Evaluation of tissue engineered models of the oral mucosa to investigate oral candidiasis

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Abstract

Candida albicans is a commensal organism that can be isolated from the majority of healthy individuals. However, in certain susceptible individuals *C.albicans* can become pathogenic leading to the mucocutaneous infection; oral candidiasis. Murine models and *in vitro* monolayer cultures have generated some data on the likely virulence and host factors that contribute to oral candidiasis but these models have limitations. Recently, tissue engineered oral mucosal models have been developed to mimic the normal oral mucosa but little information is available on their true representation. In this study we assessed the histological features of three different tissue engineered oral mucosal models compared to the normal oral mucosa and analysed both cell damage and cytokine release following infection with *C.albicans*. Models comprised of normal oral keratinocytes and a fibroblast-containing matrix displayed more similar immunohistological and proliferation characteristics to normal mucosa compared to models composed of an oral carcinoma cell line. Although all models were invaded and damaged by *C.albicabs* in a similar manner, the cytokine response was much more pronounced in models containing normal keratinocytes. These data suggest that models based on normal keratinocytes atop a fibroblast-containing connective tissue will significantly aid in dissecting the molecular pathogenesis of oral candidiasis.

Keywords: Candida albicans; Tissue engineering; oral mucosa; oral candidiasis

1. Introduction

Candida albicans is a fungal organism that forms part of the normal oral microbial flora in approximately 50% of healthy individuals [1]. *C. albicans* is a polymorphic organism and can grow as yeast, pseudohyphae or hyphae; each differing in its morphology and virulence [2]. The yeast form is commonly associated with the commensal carrier state where a balance exists between the oral microbial flora and the host innate immune response. If this balance is disrupted, for example in those with HIV infection or in immunosuppressed individuals, those receiving immunotherapy, corticosteroid or prolonged antibiotic therapy, or in individuals with uncontrolled diabetes, salivary gland dysfunction (Sjögren's syndrome) or wearing dentures, *C. albicans* may proliferate and convert to its hyphal form which penetrates into the oral mucosa causing the tissue damage that is frequently observed in oral candidiasis [3,4].

Several experimental approaches have been used in an attempt to identify the key molecules involved in the interaction of *C. albicans* with oral epithelial cells. Immunohistological analysis of biopsies from patients with oral candidiasis have been performed [5-7] but such studies are not amenable to experimental manipulation and so can only provide limited information. Murine models of oral candidiasis have identified several potential virulence factors that may play a role in pathogenesis [8-10], but since *C. albicans* does not naturally colonise the murine oral cavity [11] it is not clear how representative these studies are. Moreover, there are significant differences between the innate immune systems of humans and mice [12] which may make the results of murine studies difficult to interpret. To address these concerns numerous *in vitro C. albicans* infection studies have used monolayer cultures of human oral keratinocytes or oral epithelial cell lines [13-16]. However, the oral mucosa is a complex multi-layered, multi-cellular tissue consisting of increasingly differentiated epithelial cells and so monolayer cultures are unlikely to be representative. Recently, multi-layered organotypic three-dimensional *in vitro* culture systems have been developed to mimic the oral mucosa [17-20]. The reconstituted human oral epithelial (RHOE)

model, which is based upon the TR146 buccal carcinoma cell line cultured on a porous polycarbonate insert, has been widely used in *C. albicans* infection studies [21-25]. More advanced, alternative tissue engineered mucosal models have since been developed that consist of oral keratinocytes cultured on a fibroblast-containing extracellular matrix [20,26,27]. To date no study has examined whether these models display histological features or express immune molecules similarly to the oral mucosa. In this study we compared three tissue engineered oral mucosal models with normal healthy oral mucosa for expression of key oral mucosal markers using immunohistochemistry. We also compared these models in terms of invasion, cell damage, cytokine and innate immune molecule production in response to *C. albicans* infection in order to identify which model most accurately represents an *in vitro* experimental model of oral candidiasis.

2. Results

2.1 Oral mucosal models differ in histology and expression of differentiation markers

Sections of paraffin-embedded RHOE, EpiOral™ or Full-thickness oral mucosa (FTOM) were compared with normal oral buccal mucosa for histological similarities. Hematoxylin and eosin staining showed that all three mucosal models have a similar thickness of 10-15 cell layers which is much less than normal buccal mucosa (Fig. 1A). Like the normal oral mucosa, both FTOM and EpiOral™ models displayed a fibroblast-containing connective tissue topped by basal cells followed by maturing spinous epithelium that parakeratinizes towards the apical surface. Noticeably absent from both the FTOM and EpiOral™ models but present in the normal mucosa are rete pegs, which are characteristic of papillary ridges, a vascular component and immune cells. In contrast to these models, the RHOE model is devoid of a fibroblast and connective tissue component and displays minimal differentiation and parakeratinization of the upper layers of the epithelium (Fig. 1A).

