

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 (a 3:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 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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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 the 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 (keratinocyte serum-free medium (KSFM), minimum essential medium Eagle (MEM) and FAD (a 3:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium)), 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.

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,

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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 flucanazole. 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/cm²) 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 extensions 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

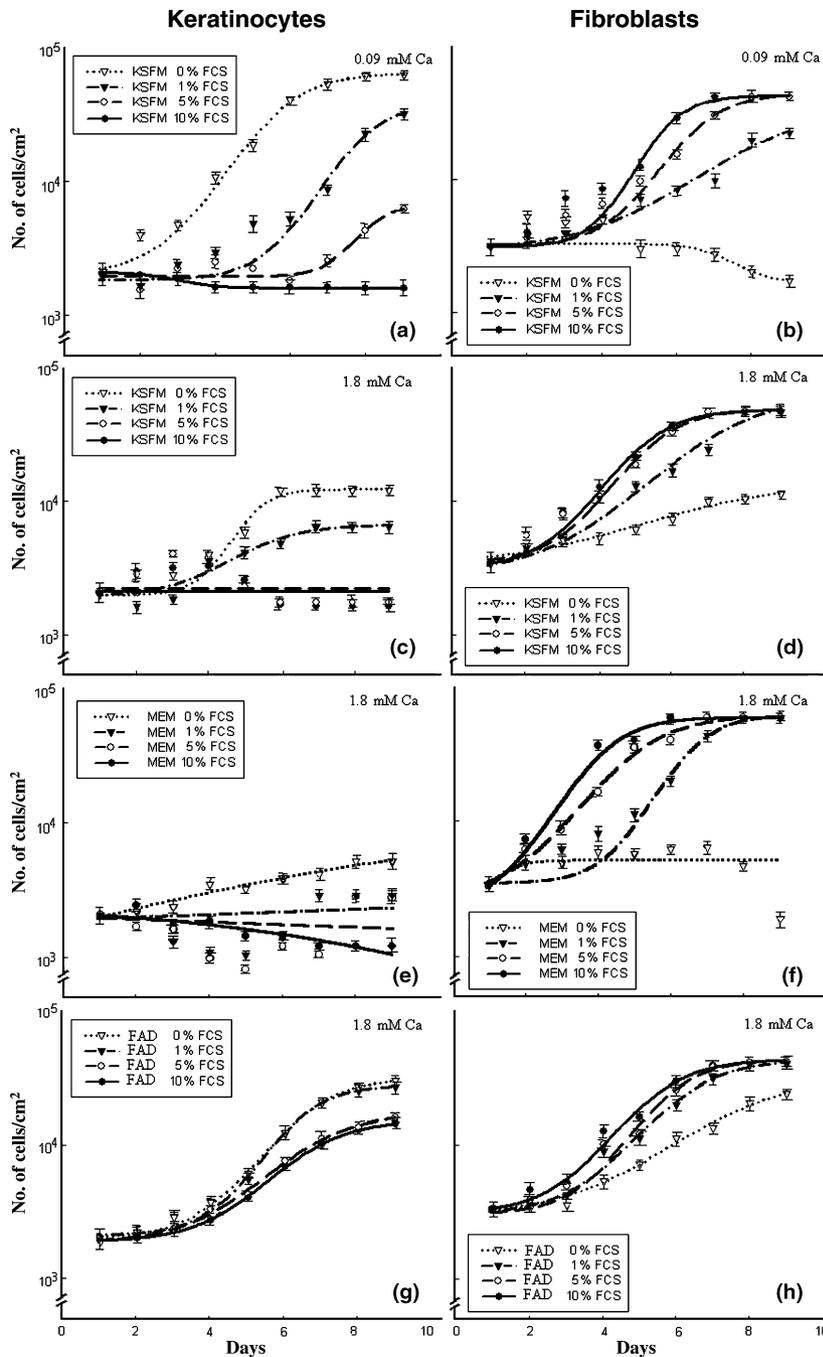


Figure 1 The influence of medium type, FCS and Ca⁺⁺ concentration on growth of oral keratinocytes and fibroblasts in monolayer cultures. 3×10^4 cells/well were seeded in 24-well dishes. The effect of different media (KSFM, MEM and FAD), two different Ca⁺⁺ concentrations (0.09 and 1.8 mM), and various concentrations of FCS (0, 1, 5 and 10%) were tested on primary NHOK and NHOE. The cells were daily counted for 10 days. Growth curves were obtained after plotting cell density (cells/cm²) against time using Sigma Plot program. The data represent mean \pm SEM from three separate batches of primary cells. The experiments were run in duplicates.

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 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,

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 \pm 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 \pm 5.7	27.5 \pm 3.8	52.9 \pm 4.6	46.8 \pm 6.0	77.3 \pm 6.6	67.9 \pm 6.6
PI basal cell layer (%)	8.1 \pm 1.0	6.9 \pm 1.3	14.5 \pm 1.9	14.1 \pm 2.2	16.9 \pm 2.3	16.1 \pm 1.9
PI suprabasal cell layers (%)	3.7 \pm 1.5	3.0 \pm 1.8	4.8 \pm 2.7	4.6 \pm 0.4	1.1 \pm 0.3	0.4 \pm 0.2
AI basal cell layer (%)	4.2 \pm 1.6	4.5 \pm 0.9	8.0 \pm 1.4	8.3 \pm 1.1	1.6 \pm 0.4	1.8 \pm 0.3
AI suprabasal cell layers (%)	3.9 \pm 1.1	4.4 \pm 1.1	7.0 \pm 1.8	6.0 \pm 0.7	5.0 \pm 0.5	5.4 \pm 0.1

