basal cell layer (%)	8.1 ± 1.0	6.9 ± 1.3	14.5 ± 1.9	14.1 ± 2.2	16.9 ± 2.3	16.1 ± 1.9
PI suprabasal cell layers (%)	3.7 ± 1.5	3.0 ± 1.8	4.8 ± 2.7	4.6 ± 0.4	1.1 ± 0.3	0.4 ± 0.2
AI basal cell layer (%)	4.2 ± 1.6	4.5 ± 0.9	8.0 ± 1.4	8.3 ± 1.1	1.6 ± 0.4	1.8 ± 0.3
AI suprabasal cell layers (%)	3.9 ± 1.1	4.4 ± 1.1	7.0 ± 1.8	6.0 ± 0.7	5.0 ± 0.5	5.4 ± 0.1

COLOUR FIG.

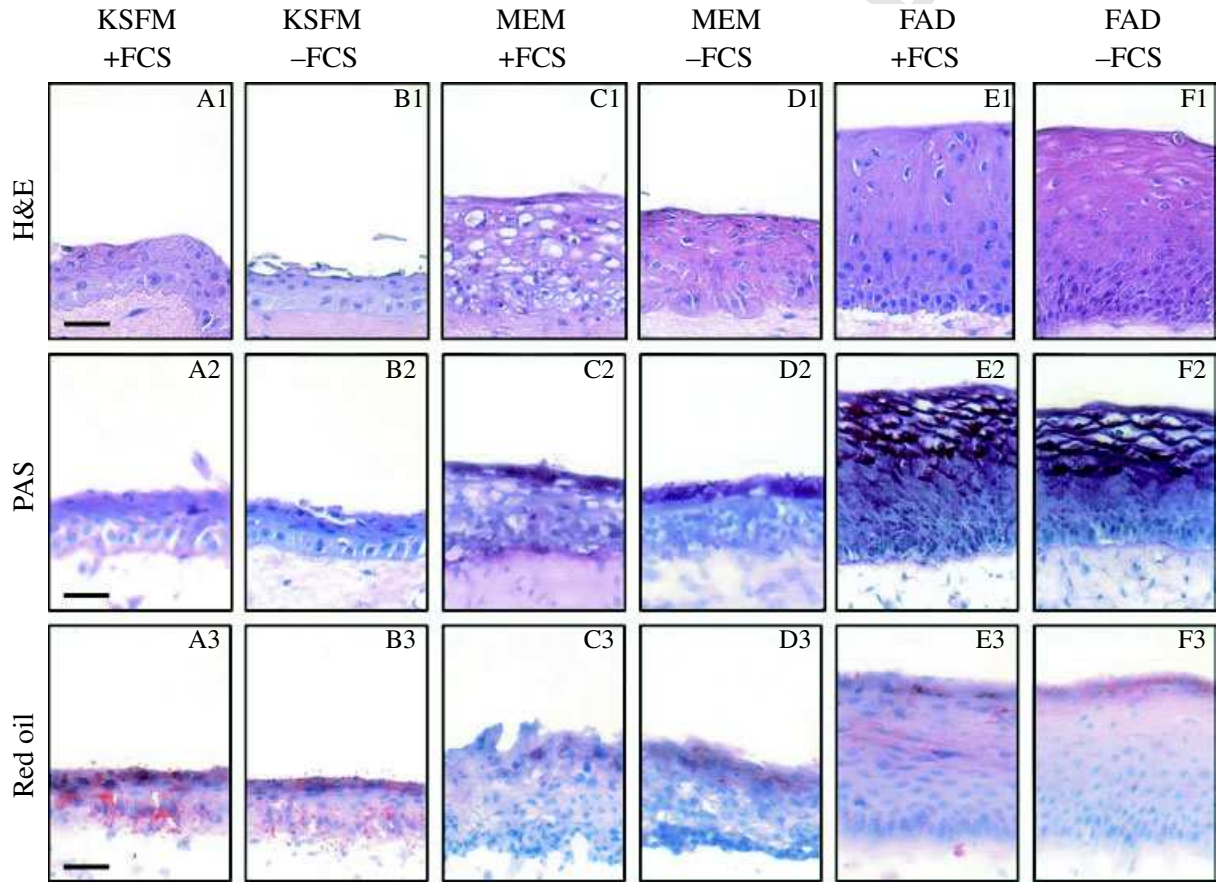

Figure 2 Culture medium and serum effect on morphogenesis of *in vitro* reconstituted human oral epithelium. The organotypic co-cultures were grown for 10 days in KSFM, MEM or FAD in absence or presence of FCS and BPE. Epithelial morphology in HE staining (panels A1–F1), PAS (panels A2–F2), and Red Oil (panels A3–F3) staining in reconstituted human oral epithelium are shown. Scale bar = 25 μm.

of 20.8 ± 2.3 and 36.4 ± 4.6 μm, respectively, and a better attachment to the collagen substrate. The variation of the epithelial thickness with the type of medium followed the same trend in both absence and presence of fibroblasts, suggesting a direct influence of the culture medium on *in vitro* epithelial growth (Fig. 3).

Differentiation of in vitro reconstituted human oral epithelium is essentially influenced by the culture medium

The morphometric analysis of *in vitro* oral epithelium grown on top of fibroblast-containing gels showed a prominent basaloid cell layer in KSFM (53.3 ± 3.2%) and MEM cultures (52.3 ± 2.6%), and a less developed spinous cell layer (28.8 ± 1.8 and 29.7 ± 1.9%, respectively), as compared with FAD cultures that showed, as previously reported by us, a more prominent spinous cell layer (46.1 ± 2.4%, $P < 0.05$), and a distribution of various epithelial cell layers comparable with native buccal mucosa (11). Expression of cytokeratin 13, a marker of differentiation for non-keratinized oral epithelium (16), was detected only scattered in the superficial cell layer of MEM cultures, and even to a lesser extent in KSFM cultures as compared with the strong CK 13 expression in all suprabasal cell layers of

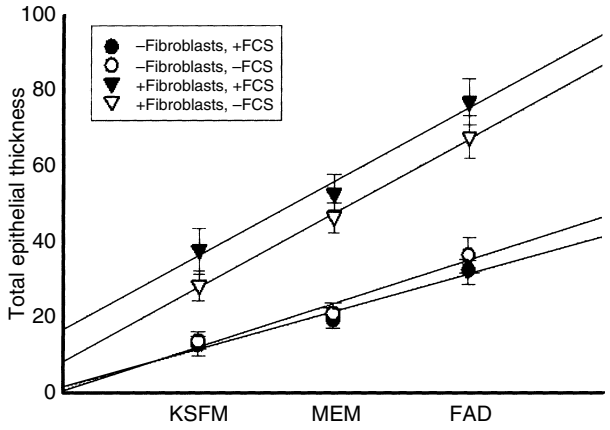

Figure 3 The effect of medium type and serum on total epithelial thickness of *in vitro* reconstituted human oral epithelium. Human oral epithelium was cultured on top of either simple collagen gel or on fibroblast-containing biomatrix in KSFM, MEM and FAD, in absence or presence of serum and BPE. Total epithelial thickness was measured by histomorphometry. Data represent the mean ± SEM (μm) of five different experiments. To assess whether the observed effects are because of the type of media or because of the presence of fibroblasts, regression lines have been drawn for cultures grown in presence and absence of fibroblasts.

FAD cultures (11). Collagen IV could not be detected in KSFM cultures. A positive staining for collagen IV was detected at the epithelial-mesenchymal interface of MEM and FAD cultures, but less confined in the MEM cultures. TUNEL positive cells were randomly distributed throughout all cell layers of the epithelium of KSFM and MEM cultures, with no significant difference between basal and suprabasal compartments ($P > 0.05$; Table 1), as compared with FAD cultures where they were located mainly in the upper epithelial layers (11). In KSFM cultures, intracellular vacuoles could be observed mainly in the basal cell layer of the reconstituted epithelium (Fig. 2 panels A1, B1). In MEM and FAD cultures, such vacuoles were present predominantly in the suprabasal layers (Fig. 2 panels C1–F1). We sought to identify whether this histological appearance was because of intracellular edema or to intracellular accumulations of lipids or carbohydrates. PAS staining showed a strong positivity in all suprabasal cell layers of FAD cultures, a weak staining of the superficial cell layer of MEM cultures, and no staining in KSFM cultures (Fig. 2 panels A2–F2). The staining disappeared after treatment with diastase, suggesting that at least part of these vacuoles, and mainly in FAD, was because of intracellular accumulation of glycogen. Small droplets of lipids were identified by Red Oil staining in the suprabasal layers of both MEM and FAD cultures, and in all layers of KSFM cultures (Fig. 2 panels A3–F3).

Epithelial phenotype was maintained in organotypic cultures grown in defined media

Defined conditions were obtained by excluding the BPE and replacing FCS with bovine serum albumin (1 mg/ml) in the culture medium. A certain restriction of epithelial growth was observed in defined conditions, as shown by a decrease in the total epithelial thickness (Fig. 2) and cell proliferation rate (Table 1) in conditions without FCS and BPE, but not statistically significant ($P > 0.05$). On the contrary, reconstituted oral epithelium grown serum free seemed to be better organized, with less intracellular vacuoles on H&E stained sections (Fig. 2 panels B1, D1, F1), less Red Oil stained vacuoles (Fig. 2 panels B3, D3, F3), and a more defined basement membrane deposition. These results were consistently reproduced in all types of medium tested.

Discussion

Serum dependency of fibroblasts has been previously reported (17), but none of the so far published reports have included FAD nutrient reach medium. We report here that the FAD medium was able to reduce the dependency on serum for optimal fibroblastic growth (Fig. 1h). Moreover, FAD medium could diminish the negative impact that high Ca^{++} concentration has on keratinocyte growth (18–20). The experiments on organotypic cultures found also the FAD medium superior to the other media tested in supporting oral epithelial morphogenesis. From the experiments run in the absence of fibroblasts it was concluded that this effect was not dependent on serum supplements, or on the presence of fibroblasts (Fig. 3). However, a full epithelial differentiation comparable with native NHOM was obtained only in the presence of both fibroblasts and FAD medium (Fig. 2). Apart from giving support to the important role of fibroblasts in epithelial morphogenesis as previously reported (7, 11), these findings also point to an essential role of medium composition for optimal growth and differentiation on *in vitro* reconstituted human oral mucosa. So far, it has been generally considered that serum is a necessary requirement for optimal growth of fibroblasts in co-culture (6, 8, 21). We demonstrate here, on both monolayers and organotypic cultures, that the serum dependency of fibroblasts can be diminished by use of FAD.

Reconstituted oral epithelium showed intracellular lipid vacuoles especially in KSFM and serum supplemented cultures. Intracellular fatty inclusions have been previously reported in reconstituted skin, and their presence has been related to an imbalanced metabolism of lipids in cultured cells (22). To reduce the amount of fat inclusions, we included linoleic acid in the media composition. Linoleic acid-albumin supported better epithelial growth, although not statistically significant, but did not reduce the amount of lipid vacuoles. Other intracellular inclusions could be identified as accumulations of glycogen. A potential reason for their occurrence could have been the supra-physiological levels of insulin that we used, as indicated for cultivation of skin keratinocytes (23), insulin being known to increase the accumulation of glycogen in hepatocytes and of tryglicerides in fat cells (24). This hypothesis, however, was not further tested in this study.