Immunohistochemical staining using the pan-cytokeratin markers MNF116 and AE1&AE3 showed that all the mucosal models consisted entirely of epithelium without contaminating cells (data not

shown). Staining for cytokeratin 13, a specific marker for cell differentiation, showed that the FTOM and EpiOral™ models contain well-differentiated cytokeratin 13-positive cells in the upper layers of the mucosa whilst the basal and supra-basal cells were negative; and this was comparable to the normal oral mucosa (Fig. 1B). In contrast, the RHOE model was cytokeratin 13-negative confirming that this model does not contain differentiated epithelium (Fig. 1B). Collagen IV staining was used to assess the presence of a basement membrane between the epithelium and the connective tissue as this plays a key role in anchoring the epithelium to the lamina propria. Similar to the normal oral mucosa, the FTOM model displayed a continuous basement membrane that separated the oral basal cells from the lamina propria. In the former, collagen IV staining of blood vessels was also observed (Fig. 1C). In marked contrast to the FTOM, both the EpiOral™ and RHOE did not display collagen IV staining (Fig. 1C).

The proliferation of basal cells that differentiate as they reach the apical surface is an important aspect of the developing oral mucosa. Ki67 staining was used to identify the location of actively proliferating cells. Both FTOM and EpiOral™ models displayed Ki67-positive cells that were restricted to the basal layers analogous to the normal oral mucosa (Fig. 1D). Although the RHOE models did display some Ki67-positve cells in the basal layers, many positive cells were also seen throughout the upper layers of the mucosal model (Fig. 1D). To confirm the immunohistochemical observations, the proliferation index of all three models along with the oral mucosa was also assessed. The FTOM and EpiOral™ models had a similar proliferation index that was not significantly different to that of the normal mucosa. However, the RHOE had a significantly ($p<0.01$) lower proliferation index to the FTOM, EpiOral™ and normal mucosa (Fig. 2).

2.2 C. albicans infection of oral mucosal models – histological examination

Mucosal models were infected with $5x10^6$CFU of a *C. albicans* wild-type strain (CAF2-1) for 24 h and, along with tissue obtained from a patient with oral candidiasis, were examined histologically.

C. albicans hyphae are clearly observed penetrating the upper epithelial layers of the oral mucosa in oral candidiasis. The epithelium also appears mildly dysplastic and the upper layers show signs of desquamation (Fig. 3A). In all three oral models, *C. albicans* formed a biofilm on top of the epithelium consisting of both yeast and hyphae. The biofilm on the RHOE was consistently not as compact as those formed on the FTOM and EpiOral™ models. Similar to the situation in oral candidiasis, hyphal penetration into the upper layers of the epithelium along with epithelial desquamation was observed in all three oral models, although this was less evident in the RHOE mucosa (Fig. 3B-D). All three models also displayed loss of intraepithelial attachments indicative of tissue damage which was confirmed by a time-dependent increase in lactate dehydrogenase (LDH) release over 24 h signifying a gradual loss of cell viability following infection (Fig. 4A).

2.3 Oral mucosal models differ in cytokine release upon infection with C. albicans

Release of the inflammatory cytokines TNF-α, IL-1β and CXCL8 was analysed to determine whether the mucosal models responded differently to *C. albicans* infection. All three uninfected models constitutively produced similar amounts of TNF-α, whereas uninfected FTOM and EpiOral™ models constitutively secreted significantly more IL-1β and CXCL8 ($p<0.05$) than the RHOE models (Fig 4B-C). Twenty-four hours following *C. albicans* infection, all three displayed at least a 5-fold increase in TNF-α secretion compared to uninfected controls. However, the FTOM model secreted significantly more TNF-α then the EpiOral™ and RHOE models (Fig. 4B; $p<0.05$). Infected FTOM and EpiOral™ models secreted significantly ($p<0.05$) more IL-1β and CXCL8 than uninfected controls and also produced 4-5 fold greater levels of both cytokines than those elicited by infected RHOE models. In fact, *C. albicans*-infection of RHOE models failed to stimulate an IL-1β response and the CXCL8 response was modest compared to that produced by the FTOM and EpiOral™ models.

