Published in final edited form as:

Mol Oral Microbiol. 2018 June ; 33(3): 212–223. doi:10.1111/omi.12214.

Chemotherapy-induced oral mucositis and associated infections in a novel organotypic model

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Summary

Oral mucositis is a common side effect of cancer chemotherapy, with significant adverse impact on the delivery of anti-neoplastic treatment. There is lack of consensus regarding the role of oral commensal microorganisms in the initiation or progression of mucositis since relevant experimental models are non-existent. The goal of this study was to develop an in vitro mucosal injury model that mimics chemotherapy-induced mucositis, where the effect of oral commensals can be studied. A novel organotypic model of chemotherapy-induced mucositis was developed based on a human oral epithelial cell line and a fibroblast-embedded collagen matrix. Treatment of organotypic constructs with 5-fluorouracil (5-FU) reproduced major histopathologic characteristics of oral mucositis, such as DNA synthesis inhibition, apoptosis and cytoplasmic vacuolation, without compromising the three-dimensional structure of the multilayer organotypic mucosa. Although structural integrity of the model was preserved, 5-FU treatment resulted in a widening of epithelial intercellular spaces characterized by E-cadherin dissolution from adherens junctions. In a neutrophil transmigration assay we discovered that this treatment facilitated transport of neutrophils through epithelial layers. Moreover, 5-FU treatment stimulated key proinflammatory cytokines that are associated with the pathogenesis of oral mucositis. 5-FU treatment of mucosal constructs did not significantly affect fungal or bacterial biofilm growth under conditions tested in this study, however it exacerbated the inflammatory response to certain bacterial and fungal commensals. These findings suggest that commensals may play a role in pathogenesis of oral mucositis by amplifying the proinflammatory signals to mucosa that is injured by cytotoxic chemotherapy.

Keywords

chemotherapy; oral mucositis; *Candida*; *Streptococcus*; cytokine; adherens junction

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Disclosure statement: Authors have no conflicts of interest to disclose

Introduction

An estimated 1.6 million patients are diagnosed with cancer in the US each year. Alimentary tract mucositis (oral and gastrointestinal) occurs in approximately 20%–40% of patients receiving standard dose cancer chemotherapy regimens for solid tumors, depending on the dose and cytotoxicity of the drug.^{1,2} In patients receiving high dose conditioning chemotherapy prior to a hematopoietic stem cell transplant, oral mucositis prevalence rises to approximately 80%.³ Mucosal ulceration can involve the oropharynx, esophagus and/or gastrointestinal tract and can compromise nutrition and speech, prolong hospital stays, predispose patients to bloodstream infections, and cause delays or dose reductions in the delivery of antineoplastic treatment.⁴ In certain cases, pain associated with oral mucositis can be of such severity that patients are not able to comply with routine daily oral care.^{5,6}

5-fluorouracil (5-FU) is a chemotherapeutic agent commonly used for treatment of a variety of metastatic neoplasms including breast, gastrointestinal tract and head & neck. 5-FU acts primarily as a thymidylate synthase inhibitor, resulting in interruption of DNA synthesis that leads to apoptotic cell death.⁷ 5-FU causes alimentary tract mucositis which is characterized by a pronounced epithelial proinflammatory cytokine response and mucosal neutrophil transmigration, while the number of peripheral blood neutrophils gradually declines due to myelosuppression.^{8,9}

It has been proposed that such chemotherapy agents disrupt the oral mucosal barrier thereby promoting local invasion and systemic dissemination of commensal microorganisms; however to our knowledge this has not been experimentally tested. Indeed, up to 50% of cases of septicemia in cancer chemotherapy patients are associated with oral microorganisms.¹⁰ Oral viridans streptococci are responsible for up to 39% of bacteremia cases in high-dose chemotherapy-treated patients, with two streptococcal species of the *mitis* group (*S. oralis* and *S. mitis*) most frequently isolated from blood cultures.^{10,11} In this patient population oral thrush and/or candidemia with disseminated multi-organ infection are also prevalent and most frequently attributed to *Candida albicans* and *Candida glabrata*, two core components of the human oral mycobiota.^{12–14} A recent report identified high levels of phospholipase production by *C. albicans* oral isolates from chemotherapy-treated cancer patients, and it was proposed that this virulence factor may elevate the risk of oral and/or systemic infection.¹⁵ In addition, although this remains controversial, commensal microorganisms have been proposed to contribute to the initiation or persistence of mucositis.^{16,17} In a gastrointestinal mucositis germ-free mouse model it was shown that the indigenous microbiota are required for chemotherapy-induced mucosal damage.¹⁷ However, the role of commensal organisms has never been examined in animal or *in vitro* models of oral mucositis.