In conclusion, this study shows that medium composition plays an essential role not only for the growth of primary cells in monolayers, but also for optimized growth and differentiation of primary *in vitro* organotypic cultures of NHOM.

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Paper II

Crucial Effects of Fibroblasts and Keratinocyte Growth Factor on Morphogenesis of Reconstituted Human Oral Epithelium

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Crucial Effects of Fibroblasts and Keratinocyte Growth Factor on Morphogenesis of Reconstituted Human Oral Epithelium

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The connective tissue is known to have a general supportive effect for the development of the overlying epithelium; however, the more specific effects of fibroblasts and the involvement of their product, keratinocyte growth factor, on oral epithelial morphogenesis have not yet been addressed. Therefore, the purpose of this study was to investigate the effects of fibroblasts and keratinocyte growth factor on human oral epithelial morphogenesis *in vitro*. Reconstituted human oral epithelium was generated from primary human oral keratinocytes and fibroblasts by use of an organotypic cell culture model in a defined medium. Addition of fibroblasts to the collagen biomatrix increased total epithelial thickness from 28.0 $\pm$ 5.0 μm to 66.1 $\pm$ 8.6 μm ($p = 0.028$), and basal cell proliferation from 3.6 $\pm$ 0.7% to 16.6 $\pm$ 1.1% ($p = 0.025$). Presence of fibroblasts profoundly influenced the pattern of epithelial differentiation, and induced a switch in the pattern of cell death, from a predominance of spontaneous cell death in the basal cell layer (from 4.7 $\pm$ 0.6% to 1.8 $\pm$ 0.3%, $p = 0.029$) to a more prevalent cell death due to terminal differentiation in the suprabasal cell layer (from 4.0 $\pm$ 0.1% to 5.4 $\pm$ 0.1%, $p = 0.034$). Keratinocyte growth factor promoted epithelial growth, but did not significantly enhance epithelial differentiation, demonstrating that fibroblasts possess additional mechanisms to keratinocyte growth factor synthesis that can modulate differentiation of reconstituted human oral epithelium.

Key words: cell culture/cell differentiation/epithelium/keratinocytes. J Invest Dermatol 121:1479–1486, 2003

Epithelial–mesenchymal interactions are thought to be essential for epithelial morphogenesis, homeostasis, and repair (Mackenzie, 1994; Werner and Smola, 2001). The mechanisms are yet to be elucidated and much of the present knowledge on this subject has been derived from studies on skin. Experimental *in vitro* models have been developed to address how mesenchymal tissue influences growth and differentiation of the overlying epithelium (Garlick and Taichman, 1993, 1994; Fusenig, 1994; Tomakidi *et al*, 1998; Grøn *et al*, 1999). In such studies, it has been suggested that the underlying mesenchyme could stimulate epithelial growth and differentiation by provision of a suitable biomatrix environment (Chung *et al*, 1997; Kim *et al*, 2001) or by synthesis of diffusible factors (Boukamp *et al*, 1990; Maas-Szabowski *et al*, 1999; Werner and Smola, 2001). The role of dermal fibroblasts in the regulation of epidermal morphogenesis and homeostasis has been investigated by several researchers (Mackenzie *et al*, 1993; El-Ghalbzouri *et al*, 2002a, b). In these studies, an important role for fibroblasts has been proposed, not only in promoting keratinocyte proliferation, but also in differentiation of the overlying epidermis. Fibroblasts are known to secrete several growth factors and interleukins, such as interleukins 1, 6, and 8, granulocyte macrophage colony stimulating factor, transforming growth factor-α and -β, platelet-derived growth factor as well as members of the fibroblast growth factor family (Fusenig, 1994). These diffusible factors have been discussed as potent mediators of keratinocyte growth and differentiation. Keratinocyte growth factor (KGF), a member of the fibroblast growth factor family, known also as fibroblast growth factor-7, has been particularly investigated and considered one major regulator of keratinocyte growth and differentiation (Maas-Szabowski *et al*, 1999). KGF is a typical paracrine acting growth factor, produced solely by underlying stromal cells, with a distinctive specificity for keratinocytes. It has been shown that KGF stimulates epidermal keratinocyte proliferation (Marchese *et al*, 1990; Rubin *et al*, 1995), but its effects on keratinocyte differentiation are more controversial (Gibbs *et al*, 2000; Andreadis *et al*, 2001).

There are few published data on epithelial–mesenchymal interactions in oral mucosa. Several studies have pointed out the importance of the intrinsic properties of keratinocytes for epithelial morphogenesis (Gibbs and Ponec, 2000; Chinnathambi *et al*, 2003), whereas others have found the connective tissue essential for the modulation of epithelial growth (Hill and Mackenzie, 1989) and differentiation (Mackenzie and Hill, 1984). Furthermore, the influence of fibroblasts on oral epithelial morphogenesis, based on mutual interactions with keratinocytes, seemed to be ambivalent, strongly supporting proliferation initially, but gradually being superimposed by signals promoting differentiation (Tomakidi *et al*, 1998). Although the connective tissue has been shown to have a general supportive effect for the morpho-

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Abbreviations: KGF, keratinocyte growth factor; OTK, organotypic monocultures; OTKF, organotypic cocultures; NHOM, normal human oral mucosa; PI, proliferation index; AI, apoptotic index; CK, cytokeratin.

genesis of the overlying epithelial tissue (Mackenzie and Dabelsteen, 1987), the more specific effects of fibroblasts on oral epithelial morphogenesis have not yet been fully addressed. It is known that oral fibroblasts synthesize KGF (Dabelsteen *et al*, 1997; Sanale *et al*, 2002), and it has been proposed that oral fibroblasts might be even more potent in producing KGF than their dermal counterparts (Okazaki *et al*, 2002; Grøn *et al*, 2002). Exogenously added KGF stimulated cell proliferation in a study done on normal murine oral keratinocyte cell lines, but did not stimulate proliferation of neoplastic oral keratinocyte cell lines (Ning *et al*, 1998).

The purpose of this study was to examine the effect of fibroblasts and exogenous KGF on morphogenesis of reconstituted human oral epithelium obtained from organotypic primary cultures. To our knowledge, this is one of the first studies on *in vitro* reconstituted human oral mucosa that addresses the effects of KGF on oral epithelial morphogenesis. Our data show that fibroblasts had crucial effects on human oral epithelial growth, differentiation, and apoptosis. In our models, KGF stimulated oral epithelial growth, but did not significantly enhance epithelial differentiation. These results demonstrate crucial effects of fibroblasts and KGF on morphogenesis of *in vitro* reconstituted oral epithelium.

MATERIALS AND METHODS

Tissue material Twenty-three samples of normal human oral mucosa (NHOM) were obtained from the superfluous oral tissue after wisdom tooth extraction. Twelve samples were formalin fixed, paraffin embedded, and served as controls; 11 samples were used for isolating the cells and growing the organotypic cultures. The study was approved by the Ethics Committee of the University of Bergen, and it included only clinically healthy donors after informed consent.

Cell culture procedures Two types of organotypic cultures were prepared and used in this study. In the first model, namely the organotypic keratinocyte monoculture (OTK), the primary oral keratinocytes were grown on top of a reconstituted collagen matrix without supplement of fibroblasts. In the second model, the organotypic keratinocyte–fibroblast coculture (OTKF), primary oral keratinocytes were grown on top of a reconstituted collagen biomatrix supplemented with primary oral fibroblasts. Primary oral keratinocytes and fibroblasts were isolated from NHOM as described elsewhere (Costea *et al*, 2002). Keratinocytes were grown serum free in KSFM medium (GibcoBRL, Grand Island, New York) supplemented with 1 ng human recombinant epidermal growth factor per mL (GibcoBRL), 25 μg bovine pituitary extract per mL (GibcoBRL), 200 U penicillin per mL (GibcoBRL), 200 μg streptomycin per mL (GibcoBRL), 0.5 μg amphotericin B per mL (GibcoBRL), 6 μg fluconazole per mL (Pfizer, Amboise, France), and 20 μg L-glutamine per mL (GibcoBRL), with no feeding layer. Fibroblasts were grown in Dulbecco's modified Eagle's medium (Sigma, St Louis, Missouri) supplemented with 10% fetal calf serum (Sigma), 200 U penicillin per mL, 200 μg streptomycin per mL, 0.5 μg amphotericin B per mL, 6 μg fluconazole per mL, and 20 μg L-glutamine per mL. Second passage primary cultured keratinocytes and fibroblasts were used for preparation of organotypic cultures. The collagen matrix (final pH 7.2) was made by mixing on ice 7 volumes (3.40 mg per mL) of rat tail collagen type I (Collaborative Biomedical, Bedford, Massachusetts), 2 volumes of reconstitution buffer pH 8.15 (2.2 g $NaHCO_3$, 0.6 g NaOH, 4.766 g HEPES in 100 mL dH_2O), and 1 volume of Dulbecco's modified Eagle's medium 10 × (Sigma). The biomatrix necessary for organotypic cocultures was further prepared by mixing the collagen matrix obtained as described above with primary fibroblasts. The fibroblasts obtained after trypsinization were resuspended in 1 volume of fetal calf serum, and added to the collagen matrix to a final concentration of 0.5×10^6 fibroblasts per mL collagen matrix. Seven hundred microliters of either collagen matrix or fibroblast-containing biomatrix were layered into each inner well of the organ culture dishes (Becton Dickinson, Franklin Lakes, New Jersey). The matrix was allowed to polymerize for 1 h in a humidified chamber at 37°C and then equilibrated with 1 mL of routine fibroblast culture medium. After 24 h of culture, the equilibration medium was removed and 1 mL of primary keratinocytes (0.5×10^6 cells per mL) in serum-free KSFM medium was layered on top of each collagen matrix. The cultures were detached from the dish walls after 24 h, at a time point when the keratinocytes had not yet reached confluency. After detachment, half of the culture medium was replenished with serum free organotypic medium: 3 volumes of Dulbecco's modified Eagle's medium (Sigma)/1 volume of Ham's F12 (Sigma), supplemented with 0.4 μg hydrocortisone per mL (Sigma), 5 μg insulin per mL (Novo Nordisk, Bagsværd, Denmark), 20 μg transferrin per mL (Sigma), 50 μg L-ascorbic acid per mL (Sigma), 10 ng human recombinant epidermal growth factor per mL (Gibco BRL), 1 mg linoleic acid–albumin per mL (Sigma), 200 U penicillin per mL, 200 μg streptomycin per mL, 0.5 μg amphotericin B per mL, 6 μg fluconazole per mL, and 20 μg L-glutamine per mL. Two days later, the cultures were layered on curved metal grids that were covered with a thin filter paper soaked in organotypic culture medium. The cultures were lifted to the liquid–air interface and grown for 7 more days. Half of the culture medium (2.5 mL) was changed every second day during the time of culture. After a total period of 10 d of coculture, the organotypic cultures were harvested and divided in two equal parts. One part was snap frozen in liquid nitrogen and the other was fixed in 4% buffered formalin pH 7.15 and embedded in paraffin. Human recombinant KGF (Sigma), diluted in nutrient medium, was added to the cultures starting at the time of seeding the keratinocytes, and continuously for the whole period of culture.