2.4 Differential expression of human β-defensin 2 (hBD2) by mucosal models

Expression of hBD2, which has been previously shown to be up-regulated in *Candida*-infected oral epithelium [28,29], was examined in the mucosal models. All three models displayed little or no staining for hBD2 in uninfected controls (Fig. 5). However, following infection with *C. albicans*, the expression of hBD2 was markedly increased in the FTOM and EpiOral™ models, particularly in the more differentiated, upper epithelial layers where intense immunostaining was observed. In contrast, the expression of hBD2 in infected RHOE models was slight and focused in one or two small patches within the epithelium (Fig. 5). Furthermore, constitutive expression of hBD 1 and 3 was observed throughout the epithelium in the normal healthy oral mucosa and in the FTOM and EpiOral™ models but was absent from the RHOE models (data not shown).

3. Discussion

One limiting factor preventing a better understanding of the interaction between *C. albicans* and the oral mucosa is the availability of a suitable *in vitro* model. Monolayer cultures of oral keratinocytes have been extensively used to examine the interaction of *C. albicans* with host cells [21-25], but such studies provide limited information because the oral mucosa is a multi-layered complex tissue and signalling between cells may be important in host defence. Recently, the increased use of the RHOE multi-layered *in vitro* mucosal model has enabled more complex studies to be performed. However, there is limited data on whether this model is representative of the normal oral mucosa. In this study we compared three *in vitro* models of the oral mucosa (FTOM, EpiOral™ and RHOE) for histology, differentiation and proliferation status, and cellular response to *C. albicans* infection to determine which would be most representative of the normal oral mucosa. Both the FTOM and EpiOral™ models are comprised of normal oral keratinocytes cultured on a fibroblast-containing matrix. Fibroblasts are essential for supplying factors that aid in the proliferation and differentiation of keratinocytes into mature squamous epithelia [30]. Therefore, it is not surprising that the FTOM and EpiOral™ models displayed similar differentiation and proliferation characteristics to that of

the normal oral mucosa. In contrast, the RHOE models, which are generated from a buccal carcinoma cell line (TR146) that was originally isolated from a neck metastasis [31], formed undifferentiated, non-para-keratinized stratified epithelium in which cell proliferation was not restricted to the basal layers. The altered phenotype of these cells due to genetic changes associated with tumor transformation and the lack of fibroblast support are the likely explanations for the lack of differentiation in this model. Interestingly, we found that when TR146 cells were cultured on a fibroblast-containing collagen support, differentiation was markedly enhanced (unpublished observations), underscoring the importance of a fibroblast-containing connective component in oral models. We found the main difference between the FTOM and EpiOral™ models is the presence of a collagen IV-containing basement membrane which may facilitate enhanced anchorage of the FTOM basal cells to the connective tissue compared to EpiOral™ models.

To assess their usefulness at mimicking oral candidiasis, the mucosal models were infected with *C. albicans* and then analysed both histologically and for inflammatory cytokine release. *Candida* biofilm formation and hyphal penetration into the upper layers of the epithelium was observed for all three models replicating the situation in oral candidiasis. Epithelial cell damage in all models included desquamation of the upper epithelial layers and marked cellular damage, which was associated with a time-dependent increase in LDH release. Such findings are consistent with clinical observations of mucosal *Candidal* infections [32] and previous studies using mucosal models [33]. We also observed breakdown of epithelial cellular junctions which was associated with loss of E-cadherin expression in all three models as assessed by immunohistochemistry (unpublished observations), mirroring the observations made by Villar *et al* in their experiments using a mucosal model constructed of immortalised epithelial cells [34].

Although the histological and viability data were similar for all three models following *C. albicans* infection, the secretion of inflammatory cytokines was markedly different. Previous studies have