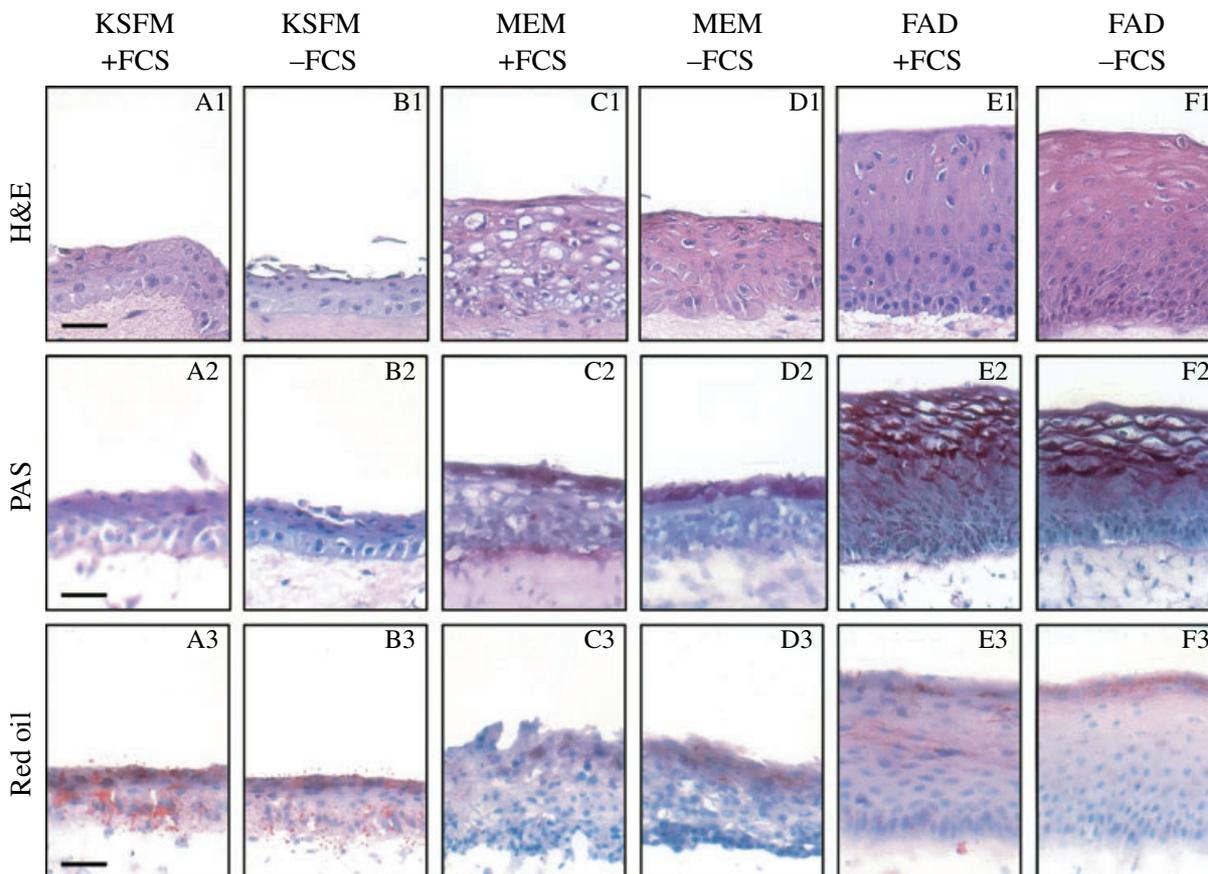


Figure 2 Culture medium and serum effect on morphogenesis of *in vitro* reconstituted human oral epithelium. The organotypic co-cultures were grown for 10 days in KSFM, MEM or FAD in absence or presence of FCS and BPE. Epithelial morphology in HE staining (panels A1–F1), PAS (panels A2–F2), and Red Oil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

we grew NHOK on top of simple collagen gels. In this culture type, KSFM did not support the growth of epithelium (total thickness $13.3 \pm 1.9 \mu\text{m}$). 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 of 20.8 ± 2.3 and $36.4 \pm 4.6 \mu\text{m}$, 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 ($53.3 \pm 3.2\%$) and MEM cultures ($52.3 \pm 2.6\%$), and a less developed spinous cell layer (28.8 ± 1.8 and $29.7 \pm 1.9\%$, respectively), as compared with FAD cultures that showed, as previously reported by us, a more prominent spinous cell layer ($46.1 \pm 2.4\%$, $P < 0.05$), and a distribution of various epithelial cell layers comparable with native buccal mucosa (11). Expression of

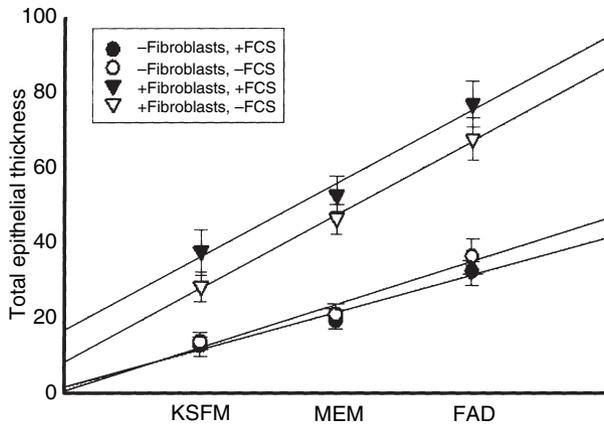


Figure 3 The effect of medium type and serum on total epithelial thickness of *in vitro* reconstituted human oral epithelium. Human oral epithelium was cultured on top of either simple collagen gel or on fibroblast-containing biomatrix in KSFM, MEM and FAD, in absence or presence of serum and BPE. Total epithelial thickness was measured by histomorphometry. Data represent the mean \pm 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.

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 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 rich 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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