Immunohistochemical staining Formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene, hydrated through a graded alcohol series and then rehydrated in distilled water (dH_2O). Samples were microwave treated in 10 mM citrate buffer (pH 6) for 9 min at 750 W and then for 15 min at 500 W. After cooling for 20 min at room temperature, endogenous peroxidase activity was quenched by immersing the slides in 0.3% H_2O_2 diluted in Tris-buffered saline (TBS; 0.05 M Tris–HCl, 0.15 M NaCl, pH 7.6) for 5 min. For Ki-67 antibody, quenching by 0.3% H_2O_2 was done after incubation with primary antibody. Prior to the application of collagen IV and laminin antibodies, the sections were pretreated with 20 μg proteinase K per mL (DAKO, Glostrup, Denmark) diluted in TBS for 10 min at room temperature. The specimens were incubated with primary antibodies in a humidified chamber at room temperature for 60 min. The primary antibodies and the titrations used in this study were as follows: Ki-67 (IgG), MIB-1clone, 1:50 (DAKO); CK13 (IgG), KS-1A3 clone, 1:400 (Novocastra Laboratories Ltd, Newcastle, Upon-Tyne, UK); CK14 (IgG), LL002 clone, 1:100 (Novocastra Laboratory Ltd); CK19 (IgG), RCK clone, 1:50 (DAKO); collagen IV (IgG), CIV221 clone, 1:25 (DAKO), and laminin polyclonal, 1:200 (DAKO). The secondary antibodies, conjugated with horseradish peroxidase labeled polymer (EnVision$^+$ System, DAKO) were applied afterwards for 30 min. After each step above, the specimens were washed twice in TBS, for 7 min each time. The presence of antigen was visualized by staining with 3,3′-diaminobenzidine (DAKO) in buffered substrate solution, pH 7.5, for 10 min. After 10 min washing in dH_2O the slides were counterstained with hematoxylin (DAKO) and mounted with aqueous mounting medium (Shandon, Pittsburgh, Pennsylvania). NHOM and normal salivary glands (for CK19) served as positive controls. Specimens incubated with antibody diluent (DAKO) instead of primary antibody were used as negative controls. Specimens stained with various monoclonal antibodies were of the same isotype, serving as a negative control for each other.

TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling) method Cell death was detected by labeling DNA fragments using biotinylated deoxyuridine triphosphate (Roche Diagnostics GmbH, Mannheim, Germany), by a modification of the TUNEL method. Sections were first deparaffinized in xylene, hydrated through a graded alcohol serie and then rehydrated in dH_2O. Samples were then immersed in citrate buffer pH 3 and microwave treated at 850 W for 5 min, followed by 500 W for 5 min. After cooling, the specimens were digested with 20 μg per mL proteinase K in TBS for 15 min at 37°C. Sections were then incubated with 3% bovine serum albumin (Sigma) for 30 min, followed by incubation with TUNEL labeling mix containing biotinylated deoxyuridine triphosphate, terminal deoxynucleotidyl-transferase (Roche) and cobalt chloride mixed in reaction buffer (Roche). Specimens were further processed as previously reported (Loro *et al*, 1999). After each step described above, the slides were washed three times in TBS for 5 min each time. For positive controls, specimens were treated with 0.5 mg per mL DNase (Roche) in TBS for 15 min at 37°C prior to incubation with bovine serum albumin. The specificity of the TUNEL reaction was tested by substituting the biotinylated deoxyuridine triphosphate in the TUNEL labeling mix with unbiotinylated deoxyuridine triphosphate (Roche) in excess.

Evaluation of samples and statistical analysis

Histomorphometry Tissue sections (5 μm) from paraffin-embedded specimens, stained with hematoxylin–eosin, were analyzed by a computer-

based optical image analyzer (analySIS 11.0 Pro Soft Imaging system, GmbH, Munster, Germany). Analysis was done at 200-fold magnification on a standard microscope (Leika DMLM, GmbH, Munster, Germany) on six consecutive fields situated 200 μm apart. The epithelium was divided into three compartments (according to the criteria proposed by Presland and Dale, 2000): (1) basal cell layer (population of columnar or cuboidal cells with their long axis perpendicular to the basement membrane); (2) spinous cell layer (population of large polygonal and rather flattened cells with their long axis parallel to the basement membrane); and (3) superficial cell layer (population of flattened cells in their terminal stage of cytodifferentiation). The spinous and superficial cell layers were evaluated sometimes jointly, as the suprabasal cell layer. The total epithelial thickness and the thickness of each epithelial cell layer were measured.

To calculate the ratio of proliferating cells, the number of Ki-67 positive cells was determined in the basal and suprabasal cell layers, under a light microscope, at a 400-fold magnification. The average percentage of positive cells, counted in six fields per section (situated at 200 μm apart), was presented as proliferation index (PI). The average percentage of TUNEL-positive cells, similarly counted in basal and suprabasal cell layers, in six fields per section (situated at 200 μm apart) was presented as apoptotic index (AI). For NHOM specimens all measurements and counts were done in suprapapillary areas. Statistical analysis was performed using the SPSS program version 11.0 (SPSS Inc., Chicago, Illinois). The comparison of experimental samples with the NHOM samples was done using the Mann–Whitney U test, and between experimental samples using the Wilcoxon test, with the level of significance set at 5%. Spearman's Rank Order Correlation (r) was used to calculate the relationship between two variables.

RESULTS

Effects of fibroblasts and KGF on growth and proliferation of human oral epithelium in culture Primary oral keratinocytes were able to proliferate and stratify in organotypic monocultures grown on a substrate of collagen, even in the absence of fibroblasts (OTK). The epithelium formed was thin (mean total epithelial thickness ± SEM: 28.0 ± 5.0 μm), however, and had a loose attachment to the underlying collagen substrate (**Fig 1*a***). In organotypic cocultures, grown in the presence of fibroblasts (OTKF), the keratinocytes formed a significantly ($p = 0.028$) thicker epithelium (66.1 ± 8.6 μm) (**Fig 1*c***). Addition of KGF to the culture media induced a concentration-dependent increase in total epithelial thickness in both OTK and OTKF cultures (**Fig 2**). The relative effect of KGF on total epithelial thickness appeared mainly independent on the presence of fibroblasts (**Fig 3*A***). Moreover, there was no tendency for a decreased slope in the interaction plot (**Fig 3*A***), so no evidence for synergy effects between KGF and fibroblasts was found. The effect of KGF on total epithelial thickness, however, was somewhat more pronounced in reconstituted epithelium in the absence of fibroblasts than in their presence (**Fig 3*A***). This was also reflected by the stronger relationship between epithelial thickness and KGF concentration in OTK cultures ($r = 0.7$, $p = 0.001$) than in OTKF cultures ($r = 0.5$, $p = 0.002$).

When reconstituted human oral epithelium was compared with NHOM, the most optimal culture condition was found in cultures grown in the presence of fibroblasts, supplemented with 10 ng KGF per mL. Grown under these conditions, the reconstituted oral epithelium was not statistically different ($p = 0.316$) from the suprapapillary areas of NHOM with respect to total epithelial thickness (**Table I**).

Immunohistochemical analysis showed that Ki-67-positive cells were found mainly within the cells of the basal cell layer of the reconstructed oral epithelium in both OTK and OTKF cultures (**Fig 1*f,h***). The PI in the basal cell layer of reconstructed epithelium was significantly lower ($p = 0.025$) in OTK cultures compared with OTKF cultures (**Table I**). Addition of KGF increased PI in both types of cultures (**Table I**), but more prominently in the basal cell layer of OTK cultures than in OTKF cultures (**Fig 3*B***). This was also reflected by a stronger correlation between exogenous KGF and PI in the basal cell layer for OTK ($r = 0.8$, $p = 0.010$) than for OTKF cultures ($r = 0.5$, $p = 0.034$). For OTKF cocultures, the values of PI in basal and suprabasal cell layers were comparable with the values found in native oral epithelium (**Table I**).

The potential influence of the basal cell proliferation rate on epithelial thickness was further tested. The relationship between PI in the basal cell layer and total epithelial thickness showed a strong positive correlation just for OTK cultures ($r = 0.71$, $p = 0.003$). In OTKF cultures and NHOM a positive, although not significant correlation was found between total epithelial thickness and PI in the basal cell layer ($r = 0.22$, $r = 0.20$, respectively). The influence of PI on the development of spinous cell layer showed the same pattern, a strong positive correlation for OTK cultures ($r = 0.67$), but a weak positive one for OTKF and NHOM ($r = 0.20$, and $r = 0.14$, respectively).

Effects of fibroblasts and KGF on cell death (apoptosis) rate in reconstituted oral epithelium TUNEL-positive cells found in the basal cell layer were considered to reflect spontaneous

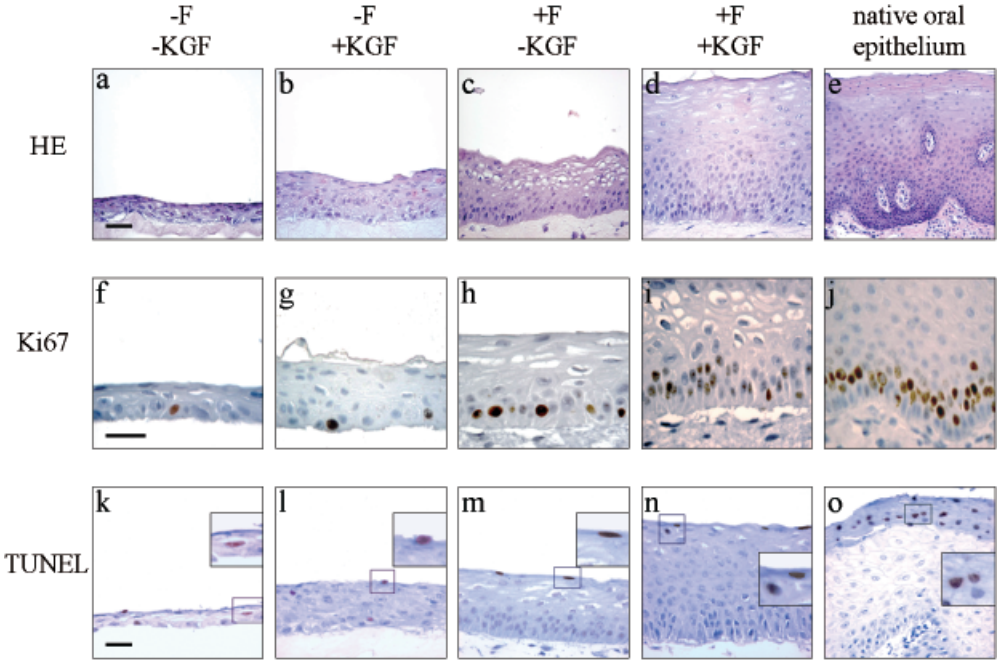

Figure 1. Effects of fibroblasts and KGF on tissue morphology, cell proliferation, and cell death in reconstituted human oral epithelium. Oral epithelium was reconstituted on a collagen matrix in the absence (*a,b,f,g,k,l*) or presence (*c,d,h,i,m,n*) of fibroblasts, without (*a,c,f,h,k,m*) or with (*b,d,g,i,l,n*) exogenously added 10 ng KGF per mL. Reconstituted oral epithelium is compared with native oral epithelium (*e,j,o*) regarding tissue morphology on paraffin embedded, hematoxylin–eosin stained sections (*a–e*), cell proliferation by Ki-67 staining (*f–j*) and cell death (apoptosis) evaluation by TUNEL staining (*k–o*). *Scale bar* = 25 μm.