shown increased expression of numerous cytokines in response to *C. albicans* infection *in vivo* and *in vitro* and these are essential for directing an appropriate immune response (reviewed by [35]). FTOM and EpiOral™ models were more responsive to infection and secreted significantly more inflammatory cytokines than the RHOE models. In fact, the RHOE models displayed no increase in IL-1β production following infection while CXCL8 secretion was only slightly elevated. Moreover, unstimulated FTOM and EpiOral™ models constitutively secreted significantly more CXCL8 than the RHOE models. Such differences have been observed previously in monolayer cultures [36]. Major differences in anti-microbial peptide expression were also observed between the oral mucosal models. Specifically, the FTOM and EpiOral™ models constitutively expressed hBD 1 & 3 in a manner similar to normal oral mucosa [29], whereas the RHOE model lacked expression of these proteins. Furthermore, when infected with *C. albicans*, hBD2 was markedly up-regulated in the FTOM and EpiOral™ models in a similar manner to that described for the normal oral mucosa [28,29], whereas the RHOE model showed only very minor increases in expression. The disparities in the expression of these immune molecules between models are likely to be due to genetic differences between normal and tumor cells where gene expression of cytokines, pattern recognition receptors, anti-microbial peptides and cell signaling molecules are often dysregulated [37,38].

Taken together, our data show that compared to RHOE models, the FTOM and EpiOral™ models are histologically much closer to and express the same pattern of innate immune molecules as normal oral mucosa. These data suggest that FTOM and EpiOral™ models represent a more advanced model of the oral mucosa that should significantly aid in dissecting the molecular mechanisms involved in the innate immune response against *C. albicans*. Currently, the main limitation of these models is that they currently do not include immune cells such as neutrophils, Langerhans cells or T lymphocytes that are important in the host response to *Candida* infections [39,40]. However, as with the RHOE models [25], the introduction of these immune cells into the mucosal models is possible and the likelihood of generating more complex tissue engineered oral

mucosal models containing several cell types is imminent.

4. Materials and methods

4.1 Isolation and culture of normal oral keratinocytes and fibroblasts

Normal oral keratinocytes and oral fibroblasts were isolated from biopsies of normal buccal mucosa removed from healthy individuals with written, informed consent (ethical approval 09/H1308/66) as previously described [27]. To isolate keratinocytes, oral biopsies were incubated with 0.1% w/v Difco-Trypsin (Difco Laboratories, Detroit, MI) overnight at 4°C, followed by mechanical removal of the epidermal layer from the dermis. Keratinocytes were collected by centrifugation and cultured with a feeder layer of irradiated 3T3 murine fibroblasts in keratinocyte culture media (DMEM and Ham's F12 medium in a 3:1 ratio, supplemented with 10% FBS, 10ng/ml epidermal growth factor (EGF), 0.4μg/ml hydrocortisone, $1.8x10^{4}$ mol/l adenine, 5μg/ml insulin, 1.7ng/ml cholera toxin, $2x10^{-7}$ M tri-iodothyronine, 0.625 μg/ml amphotericin B, 100 IU/ml penicillin and 100μg/ml streptomycin (all from Sigma, Poole, UK). Oral fibroblasts were isolated from the dermal tissue following overnight treatment with collagenase A (Sigma, Poole, UK) at 37°C in a 5% CO_2 humidified incubator. Fibroblasts were collected by centrifugation and cells cultured in DMEM supplemented with 10% FBS, 100 IU/ml penicillin and 100μg/ml streptomycin at 37°C, 5% CO_2.

4.2 Oral mucosal models

RHOE models based upon TR146 cells were purchased from SkinEthic Laboratories (Nice, France) and maintained as described by the manufacturer's instructions. EpiOral™ models based on primary buccal keratinocytes cultured on a fibroblast-containing collagen-based matrix were purchased from MatTek Corporation (Ashland, MA) and maintained as described by the manufacturer. Full-thickness oral mucosal (FTOM) models were generated as previously described [27]. Briefly, dermis was prepared from glycerol-preserved skin from human cadavers (Euroskin

Bank, Beverwijk, Netherlands). The skin was washed several times with PBS and decellularised by incubation with 1M NaCl overnight at 37°C. The epidermis was removed from the dermal layer, which was then washed extensively in PBS, cut into 10mm diameter circular pieces and placed basement membrane uppermost into a 10mm, 0.4μm pore polycarbonate snapwell tissue culture insert (Corning, New York, NY). Five x 10^5 fibroblasts (between passages 2-6) and $5x10^5$ oral keratinocytes (between passages 2-4) were seeded onto the dermis in 200μl of keratinocyte medium. The lower compartment of the snapwell insert was filled with 2ml of keratinocyte medium and the composites incubated at 37°C in a 5% CO_2 humidified incubator. Seventy-two hours later composites were taken to an air liquid interface by aspirating the medium from the centre of the dermis-containing insert. Mucosal models were maintained at 37°C for 14 days at an air liquid interface with fresh medium added every 2 days.