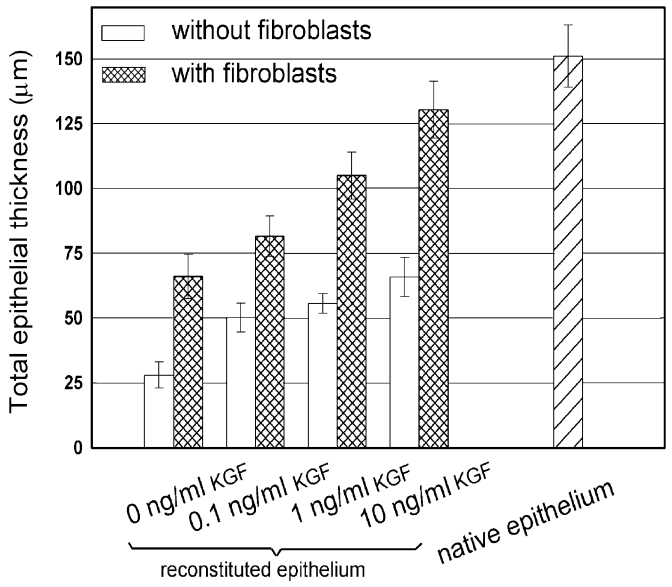

Figure 2. Effects of fibroblasts and KGF on total epithelial thickness in reconstituted human oral epithelium. Human oral epithelium reconstituted on a collagen matrix in the absence (□) or presence (⊠) of primary human oral fibroblasts, was supplemented with increasing concentrations of KGF. Total epithelial thickness was measured by histomorphometry. Data represent mean ± SEM (in µm) of five or more different experiments. Native oral epithelium was included for comparison (n = 12).

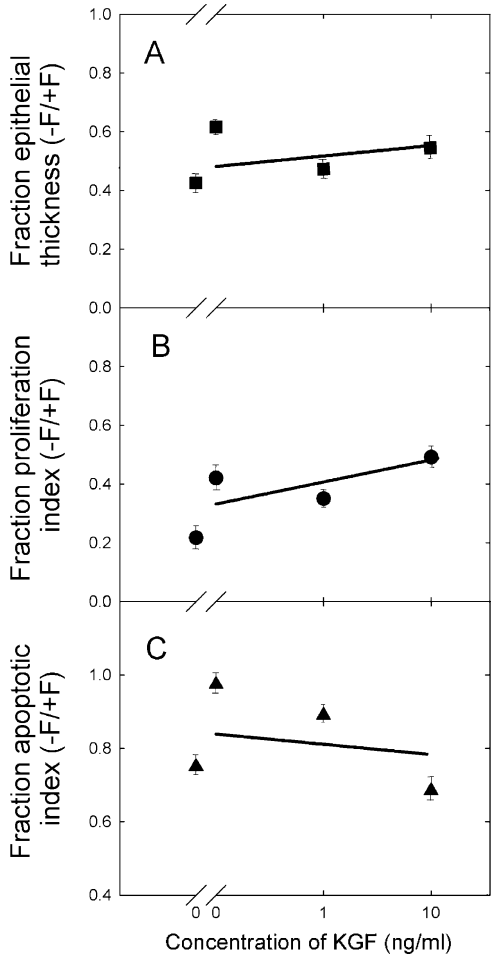

Figure 3. Test on potential fibroblast independency of KGF-induced effect on epithelial thickness, proliferation, and apoptosis in reconstituted human oral epithelium. The graphs were obtained by plotting the ratio of the values obtained from cultures grown in the absence of fibroblasts (–F) to the values obtained from the cultures grown in the presence of fibroblasts (+F), using SigmaPlot software (version 8.0). The slope of the line is interpreted as a measure of the synergy between KGF and fibroblasts, with respect to their effects on total epithelial thickness (*A*), basal cell proliferation (*B*), and suprabasal cell death (*C*). Data represent mean ± SEM of five different experiments.

apoptotic cell death, whereas TUNEL-positive cells on the shedding surface of the epithelium were considered as terminally differentiated cells through the process of epithelial maturation (Loro *et al*, 2003). In OTK cultures, scattered TUNEL-positive cells were observed throughout all epithelial cell layers, with no obvious zonal distribution (**Fig 1*k***). In OTKF cultures, TUNEL-positive cells were mainly detected in the superficial cell layer (**Fig 1*m***), similar to NHOM (**Fig 1*o***). AI of the basal cell layer was higher ($p = 0.029$) in cultures grown in the absence of fibroblasts (mean ± SEM: 4.7 ± 0.6%) than in the presence of fibroblasts (1.8 ± 0.3%) (**Table I**). AI of the superficial cell layer was lower ($p = 0.034$) in OTK (4.0 ± 0.1%) than in OTKF (5.4 ± 0.1%). These data showed a switch in the cell death pattern from a high basal spontaneous cell death to a more predominant cell death in the suprabasal cell layer with the addition of fibroblasts.

The importance of the mode of cell death on the development of epithelium thickness was further investigated by running Spearman's correlation tests. The relationship between AI in the

Table I. Histomorphometric analysis, proliferation and apoptotic indexes of reconstituted human oral epithelium and native oral epithelium. Oral epithelium was reconstituted on a collagen matrix in the absence or presence of fibroblasts, and the culture media was supplemented or not with 10 ng KGF per mL. Data represent mean ± SEM of 5 or more independent experiments and 12 native human oral normal mucosa specimens

	– Fibroblasts 0 ng KGF per mL n = 5	– Fibroblasts 10 ng KGF per mL n = 5	+ Fibroblasts 0 ng KGF per mL n = 11	+ Fibroblasts 10 ng KGF per mL n = 11	Native oral epithelium n = 12
Thickness of basal cell layer (µm)	15.2 ± 2.2	43.9 ± 4.1	26.0 ± 2.8	37.3 ± 3.5	42.9 ± 3.4
Thickness of spinous cell layer (µm)	6.5 ± 1.5	11.4 ± 2.9	27.5 ± 4.9	68.5 ± 7.8	82.9 ± 8.0
Thickness of superficial cell layer (µm)	6.3 ± 1.5	10.2 ± 1.9	12.1 ± 1.7	21.6 ± 2.7	25.2 ± 2.6
Total epithelial thickness (µm)	28.0 ± 5.0	65.6 ± 7.6	66.1 ± 8.6	127.4 ± 11.0	151.1 ± 12.0
PI basal cell layer (%)	3.6 ± 0.7	11.9 ± 1.0	16.6 ± 1.1	24.3 ± 1.3	23.8 ± 1.5
PI suprabasal cell layer	0.8 ± 0.8	1.7 ± 0.5	0.5 ± 0.2	0.63 ± 0.2	0.01 ± 0.0
AI basal cell layer (%)	4.7 ± 0.6	4.1 ± 0.3	1.8 ± 0.3	1.1 ± 0.4	0.3 ± 0.1
AI suprabasal cell layer	4.0 ± 0.1	5.3 ± 0.4	5.4 ± 0.1	7.8 ± 0.4	8.1 ± 0.6

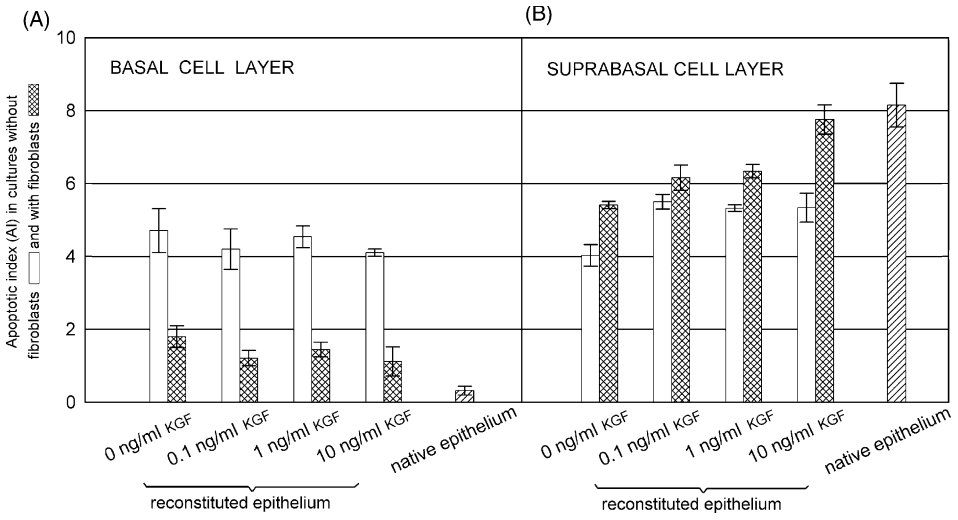

Figure 4. Effects of fibroblasts and KGF on spontaneous cell death (apoptosis) in basal and suprabasal cell layers of reconstituted human oral epithelium. Apoptotic index (AI) was determined in the basal cell layer (*A*) and suprabasal cell layer (*B*) in artificial epithelium reconstituted in the absence (□) or presence (⊠) of fibroblasts, and grown in culture medium supplemented with increasing concentrations of KGF. Data represent mean ± SEM (in percentage) of five or more separate experiments. AI determined in normal human oral epithelium are included for comparison (n = 12).

basal cell layer and total epithelial thickness was a negative but rather weak correlation for both OTK and OTKF cultures (r = – 0.07 and r = – 0.17, respectively), and for NHOM as well (r = – 0.2). A stronger negative correlation was found between AI in the basal cell layer and the thickness of the spinous cell layer for OTKF cultures (r = – 0.46) and NHOM (r = – 0.80). Although the role of spontaneous cell death rate in the basal cell layer for the development of epithelium is not clearly explained by simple correlations, it seems to be part of the delicate balance between cell proliferation, spontaneous cell death, and terminal differentiation in oral mucosa tissue, both *in vitro* and *in vivo*.

Addition of KGF decreased spontaneous cell death in the basal cell layers, and induced a slight increase of AI in the uppermost epithelial cell layers in both OTK and OTKF cultures (**Fig 4**). Although this effect was not statistically significant (**Table I**), we noticed that it was more prominent in the presence of fibroblasts (**Fig 3*C***).

Effects of fibroblasts and KGF on human oral epithelial differentiation in organotypic culture In OTK monocultures there was a predominance of cells with a basaloid appearance, the thickness of basal cell layer accounting for 56.3 ± 3.5% of the total epithelial thickness, as compared with 28.9 ± 1.7% in NHOM (**Fig 1*a,b*, Table I**). In the same type of cultures (OTK without KGF addition), the spinous cell layer was relatively poorly developed, accounting for 22.4 ± 2.9% of the total epithelial thickness as compared with 55.0 ± 1.2% in NHOM. In contrast, the distribution of the various cell layers in the epithelium of OTKF cocultures showed a predominance of more differentiated cell layers, thus more closely resembling the native oral epithelium (**Fig 5**).