4.3 Infection of mucosal models with *C. albicans*

C. albicans (wild-type strain CAF2-1) was cultured in yeast-peptone-dextrose (YPD) medium overnight at 25°C. Yeast cells were pelleted by centrifugation, washed twice with PBS and resuspended in PBS at $5x10^7$CFU/ml. FTOM, RHOE and EpiOral™ models were infected with 100μl (5 x 10^6CFU) CAF2-1 for up to 24 h at 37°C at 5% CO_2; PBS alone was added to the uninfected control. Prior to infection, 1.5 ml fresh keratinocyte medium was placed in the lower chamber of the snapwell. After 24 h the medium from the lower chamber was collected for further analysis and the models washed twice in PBS, fixed in 10% PBS-buffered formalin and then embedded in paraffin.

4.4 Cytokine production and cell damage analysis

Release of the cytokines TNF-α, IL-1β and CXCL8 was quantified by ELISA (OptiEIA, BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Cell damage caused by *C. albicans* was assessed by measuring release of cellular lactate dehydrogenase (LDH) into the culture

medium using the CytoTox96 enzyme assay kit (Promega, Madison, WI).

4.5 Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed paraffin-embedded mucosal models and oral biopsies from patients with oral candidiasis (with written, informed consent and ethical approval 07/H1309/105). Tissue sections (4 μm) were de-waxed with xylene, rehydrated through a series of alcohols and immersed in 3% H_2O_2 in ethanol to quench endogenous peroxidase activity. Antigen retrieval was performed by microwaving for 8 min 10 mM citrate buffer (pH 6.0). Sections were blocked with normal rabbit serum for 30 min at room temperature and then incubated with primary antibody (Ki67, cytokeratin 13 (Dako, Copenhagen, Denmark), Collagen IV (Sigma, Poole, UK), human β defensin-2 (Peprotech, London, UK) for 60 min at room temperature, followed by incubation with biotinylated secondary antibody and streptavidin-HRP for 30 min at room temperature (Vectastain Elite ABC kit, Vector Laboratories, Peterborough, UK). Staining was visualised using 3,3'-diaminobenzidine chromagen substrate followed by counterstaining with haematoxylin. Sections were mounted and analysed by light microscopy. Control sections were prepared in the same manner but incubated with isotype-matched murine IgG antibody. Proliferation index was calculated as a percentage of Ki67-positive stained cells within the basal and supra-basal cell layers within five randomly selected fields of view (x40).

4.6 Periodic Acid Schiff (PAS) staining of *C. albicans*-infected composites

Tissue sections (4 μm) were cut, de-waxed with xylene, rehydrated through a series of alcohol solutions, treated with 1% periodic acid (BDH, Leicestershire, UK) for 5 min, washed with distilled water and then incubated in Schiff's reagent (BDH, Leicestershire, UK) for 15 min. After washing under running tap water for 10 min, sections were counterstained with Mayer's haematoxylin, passed through Scott's tap water, de-hydrated, mounted and analysed by light microscopy.

4.7 Statistical analysis

To determine significances between groups, comparisons were made using Mann-Whitney U or ANOVA using GraphPad Prism (San Diego, CA). For all statistical tests a p-value of at least <0.05 was considered statistically significant.

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Figure Legends

Figure 1. Differences and similarities in differentiation, keratinization, basement membrane composition and proliferation of three oral mucosal models compared to normal oral mucosa. Hematoxylin and eosin stained sections (A). Sections stained by immunohistochemistry for cytokeratin 13 (B), collagen IV (C) and the proliferation marker ki67 (D). Each panel consists of (i) normal healthy oral mucosa, (ii) FTOM, (iii) EpiOral™ and (iv) RHOE. Images are representative of models from three individual experiments each performed in triplicate. Scale bar = 100μM.

Figure 2. The proliferation index of FTOM and EpiOral™ mucosal models are similar to the normal oral mucosa. Sections of FTOM, EpiOral™ and the RHOE mucosal models and normal oral mucosa were stained for Ki67 and the number of Ki67-positive cells within the basal and supra-basal layers calculated as an overall percentage of proliferative cells. Data shown represent mean ± SEM of three independent experiments each performed in triplicate with 5 randomly selected fields of view (x40) analysed per group. *$p<0.01$ determined by ANOVA compared to the RHOE model.