KGF increased the thickness of all epithelial cell layers (**Table I**) in organotypic cell cultures, but their relative distribution was not significantly changed (**Fig 6**).

Epithelial differentiation was further evaluated by the expression of several cytokeratin isoforms: CK14, CK19, and CK13. In OTKF cocultures CK13 showed a strong and uniform staining of the suprabasal cell layers (**Fig 7*c***), whereas in OTK monocultures only few scattered cells of the suprabasal cell layers were positive (**Fig 7*a***). The expression of CK13 was not changed by the addition of KGF in culture media (**Fig 7*b,d***). The expression of CK13 in OTKF cultures closely resembled the CK13 expression in NHOM (**Fig 7*e***). CK14 was strongly and uniformly expressed throughout all the cell layers of the epithelium in OTK monocultures. In OTKF cocultures, CK14 was preferentially expressed in the basal cell layer. The pattern of CK14 staining was not affected by the addition of KGF (data not shown). CK19 was strongly expressed in all epithelial cell layers in OTK monocultures. In OTKF cocultures, the expression of CK19 was less strong, but still present heterogeneously in all cell layers. Addition of KGF had no effect on the CK19 staining pattern of either OTK or OTKF cultures (data not shown).

The major components of the basal membrane, collagen IV and laminin, were detected only in OTKF cocultures (**Fig 7*h,i,m,n***), and the basement membrane structure seemed to be less developed when compared with NHOM (**Fig 7*j,o***). Although this does not prove the existence of a completely developed or functional basement membrane, the presence and deposition of laminin and collagen IV did not seem to be dependent on exogenously added KGF. In OTK monocultures the staining for laminin revealed that the protein was expressed intracellularly in the basal cells, but no deposition of extracellular components of the basal membrane could be detected (**Fig 7*k,l***).

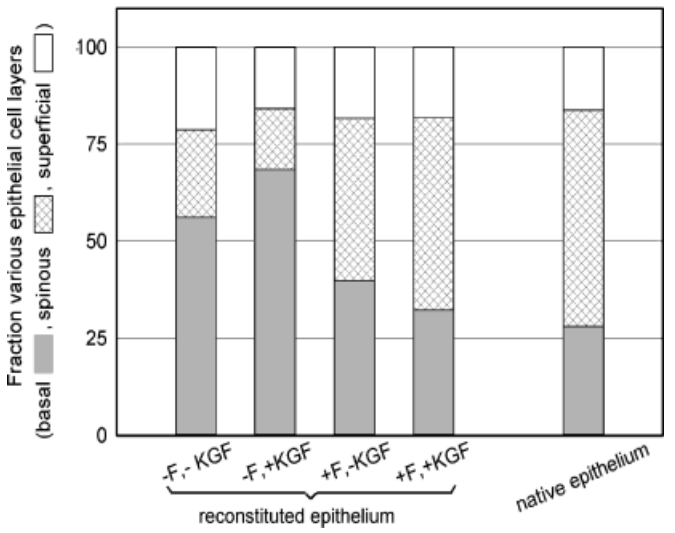

Figure 5. Noninteractive effects of fibroblasts and KGF on the relative distribution of various cell layers in reconstituted human oral epithelium. Reconstituted epithelium was cultivated in the absence (–F) or presence (+F) of fibroblasts, in the absence (–KGF) or presence (+KGF) of KGF for 10 d. The thickness of the basal (■), spinous (▨) and superficial (□) epithelial cell layers were analyzed by histomorphometry and the relative values were calculated (data from five or more separate experiments). Measurements of native oral epithelium (n = 12) were included for comparison.

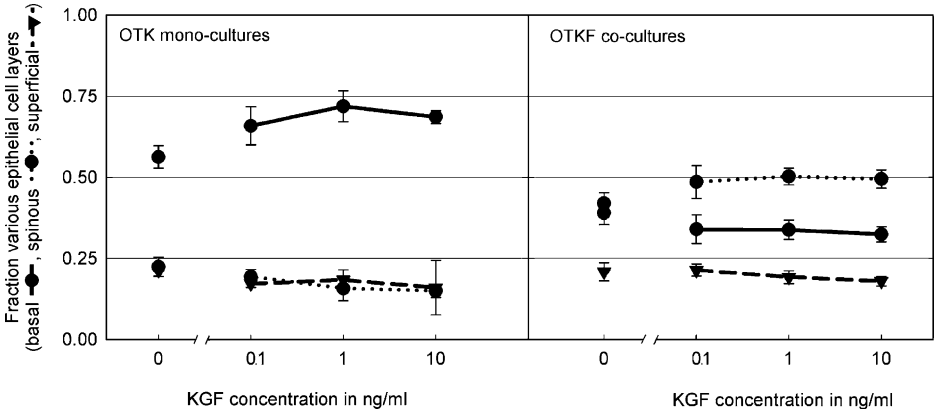

Figure 6. Effects of KGF on epithelial differentiation in reconstituted human oral epithelium. The relative distribution of basal, spinous, and superficial epithelial cell layers was evaluated after 10 d organotypic culture of keratinocytes in the absence (OTK monocultures) or presence (OTKF cocultures) of primary oral fibroblasts, in the absence or presence of various concentrations of KGF. Data represent mean ± SEM of five separate experiments.

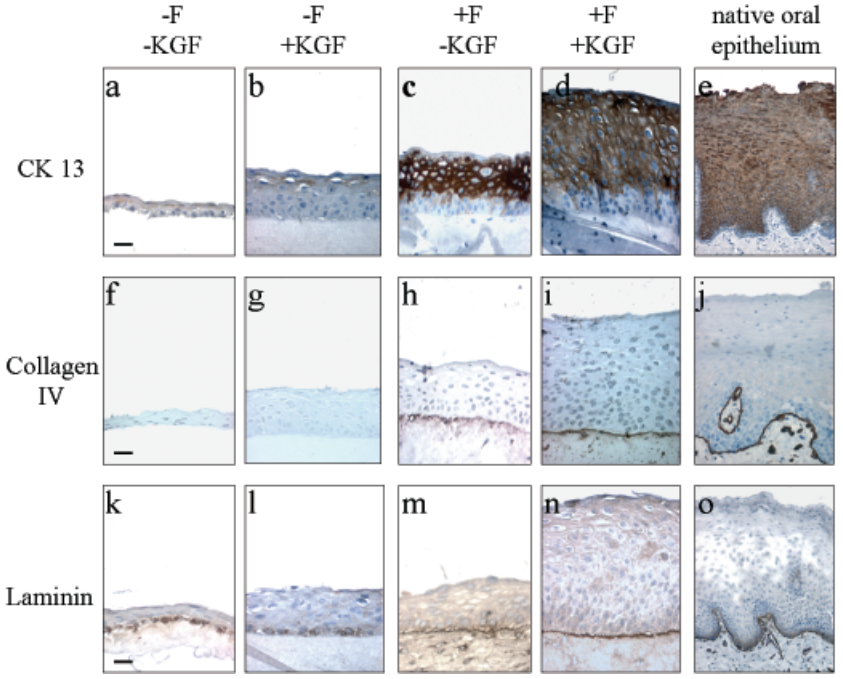

Figure 7. Expression of differentiation marker (CK13) and markers of basement membrane (collagen IV and laminin) in reconstituted human oral epithelium. The cultures were grown for 10 d in the absence (*a,b,f,g,k,l*) or presence (*c,d,h,i,m,n*) of fibroblasts, supplemented (*b,g,l,d,i,n*) or not (*a,f,k,c,h,m*) with 10 ng KGF per mL. Normal oral mucosa is shown for comparison (*e,j,o*). *Scale bar* = 25 μm.

DISCUSSION

In this study, the major effect of fibroblasts was to promote epithelial differentiation of reconstituted oral epithelium. Addition of fibroblasts in the collagen matrix determined a switch in the pattern of stratification, considered an indicator of the degree of epithelial maturation (Schroeder, 1981), from a predominance of basal cell layer in OTK to a predominance of the spinous cell layer in OTKF (**Figs 1** and **5**). The effect on epithelial maturation was also shown by strong expression of CK13 in the suprabasal cell layer, decreased expression of CK14 and CK19 in the suprabasal cell layer, and increased apoptotic rate in the superficial cell layer of OTKF (**Fig 4**), considered an indicator of terminal differentiation (Loro *et al*, 2003). Moreover, in cultures supplemented with fibroblasts, laminin, and collagen IV, two major components of the basement membrane, were expressed at the epithelium–collagen biomatrix interphase (**Fig 7**), suggesting a high competence of epithelial cells to produce specialized proteins in the presence of fibroblasts.

Tissue homeostasis requires cell proliferation of the basal cell layer to be matched by differentiation and desquamation at the epithelial surface (Squier and Kremer, 2001). When we investigated this balance, a positive correlation between total epithelial thickness and proliferation index of the basal cell layer was observed only for cultures grown in the absence of fibroblasts. For cultures grown in the presence of fibroblasts, as for native oral epithelium, no such strong correlation was found. This suggested that in cultures grown in the absence of fibroblasts, cell proliferation was the major determinant of epithelial thickness, whereas in the presence of fibroblasts additional factors to cell proliferation were important for modulating the thickness of oral epithelium. One such factor could be spontaneous cell death (Squier and Kremer, 2001). The presence of fibroblasts induced a marked inhibition of spontaneous cell death in the basal cell compartment. This was compensated for by an increased terminal differentiation of cells shedding off from the superficial cell layer (**Table I**, **Figs 1** and **4**). Thus, a major observation in this study was that fibroblasts circumvent the cell death program of reconstituted epithelium by decreasing spontaneous cell death in the basal cell layer and by promoting terminal differentiation in superficial cell layers, a pattern also normally found in native oral mucosa (**Table I**) (Presland and Dale, 2000; Squier and Kremer, 2001).

KGF had important effects on cell proliferation and showed a growth promoting effect in both OTK and OTKF cultures (**Figs 1** and **2**). These results are compatible with recent data from *in vivo* experiments on murine ventral tongue epithelium, in which KGF stimulated keratinocyte proliferation (Potten *et al*, 2002). We noticed that the effect of KGF was even more prominent on keratinocyte proliferation in the absence of fibroblasts (**Fig 3*B***). A likely explanation for this observation might be the presence of endogenously secreted KGF by fibroblasts in OTKF cocultures. Previous studies have reported synthesis of KGF in organotypic

skin cultures by dermal fibroblasts (Maas-Szabowski *et al*, 1999) and by oral fibroblasts in monocultures (Okazaki *et al*, 2002) and organotypic cultures (Grøn *et al*, 2002), in even higher amounts than dermal cultured fibroblasts. The issue of KGF synthesis by fibroblasts in different culture conditions is, however, still controversial, some authors have suggested that the ability of cultured fibroblasts to synthesize KGF is lower when the fibroblasts are embedded in collagen matrix (Le Panse *et al*, 1996; William McKeown *et al*, 2003), whereas others have found no modification in stimulated KGF production with modification of the culture substrate (Grøn *et al*, 2002). Our results are compatible with a moderate endogenous synthesis of KGF that is not sufficient to support epithelial growth fully. In native oral mucosa, KGF could also be provided by other neighboring cells, such as γδ T lymphocytes (Boismenu and Havran, 1994; Jameson *et al*, 2002), which are absent in our model. In a more recent study (William McKeown *et al*, 2003), epidermal growth factor was found to inhibit the synthesis of KGF by oral fibroblasts, especially when embedded in collagen. In our hands, epidermal growth factor (10 ng per mL) was not found to have a significant effect on epithelial growth or differentiation (data not shown).

Controversial results have been reported regarding the effects of KGF on epidermal differentiation in skin models, either that KGF could promote differentiation (Gibbs *et al*, 2000), or delay it (Andreadis *et al*, 2001). In our study, KGF did not significantly enhance epithelial differentiation (**Figs 5–7**). Moreover, KGF did not delay or impair the differentiation pattern induced by fibroblasts. That KGF is unable by itself to support normal epithelial morphogenesis and epithelial differentiation fully has also been suggested by Maas-Szabowski *et al* (2000) in skin models.

Although addition of KGF did not induce any significant change in the relative distribution of the various cell layers, a more prominent spinous cell layer with addition of KGF was noted in the presence of fibroblasts (**Fig 5**). An increased thickness of the spinous cell layer by KGF could be due to the fact that more cells enter into the spinous compartment (Squier and Kremer, 2001) in the presence of KGF. In fibroblast-depleted cultures, a strong positive correlation was found between KGF stimulated basal cell proliferation and the development of the spinous cell layer. In fibroblast supplemented cultures as well as in native mucosa, we did not find such a simple correlation that could have explained the development of the spinous cell layer. In these conditions, the development of the spinous cell layer was more closely correlated to a reduced rate of spontaneous cell death in the basal cell layer. On the other hand, the better development of the spinous cell layer observed with the addition of KGF in the presence of fibroblasts could suggest that KGF might have an indirect effect on fibroblasts. The ability of KGF to stimulate mesenchymal cells has recently been suggested in skin models (Jeschke *et al*, 2002).

In conclusion, this study shows that fibroblasts had a crucial effect on cell proliferation, differentiation, and apoptosis of human oral epithelium reconstituted *in vitro*, and especially on the development of stratum spinosum. KGF promoted epithelial growth, but did not significantly enhance epithelial differentiation. Our data suggest that fibroblasts possess additional mechanisms to KGF synthesis that can modulate differentiation of reconstituted human oral epithelium.

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Paper III

Fibroblast Control on Epithelial Differentiation is Gradually Lost During *In Vitro* Tumour Progression

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TITLE

Fibroblast Control on Epithelial Differentiation is Gradually Lost During *In Vitro* Tumour Progression

Running title: *fibroblasts and tumour progression*

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ABSTRACT

This study aimed to investigate the role of underlying fibroblasts on morphogenesis of *in vitro* epithelium reconstituted with normal and neoplastic human oral keratinocytes at various stages of malignant transformation. Primary normal human oral keratinocytes (NOK), early neoplastic human oral keratinocytes (DOK cell line), and neoplastic human oral keratinocytes (PE/CA-PJ 15 cell line) were organotypically grown on top of a collagen type I matrix with or without primary normal human oral fibroblasts. Morphogenesis of the reconstituted epithelia was assessed by histomorphometry, immunohistochemistry (Ki-67, cyclin D1, CK13, collagen IV, E-cadherin, p53, CD40), and the TUNEL method. Reproducible *in vitro* models of multistage oral carcinogenesis were established. Presence of fibroblasts in the collagen matrix significantly increased cell proliferation in all three models ($p<0.05$), and induced an invasive pattern of growth in the neoplastic cell lines ($p<0.05$). In normal, but not in neoplastic oral keratinocytes fibroblasts induced the expression of CD40, and polarised the expression of E-cadherin and p53 to the basal cell layer. In both normal and early neoplastic keratinocytes (DOK cell line) fibroblasts induced the expression of CK13 and collagen IV. In the neoplastic oral keratinocytes (PE/CA-PJ 15 cell line) the presence of underlying fibroblasts did not change the expression of any of the protein markers assessed. This study showed that (1) major steps of oral carcinogenesis can be reproduced *in vitro*, and (2) the tight control exerted by fibroblasts on epithelial morphogenesis of *in vitro* reconstituted normal human oral mucosa is gradually lost during neoplastic progression.

Key words: keratinocytes, tumour progression, oral,
organotypic cell culture, fibroblasts.

INTRODUCTION

The importance of stromal fibroblasts for tumour development has begun to emerge from both descriptive reports on archival material and experimental work (Fusenig and Boukamp, 1998; Tlsty and Hein, 2001; Mueller and Fusenig, 2002; van Kempen et al., 2002; Rubin, 2003; De Wever and Mareel, 2003; Radisky and Bissell, 2004). However, while several studies have pointed out the growth-promoting and invasive effects of activated fibroblasts on carcinoma cells (Atula et al., 1997; Olumi et al., 1999; Berndt et al., 2001; Mueller et al., 2001; De Wever et al., 2004), only few studies have addressed the question of differentiation control exerted by fibroblasts on epithelial tissue during tumour development (Kaur and Carter, 1992; Tomakidi et al., 2003). An important role for underlying fibroblasts on differentiation of normal epithelium has been previously reported (Maas-Szabowski et al., 2000; El Ghalbzouri et al., 2002; Costea et al., 2003). Loss of the fibroblast control on epithelial differentiation has been suspected to occur during tumour development, but the concept needs experimental evidence (Fusenig et al., 1995). A better understanding of the mechanisms of normal differentiation would also be of help to identify the significance of their alterations during tumour progression (Fusenig et al., 1995; Guarino et al., 2004).

In order to investigate the role of fibroblasts on epithelial morphogenesis during tumour progression, we have initially established a multistage model of oral epithelial carcinogenesis. This model has been obtained by growing organotypically (1) primary normal human oral keratinocytes (NOK), (2) an established oral early neoplastic / dysplastic cell line (DOK), and (3) an established oral neoplastic cell line (PE/CA-PJ 15) on top of collagen type I biomatrices (Chang et al., 1992; Berndt et al., 1997; Costea et al., 2004). We report here that while normal oral fibroblasts could tightly control the differentiation of the epithelium constructed with primary normal

keratinocytes, they gradually lost this ability in the neoplastic models of human oral mucosa.

MATERIALS AND METHODS

Patient material: Twenty-five samples of normal human oral mucosa (NHOM) were obtained from the superfluous oral tissue after wisdom tooth extraction. Seven samples were snap frozen in isopentane pre-chilled in liquid nitrogen, and six samples were formalin fixed, and embedded in paraffin. They served as positive controls of native NHOM for immunohistochemical staining. Twelve samples were used for isolating primary normal oral cells and growing of organotypic cultures. The study was approved by the Ethics Committee of the University of Bergen, and included only clinically healthy donors after informed consent.

Cell lines: Primary human normal oral fibroblasts (NOF) and keratinocytes (NOK) were isolated from biopsy samples of NHOM as described elsewhere (Costea et al., 2002). NOK were routinely grown on plastic surfaces (Nunc, Naperville, I.L., USA) with no feeding layers, in keratinocyte serum free medium (KSFM) (GibcoBRL, Grand Island, N.Y., USA) supplemented with 1 ng/ml human recombinant epidermal growth factor (GibcoBRL), 25 μg/ml bovine pituitary extract (GibcoBRL), 2 mM L-glutamine (GibcoBRL), 100 U/ml penicillin (GibcoBRL), 100 μg/ml streptomycin (GibcoBRL), 0.25 μg/ml amphotericin B (GibcoBRL). NOF were grown in Minimum Essential Medium Eagle (MEM) (Sigma, St. Louis, M.O., US) supplemented with 10% foetal calf serum (FCS) (Sigma), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B. The following cell lines were obtained from the European Cell Culture Bank (ECACC): human (caucasian) dysplastic oral keratinocytes (DOK cell line, accession no. 94122104, (Chang et al., 1992), and human (caucasian) neoplastic oral keratinocytes (PE/CA-PJ 15 cell line, accession no. 961211230,

(Berndt et al., 1997). DOK cells were routinely maintained in Dulbecco's Modified Eagle's Medium (DME) (Sigma) supplemented with 10% FCS, 2 mM L-glutamine, and 13.7 μM hydrocortisone, as indicated by ECACC. PE/CA-PJ 15 cells were routinely grown in Iscove's Modified Dulbecco's Medium (IMDM) (Sigma) supplemented with 10% FCS and 2 mM L-glutamine, as also indicated by ECACC.

Organotypic cell culture procedures: Various *in vitro* models of human oral mucosa were constructed using the organotypic co-culture method as previously described (Costea et al., 2003). NOK in the 2nd passage, DOK cells in the 29th passage, and PE/CA-PJ 15 cells in the 9th passage were used to construct normal, early neoplastic and neoplastic human oral mucosa models, respectively (0.5x10^6 cells/culture). The keratinocytes were grown on top of collagen gels containing or not NOF. The simple collagen gels (700 μl for each culture) were prepared on ice, by mixing 7 vol. (3.40 mg/ml) of rat tail collagen type I (Collaborative Biomedical, Bedford, M.A., USA) with 2 vol. reconstitution buffer (261 mM $NaHCO_3$, 150 mM NaOH, 200 mM HEPES) pH 8.15, and 1 vol. DMEM 10x (Sigma). The fibroblast-containing collagen matrices were prepared by mixing 10 vol. of the mixture described above with 1 vol. FCS containing 0.5x10^6/ml NOF in passages 2-4. The organotypic cultures were grown in serum free FAD medium (DMEM:Ham's F-12 / 3:1) supplemented with 1 μM hydrocortisone, 0.8 μM insulin, 0.25 mM transferrin, 0.25 mM L-ascorbic acid, 15-30 μM linoleic acid, 15 μM bovine serum albumin, 2 mM L-glutamine (all from Sigma). The cultures were harvested on day 10 of co-culture. One half of each culture was snap frozen in isopentane pre-chilled in liquid nitrogen and the other fixed in 4% buffered formalin pH 7.15 and embedded in paraffin.

Immunohistochemical staining: The immunohistochemical staining was carried out using the DAKO autostainer – Universal Staining System (DAKO-USA, Carpinteria, California, US). Five μm thick fresh or formalin fixed, paraffin embedded sections were

used. The staining for CD40 and E-cadherin was carried on fresh frozen sections fixed for 30 sec in 50% cold acetone, and afterwards for 5 min in 100% acetone before washing in distilled water. All sections were processed then as previously reported (Costea et al., 2003). The sections were incubated with the primary antibody for 60 min, and afterwards with the secondary antibody conjugated with horseradish peroxidase labelled polymer (EnVision+ System, DAKO) for 30 min. The type of the primary antibodies used and their titrations are shown in Table 1. The presence of antigen was visualised with DAB (3,3'-diaminobenzidine, DAKO). Biopsies of NHOM served as reference controls. Specimens incubated with antibody diluent (DAKO) instead of primary antibody were used as negative controls. Specimens stained with CD 3 antibody (having the same isotype as the antibodies tested in the study) served as control for the specificity of the staining reactions.