Figure 3. Penetration of hyphal *C. albicans* into the epithelium of oral mucosal models is similar to oral candidiasis *in vivo*. Representative images of PAS stained sections from (A) oral candidiasis *in vivo*, (B) FTOM, (C) EpiOral™ and (D) RHOE show hyphal penetration into the upper layers of the epithelium in all three models which is analogous to oral candidiasis *in vivo*. All three models have a Candida biofilm and tissue damage due to loss of inter-epithelial contacts is observed. Images are representative of models from three individual experiments each performed in triplicate Scale bar = 100μM, except panel 3B where scale bar = 50μM.

Figure 4. *C. albicans* induces cell damage and differential cytokine release in oral mucosal models. FTOM, EpiOral™ and RHOE mucosal models were infected with 5 x 10^6CFU *C. albicans* for up to 24 h and LDH and cytokine release (at 24 h) measured. (A) *C. albicans* caused a time-dependent increase in cell damage as determined by LDH release in all three mucosal models. *In vitro* oral mucosal models elicited different levels of TNF-α (B), IL-1β (C) and CXCL8 (D) release upon *C. albicans* infection. Data are mean ± SEM of three independent experiments each performed in triplicate. *$p<0.05$ determined by Mann Whitney U test compared to *C. albicans*-infected RHOE. ^$p<0.05$ determined by Mann Whitney U test compared to uninfected RHOE.

Figure 5. Human β-defensin 2 (HBD2) is upregulated in FTOM and EpiOral™ but not in RHOE upon *C. albicans* infection. Sections from uninfected and *C. albicans*-infected (5 x 10^6CFU) FTOM, EpiOral™ and RHOE oral mucosal models were stained for hBD2 expression by immunohistochemistry. hBD2 expression (brown staining) was markedly increased in the FTOM and EpiOral™ models but not the RHOE models. Images are representative of models from three experiments each performed in triplicate. Scale bar = 100μM.

Fig 1

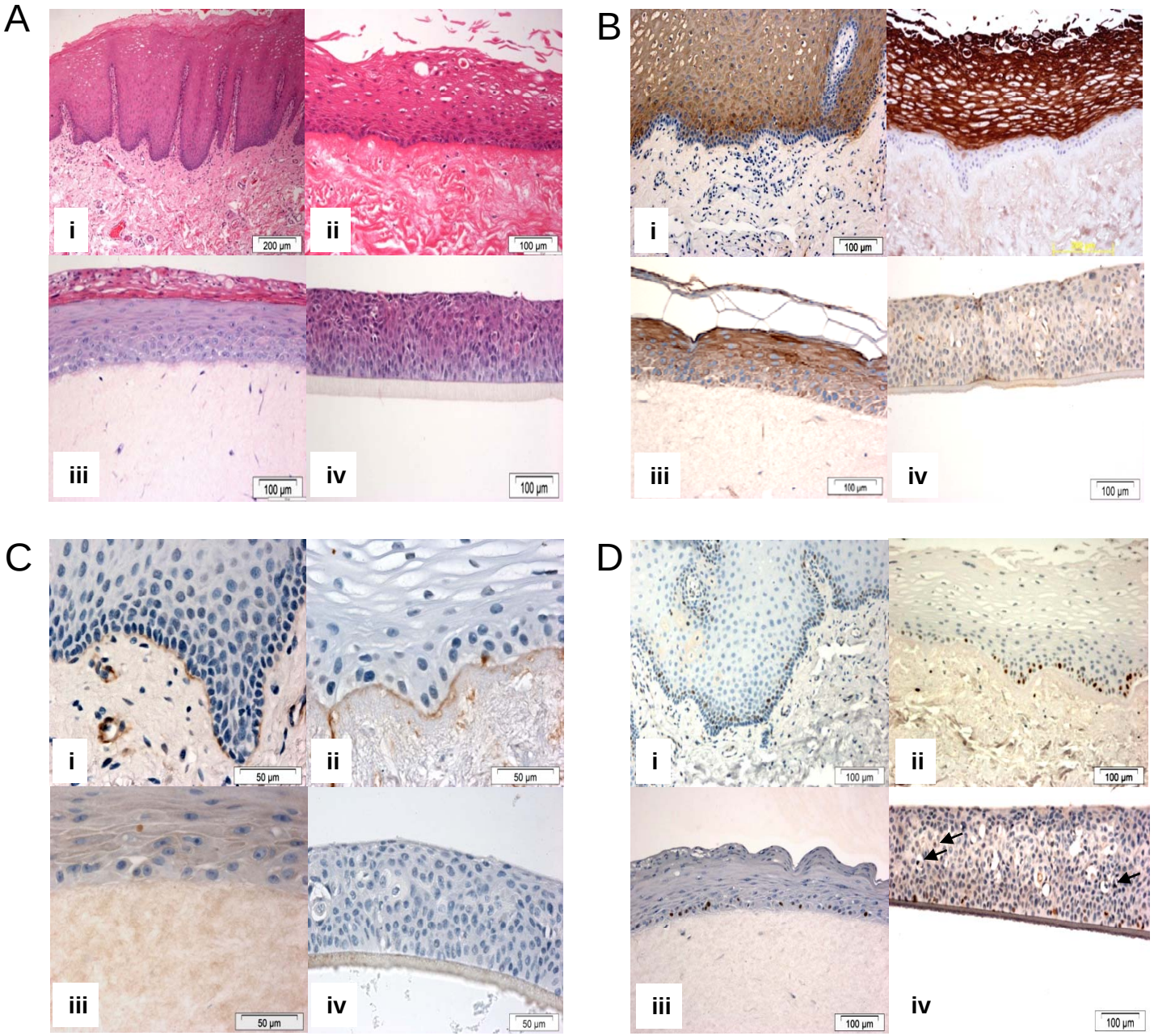
A
i
200 µm
ii
100 µm
iii
100 µm
iv
100 µm
B
i
100 µm
iii
100 µm
iv
100 µm
C
i
50 µm
ii
50 µm
iii
50 µm
iv
50 µm
D
i
100 µm
ii
100 µm
iii
100 µm
iv
100 µm