TUNEL method: Cell death was detected by the TUNEL method (terminal deoxynucleotidyl transferase-mediated dUTP in situ nick end-labelling) on formalin fixed paraffin embedded sections, as previously described (Loro et al., 2000). For positive controls, specimens were treated with 0.5 mg/ml DNase (Roche Diagnostics GmbH, Mannheim, Germany) in Tris-buffered saline (TBS) for 15 min at 37°C prior to incubation with bovine serum albumin. The specificity of the TUNEL reaction was tested by substituting the biotinylated dUTP in the TUNEL labelling mixture with unbiotinylated dUTP (Roche) in excess.

Evaluation of samples and statistical analysis:

Tissue sections (5μm) from paraffin embedded specimens, stained with Haematoxylin-eosin, were morphometrically analysed by a computer based optical image analyser (analySIS 11.0 Pro Soft Imaging system, GmbH, Munster, Germany). An arbitrary straight line was drawn at the interface between the epithelial and the connective tissue compartment. For DOK and PE/CA-PJ 15 cells this line was drawn

through the upper remnants of the collagen gel in the epithelial tissue. Epithelial thickness was measured from that line to the surface of the epithelium. The degree of local invasiveness was determined by measuring the area of the epithelial nests that grew invasive in the collagen matrix below the above described line. The area of invasion was determined as the percentage of the matrix area that was invaded by epithelial cells in a standard square of 30 000 μm^2 (100 µm depth x 300 µm length) measured in the matrix layer immediately under epithelium, starting from the arbitrary line described above. The counts for Ki-67 (proliferation index), cyclin D1, p53 and TUNEL (apoptotic index - AI) were calculated as percentage of positive cells among all keratinocyes in the epithelium. At least 500 cells were counted per field. The measurements and counts were done at 200 fold magnification on a standard microscope (Leika DMLM, GmbH, Munster, Germany), on 6 consecutive fields situated 200 µm apart.

The cultured tissues were obtained from 12 separate experiments with NOK, 11 experiments with DOK, and 9 experiments with PE/CA-PJ 15 cells. Each experiment was run in duplicates, with a different strain of fibroblasts (isolated from separate patients). The effect of fibroblasts on epithelial growth and cell death was analysed by the use of a paired statistical test (Wilcoxon, SPSS 11.0, Chicago, Illinois). Thus, the statistical results shown in Table 2 include only data from parallel cultures of NOK, DOK, and PE/CA-PJ 15 cells grown on collagen gels with fibroblasts isolated from the same patient (n = 5).

RESULTS

Role of fibroblasts on epithelial thickness and cell proliferation

The majority of the nucleated cells found in the matrix had a stellate morphology with oval contoured nuclei, were smaller and had much less cytoplasm than the overlaying

cells positive for the epithelial marker pancytokeratin (keratinocytes) (Fig. 1). Together with their positive staining for vimentin and negative staining for pancytokeratin (data not shown) these features were suggestive for a fibroblastic phenotype. The number of these matrix cells, identified as fibroblasts, varied between different types of cultures, from 14.7 ± 4.3 fibroblasts / per 100 μm^2 of collagen matrix (mean ± SD) in the NOK model to 22.8 ± 6.0 fibroblasts / per 100 μm^2 of collagen matrix in the PE/CA-PJ 15 cell line model. The number of Ki67 positive cells among the matrix fibroblasts increased from 4.3 ± 2.1 % (mean ± SD) in the NOK model to 11.8 ± 4.5 % in the PE/CA-PJ 15 cell line model. This indicated that at least some of the fibroblasts maintained their ability to proliferate until the end of the culturing period, and most prominently in the PE/CA-PJ 15 cell model.

The cultures grown in absence of fibroblasts showed a mean epithelial thickness of 45.5 μm (SD = 15.6 μm, SEM = 3.2 μm), 50.0 μm (SD = 9.7 μm, SEM = 4.0 μm) and 56.5 μm (SD = 17.7 μm, SEM = 7.2 μm) for the NOK, DOK and PE/CA-PJ 15 cell models, respectively. Although some variation was observed between different experiments with respect to epithelial thickness both in absence and presence of fibroblasts, the presence of fibroblasts induced repeatedly an increase in epithelial thickness in all NOK (n = 12), DOK (n = 11), and PE/CA-PJ 15 cell (n = 9) models (Fig. 1). In those experiments, the mean epithelial thickness in presence of fibroblasts was 92.8 μm (SD = 29.4 μm, SEM = 6.7 μm), 88.6 μm (SD = 22.0 μm, SEM = 7.3 μm), and 117,8 μm (SD = 28.1 μm, SEM = 8.9 μm) for the NOK, DOK and PE/CA-PJ 15 cell models, respectively.

In all three models a significant increase in Ki-67 and cyclin D1 counts was also observed with the addition of fibroblasts in the collagen matrix (Table 2). In both NOK and DOK models proliferating cells (Ki-67 and cyclin D1 positive) were localised mainly in the basal cell layer (Fig. 2 Panels A, B, D, E). No preferential localisation of

Ki-67 and cyclinD1 positive cells could be observed in PE/CA-PJ 15 models (Fig. 2 Panels C, F). Vertical strands of epithelial cells could be observed growing into the fibroblast-containing collagen matrix of DOK model, occupying 50.8 ± 4.1% of the nearest underlying matrix (Table 2 and Fig. 1 Panel E). PE/CA-PJ 15 cells grew invasive in a front like pattern, occupying 73.6 ± 4.7% of the nearest fibroblast-containing collagen matrix (Table 2 and Fig. 1 Panel F). The invasive pattern of growth of DOK and PE/CA-PJ 15 cells was not observed on collagen type I matrices in the absence of fibroblasts (Table 2 and Fig. 1 Panels B, C).

Effect of fibroblasts on epithelial morphology

DOK grown on top of simple collagen matrices formed a non-polarised epithelium, with cellular pleomorphism and hyperchromatic nuclei (Fig. 1 panel B). A certain degree of epithelial polarisation, as suggested by the presence of flattened cells in the uppermost cell layers, was seen in the DOK model in the presence of fibroblasts (Fig. 1 Panel E), although to a lesser degree when compared with the polarisation induced by fibroblasts in the NOK model (Fig. 1 Panels A, D). PE/CA-PJ 15 cells formed an epithelium that lacked polarisation and showed a heterogenous appearance with spongiosis, intracellular vacuolisation, cellular pleomorphism and hypercromatic nuclei (Fig. 1 Panel C). This appearance was not affected by the presence of fibroblasts (Fig. 1 Panels C, F).

Effect of fibroblasts on expression of cytokeratin 13 (CK13) and collagen IV

The presence of fibroblasts induced the expression of CK 13 in the suprabasal epithelial cell layers of both NOK and DOK models, although to a lesser extent in the DOK model (Table 3 and Fig. 2 Panels G, H). Expression of CK 13 was not observed in any of the PE/CA-PJ 15 cell cultures (Table 3 and Fig. 2 Panel I). In both NOK and DOK models, collagen IV was not expressed when the cells were grown on a simple collagen matrix, but it was induced by the presence of normal oral fibroblasts (Table 3

and Fig. 2 Panels J, K). In the PE/CA-PJ 15 cell model collagen IV was expressed at the epithelial - matrix interface in both presence and absence of fibroblasts (Table 3 and Fig. 2 Panel L).

Effect of fibroblasts on expression of E-cadherin

A strong expression of E-cadherin was observed in all cell layers of the reconstituted NOK epithelium on simple collagen gels (Table 3). The expression was confined to the cell membrane (Fig. 3 Panel A). Addition of fibroblasts to the collagen matrix induced a polarised expression of E-cadherin to the basal and spinous cell layers in this model (Fig. 3 Panels A, B). E-cadherin was expressed in all cell layers of both DOK and PE/CA-PJ 15 cell models irrespective of the presence of fibroblasts (Table 3). A weaker and more diffuse cytoplasmic staining was observed in the neoplastic cells in presence of fibroblasts, especially in the cells growing invasive into the fibroblast-containing collagen matrix (Fig.3 Panels C, D).

Modulation of apoptosis and apoptosis related proteins by fibroblasts

The number of dead cells, as visualised by the TUNEL method, increased from NOK to DOK and PE/CA-PJ 15 cell models (Tables 2 and 3). The previously reported effect of normal oral fibroblasts on polarisation of TUNEL positive cells in the NOK epithelium (Costea et al., 2003) was not observed in DOK or PE/CA-PJ 15 cell models (Table 4 and Fig. 2 Panels M, N, O). The epithelium reconstituted by NOK cells in the absence of fibroblasts showed p53 positive cells scattered throughout the whole epithelium (Fig. 3 Panel E). In presence of fibroblasts, p53 positive cells occurred less frequently ($p<0.05$, Table 2), and were localised in the basal cell layers (Fig. 3 Panel F). In DOK and PE/CA-PJ 15 cell models almost all cell nuclei were positive for p53 (Table 2 and Fig. 3 Panels G, H). CD40, another apoptosis-related protein, was not expressed in NOK grown on collagen matrices in the absence of fibroblasts, but became expressed in the basal cell layer in their presence (Fig.3 Panels I, J). In DOK

and PE/CA-PJ 15 cell models CD40 was expressed in almost all epithelial cells in both absence and presence of fibroblasts (Table 3 and Fig.3 Panels K, L).

DISCUSSION

To study the role of fibroblasts on epithelial morphogenesis at different stages of tumour progression, we reconstructed oral epithelium from normal (NOK), dysplastic (DOK cell line), and neoplastic (PE/CA-PJ 15 cell line) human oral keratinocytes in organotypic cultures on the hypothetical basis that these models may reflect different stages of oral carcinogenesis. The DOK cell line is a spontaneously immortalised cell line isolated from a dysplastic lesion of oral mucosa which did not form tumours in nude mice (Chang et al., 1992). Therefore, this cell line was considered transformed, but not malignant, and it has been classified by ECACC as "dysplastic". However, in our hands DOK cells grew invasive into the human fibroblast-containing collagen biomatrices. This observation made us to refer to this cell line as neoplastic rather than dysplastic. On the other hand, the DOK cells formed an epithelium with (1) a certain degree of epithelial maturation, (2) a polarised expression of Ki-67 and Cyclin D1 to the basal cell layer, and (3) expressing CK 13 in the spinous cell layer. Therefore, in comparison to PE/CA-PJ15 cells, which formed an epithelium that (1) lacked epithelial differentiation, (2) showed no sign of polarised proliferation, (3) did not express CK13, and (4) grew more invasive into the collagen matrix, DOK cells showed proofs for a less transformed phenotype. These observations would also fit with previous studies in which PE/CA-PJ 15 cells, unlike DOK cells, have been shown to form tumours when xenotransplanted into nude mice (Berndt et al., 2001). When comparing all the three models, the polarisation in tissue morphology, cell proliferation and cell death patterns, as well as the expression of differentiation markers, were all gradually lost from NOK to DOK and then to the PE/CA-PJ 15 cell

model (Fig. 2 and Table 3). We thus consider the NOK, DOK and PE/CA-PJ 15 cells as models of progressive stages of *in vivo* oral carcinogenesis.

The tests done on these *in vitro* step-wise models of oral carcinogenesis showed that fibroblasts had an important role in normalising the pattern of expression of CK13, collagen IV, E-cadherin, p53, CD40, and the pattern of cell death in NOK cells. This effect was observed only for the expression of CK 13 and collagen IV in DOK cells, and could not be detected for any of these markers in PE/CA-PJ cells (Table 3). These findings indicate that keratinocyte sensitivity to fibroblast differentiation signals was gradually lost with the transformation stage of the keratinocytes. Such suggestion is in line with previous reports on skin models which showed that the expression of integrins in virally immortalised skin keratinocytes (considered as a model of early transformed cells) was still under the differentiation control of the fibroblasts (Kaur and Carter, 1992), while malignant skin keratinocytes were resistant to stromal differentiation signals (Tomakidi et al., 2003).

Recently it has been suggested that the mechanisms of fibroblast-induced invasion of oral carcinoma cells could involve disruption of E-cadherin junctions (Murai et al., 2004). In DOK and PE/CA-PJ 15 cell models the expression of E-cadherin was mainly confined to the cell membrane in absence of fibroblasts, but in their presence became more diffuse and cytoplasmic, especially in the neoplastic cells at the invasive front. Although the expression of E-cadherin is generally reported to be lost during tumour progression (Thomas and Speight, 2001), our latter observation could fit with more recent observations in which the expression of E-cadherin showed an intracellular granular expression in tumour cells at the invasive front of oral squamous cell carcinomas (Bankfalvi et al., 2002).

We have previously reported that fibroblasts normalise the cell death program during the *in vitro* morphogenesis of normal human oral epithelium (Costea et al.,

2003). In this study we have tested whether fibroblasts could have a role in controlling the expression of the apoptosis-related proteins p53 and CD40. In reconstituted epithelium with NOK cells the expression of p53 was higher than normally observed in the native oral mucosa (Table 2) (Nylander et al., 2000). In presence of fibroblasts a polarised expression of p53 to the basal cell layer was observed (Fig 3 Panels E, F). In this way, the cells expressing p53 became co-localised with proliferating cells (positive for Ki-67 and Cyclin D1), but not with the dying cells identified by the TUNEL method. This could suggest a closer association for the expression of p53 with the regulation of cell proliferation than with the regulation of cell death in artificial NHOM. CD40 receptor was not expressed in NOK in the absence of fibroblasts, but was induced in their presence. The expression was mainly confined to the cell membrane and restricted to clusters of cells in the basal layer of the NOK epithelium, in this way mirroring the expression of CD40 in native NHOM (Loro et al., 2001). The finding that CD40 expression was induced by the presence of fibroblasts (Fig. 3 Panels I, J) suggests an important role for epithelial-mesenchymal interactions in regulation of CD40 expression in normal human oral keratinocytes. That CD40 expression was not modified by the presence of fibroblasts in the neoplastic models might suggests that the expression of CD40 receptor is another example of the loss of fibroblast control during tumour progression in oral kerationocytes.

In conclusion, we were able (1) to establish *in vitro* experimental models of human origin that could reproduce major steps of *in vivo* oral carcinogenesis, and (2) to bring experimental evidence for the concept of loss of fibroblast control on epithelial differentiation during tumour progression.

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FIGURE LEGENDS

Fig. 1 Tissue morphology of organotypic cultures of NOK, DOK and PE/CA-PJ 15 cells. The organotypic cultures were constructed with primary NOK (Panels A, D), DOK (Panels B, E), and PE/CA-PJ 15 oral keratinocytes (Panels C, F) on top of either collagen type I gels (Panels A-C) or human fibroblast-containing collagen gels (Panels D-F). The cultures were grown for 10 days in FAD medium at the liquid-air interface. Sections of representative cultures stained with Haematoxilin & eosin are shown. Scale bar = 50 μm.

Fig. 2 Expression patterns of growth and differentiation of NOK, DOK and PE/CA-PJ 15 cells organotypically grown in presence of fibroblasts.

Reconstituted oral mucosa was obtained by growing organotypically primary NOK (Panels A, D, G, J, M), DOK (Panels B, E, H, K, N), and PE/CA-PJ 15 keratinocytes (Panels C, F, I, L, C) on top of a collagen type one matrix supplemented with primary normal oral fibroblast. Sections stained for Ki-67 (Panels A-C), cyclin D1 (Panels D-F), CK 13 (Panels G-I), collagen IV (Panels J-L), and apoptotic cell death (TUNEL Panels M-O) are shown. Scale bar = 50 μm.

Fig. 3 Effect of fibroblasts on the expression pattern of p53, CD40 and E-cadherin in NOK, DOK and PE/CA-PJ 15 cells.

The expression of E-cadherin (Panels A-D), p53 (Panels E-H), and CD40 (Panels I-L) is shown for NOK organotypically grown in absence (Panels A, E, I) or presence of fibroblasts (Panels B, F, G), DOK organotypically grown in presence of fibroblasts (Panels C, G, K), and PE/CA-PJ 15 cells organotypically grown in presence of fibroblasts (Panels D, H, L). Scale bar = 50 μm.

Table 1. Antibodies and their titrations used for immunohistochemical staining.

Antibody	*Type*	*Clone*	*Source*	*Titration*	*Type of tissue*
Ki-67	IgG1	Mib-1	DAKO Cytomation Glostrup, Denmark	1:50	formalin fixed
Cyclin D1	IgG1	DCS-6	Novocastra Lab. Newcastle, UK	1:100	formalin fixed
CK 13	IgG1	KS-1A3	Novocastra Lab. Newcastle, UK	1:400	formalin fixed
Collagen IV	IgG1	CIV221	DAKO Glostrup, Denmark	1:25	formalin fixed
E-cadherin	IgG1	HECD-1	R&D Systems Abingdon, UK	1:600	fresh frozen
p53 optimised	IgG2b	DO-7	DAKO Glostrup, Denmark	Ready to use	formalin fixed
CD 40	IgG1	EA-5	Calbiochem Darmstadt, Germany	1:200	fresh frozen
CD 3 optimised	IgG1	F7.2.38	DAKO Glostrup, Denmark	Ready to use	fresh frozen formalin fixed

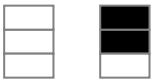
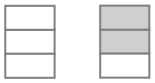
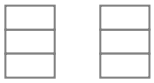
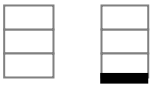
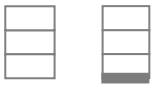
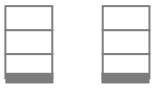
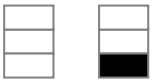
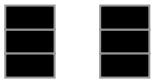
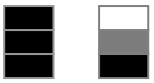
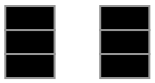
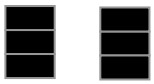
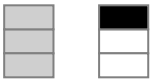
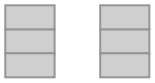
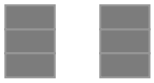
Table 2. The effect of fibroblasts on epithelial thickness, local cell invasion, cell proliferation and cell death in normal and neoplastic models of human oral mucosa.

In vitro models of oral mucosa were developed by growing normal human oral keratinocytes (NOK), a dysplastic cell line (DOK), and a neoplastic cell line (PE/CA-PJ 15) on top of a collagen matrix with or without fibroblasts. Epithelial thickness, are of invasion, cell proliferation (Ki-67 and cyclin D1), expression of p53, and cell death (AI%) were determined. The data represent mean ± SEM of 5 independent experiments run in duplicates. The counts given represent average data for the whole epithelium.

	NOK			***DOK***			***PE/CA-PJ 15***		
	-Fibs.	*+Fibs.*	*p*	*-Fibs.*	*+Fibs.*	*p*	*-Fibs.*	*+Fibs.*	*p*
Epithelial thickness (µm)	41.9 ± 3.4	84.1 ± 5.6	0.002	53.37 ± 4.7	97.3 ± 6.7	0.018	65.7 ± 2.9	122.9 ± 7.2	0.004
Area of invasion (%)	0.04 ± 0.03	0.17 ± 0.09	0.068	3.5 ± 0.8	50.8 ± 4.1	0.000	4.8 ± 0.6	73.6 ± 4.7	0.000
Ki-67 (PI %)	4.5 ± 0.5	9.7 ± 2.5	0.005	15.3 ± 0.3	21.0 ± 1.2	0.003	26.5 ± 3.1	42.5 ± 3.4	0.002
Cyclin D1 (%)	9.8 ± 0.6	15.8 ± 2.8	0.016	20.8 ± 2.3	24.3 ± 2.4	0.010	31.3 ± 1.7	48.4 ± 1.2	0.019
p53 (%)	27.5 ± 0.3	17.7 ± 2.5	0.016	98.2 ± 0.6	98.7 ± 0.4	1.000	87.7 ± 4.6	88.3 ± 2.4	1.000
AI (%)	3.1 ± 1.2	1.6 ± 0.6	0.116	4.2 ± 0.8	3.8 ± 0.3	0.214	6.6 ± 0.6	6.3 ± 0.4	0.721

Table 3. Differential effect of fibroblast on various protein markers in normal and neoplastic models of human oral mucosa.

NOK, DOK and PE/CA-PJ 15 cells were organotypically grown on top of collagen matrix in the presence and absence of fibroblasts. The expression patterns for CK 13, collagen IV, CD40, E-cadherin, and apoptotic cell death (TUNEL) are schematically shown. In this qualitative assay the epithelium was divided into the lower third (Sb), middle third (Sp), and upper third (Ss), and the level of expression for each parameter was given as negative ☐ ,low ▭ ,moderate ▭ , or high expression ■ .

	NOK			***DOK***			***PE/CA-PJ 15***		
	-Fibs.	*+Fibs.*	*change*	*-Fibs.*	*+Fibs.*	*change*	*-Fibs.*	*+Fibs.*	*change*
CK 13 Ss Sp Sb			yes			yes			no
Collagen IV Ss Sp Sb			yes			yes			no
CD 40 Ss Sp Sb			yes			no			no
E cadherin Ss Sp Sb			yes			no			no
TUNEL Ss Sp Sb			yes			no			no

Paper IV

Species-Specific Fibroblasts Trigger Invasiveness of Early Neoplastic Oral Keratinocytes

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Species-Specific Fibroblasts Trigger Invasivene

ABSTRACT

This study tests the hypothesis that invasion of early neoplastic oral keratinocytes is triggered by diffusible, proinvasive signals provided by species-specific oral fibroblasts. In vitro organotypic models were developed by growing the DOK cell line (ECACC94122104) on top of various types of matrices. Depth and area of invasion (Dinv and Ainv), epithelial thickness (histomorphometry), and cell proliferation (Ki67 immunostaining) were assessed. Presence of human fibroblasts in the matrix induced local invasion of DOK cells (Dinv=95.6±7.1μm, Ainv=50.8±3.5%). A minimal invasion was observed when DOK were grown on simple collagen matrix (Dinv=14.1±2.1μm, Ainv=3.7±0.8%, $p<0.05$), or on mouse fibroblast-containing matrix (Dinv=11.5±4.0μm, Ainv=4.3±1.0%, $p<0.05$). Local invasion was induced by the presence of human fibroblasts in the lower layer of sandwich models ($p<0.05$), or by conditioned medium from parallel organotypic co-cultures of DOK with human fibroblast-containing matrix ($p<0.05$). In conclusion, local invasiveness of early neoplastic oral keratinocytes was triggered by fibroblast-derived diffusible factor(s) in a species-specific manner.

KEY WORDS: invasion, fibroblast, organotypic cell culture, species-specificity, oral