Fig 2

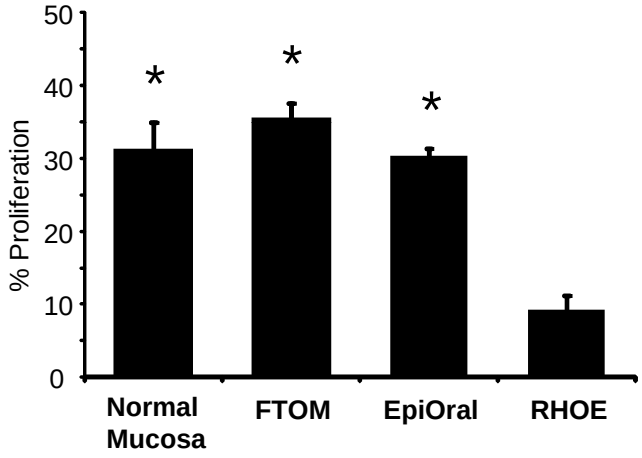
50
40
30
20
10
0
% Proliferation
*
*
*
Normal Mucosa
FTOM
EpiOral
RHOE

Fig 3

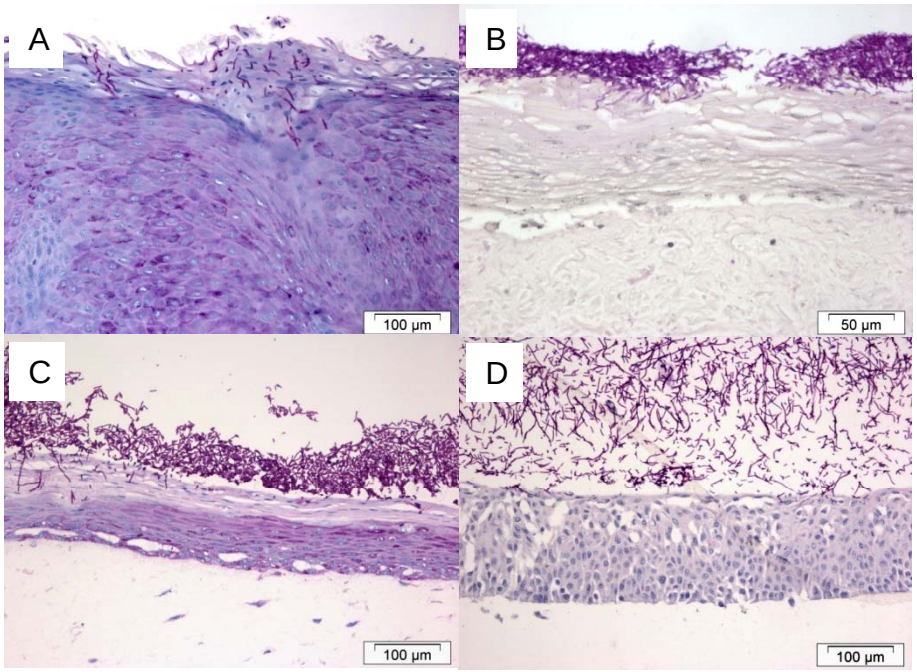

Fig 4

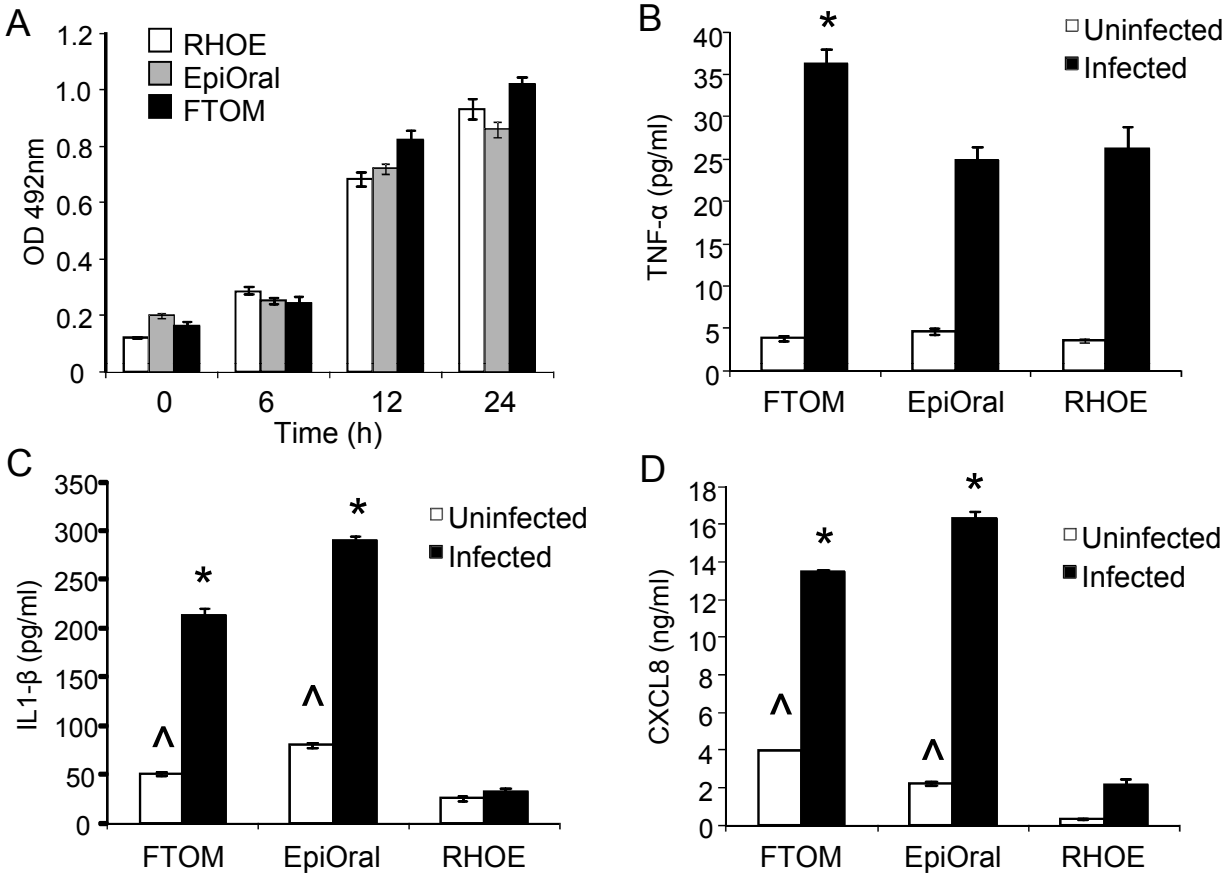
A
RHOE
EpiOral
FTOM
OD 492nm
1.2
1.0
0.8
0.6
0.4
0.2
0
0
6
12
24
Time (h)
B
Uninfected
Infected
TNF-α (pg/ml)
40
35
30
25
20
15
10
5
0
*
FTOM
EpiOral
RHOE
C
Uninfected
Infected
IL1-β (pg/ml)
350
300
250
200
150
100
50
0
*
*
^
^
FTOM
EpiOral
RHOE
D
Uninfected
Infected
CXCL8 (ng/ml)
18
16
14
12
10
8
6
4
2
0
*
*
^
^
FTOM
EpiOral
RHOE

Fig 5