Oral mucosal injury caused by high dose cancer therapies can thus result in substantial adverse impact on the clinical course of oncology patients as well as on health resource utilization.^{4,18} Despite this impact the mechanisms involved in the pathobiology of oropharyngeal mucositis are poorly understood. New research models of chemotherapy-induced mucositis in which the role of microorganisms in pathogenesis can be fully characterized are currently needed. In this context, the aims of this study were to: a) develop

an organotypic model that recapitulates the mucosal histopathologic changes that occur in oral mucositis; b) test the hypothesis that mucositis promotes biofilm formation by opportunistic fungal pathogens or commensal *mitis* group streptococci; and c) test the hypothesis that 5-FU treatment modifies the mucosal inflammatory responses to these organisms.

Materials and Methods

Organotypic constructs

The oral mucosal tissue analog has been described in detail elsewhere.¹⁹ Briefly, organotypic cultures are grown on transwell inserts that allow limited diffusion of culture media from the bottom of the well. An airlifting growth phase promotes epithelial differentiation and stratification. In this study cultures consisted of a human oral keratinocyte cell line (SCC15 cells, ATCC), seeded at 5×10^5 cells per well, over a collagen type I matrix, embedded with fibroblasts (3T3 cell line, ATCC). This cell line was originally derived from a squamous cell carcinoma of the tongue. Using SCC15 cells, mucosal constructs take approximately 2 weeks to form, resulting in a non-keratinizing, multilayer squamous epithelium.

5-FU treatment of organotypic constructs

In preliminary experiments the effect of 5-FU dose and duration was tested in cell proliferation assays using monolayer SCC15 cultures. Briefly cells were seeded at 2.5×10^4 cells/well in 96 well polystyrene plates in complete Keratinocyte Serum Free Media (KSFM®, Thermo Fisher scientific, Waltham, MA) and incubated at 37°C, in a 5% CO₂ incubator for 24 hrs. Fresh KSFM® supplemented with 1, 10 or 100 µM 5-FU (Sigma-Aldrich, St. Louis, MO) was added and cells were further incubated for 24–72h before assessing active DNA synthesis (see below), as a marker for proliferation. In some experiments, after exposure to increasing concentrations of 5-FU for 24h, cells were washed in PBS and fresh media were added to assess cell recovery. Recovery was assessed by comparing epithelial cell BrdU incorporation at 2h (baseline), 24h or 48h after removal of the chemotherapy agent.

Organotypic constructs were exposed to 5-FU (1 or 10 µM) for 16 hours. 5-FU solution was prepared in media consisting of Dulbecco's Modified Eagle's medium, supplemented with L-glutamine, hydrocortisone, insulin-transferrin ethanolamine-selenium, O-phosphorylethanolamine, adenine and triiodothyronine.²⁰ 5-FU-supplemented media were added to tissue inserts basolaterally only, to simulate drug delivery through the vasculature in the submucosal compartment.

Evaluation of epithelial cell proliferation, apoptosis and cytotoxicity

In preliminary experiments the effect of 5-FU on cell proliferation was assessed in monolayer SCC15 cultures by labeling cells during the last 2h of 5-FU exposure with 5-Bromo-2'-deoxyuridine (BrdU), a synthetic analog of thymidine. BrdU incorporation was measured using a colorimetric immunoassay kit, according to manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). To assess DNA synthesis in organotypic construct cell

layers, 10 μ M BrdU was added in the media for 16 hours. Tissues were fixed in 4% paraformaldehyde, paraffin-embedded and 5 μ m sections were stained with an anti-BrdU monoclonal antibody (1:40 dilution, Ab6326, Abcam, Cambridge, MA), followed by an Alexa Fluor 555 labelled secondary antibody (1:200 dilution, A21434, Thermo Fisher scientific, Waltham, MA), and counter-stained with nucleic acid stain Hoechst 33258 (Invitrogen, Carlsbad, CA) to visualize cell nuclei. Early stage cell apoptosis was evaluated by immunofluorescence staining for active caspase-3, an early apoptosis marker, as previously described.²¹ Briefly, tissue sections were subjected to heat-mediated antigen retrieval (10 mM citric acid and 0.05% Tween 20, pH 6.0), followed by blocking with 5% milk. Sections were subsequently incubated with a rabbit polyclonal antibody to active caspase-3 (1.5 h, 1:100 dilution, Abcam, Cambridge, MA) and a goat anti-rabbit secondary antibody conjugated with Alexa-Fluor 555 (1h, 1:500 dilution, Abcam). Late stage cell apoptosis was also evaluated with the DeadEnd™ Colorimetric TUNEL® System, according to manufacturer's instructions (Promega, Madison, WI). The cytotoxicity of 5-FU treatment in oral mucosal constructs was assessed in tissue-conditioned media by the CytoTox-96® assay (Promega, Madison, WI), which measures the release of lactate dehydrogenase (LDH) from dying cells. LDH released was quantified spectrophotometrically, as described previously.²² Spontaneous release of LDH by untreated mucosal constructs was included as a negative control in each experiment.

Evaluation of E-cadherin in epithelial junctions

To evaluate the integrity of the mucosal barrier after exposure of organotypic constructs to 5-FU, E-cadherin was assessed by immunofluorescence staining, as described previously.⁸ Briefly, paraffin-embedded tissue sections were stained using an anti-E-Cadherin polyclonal antibody (BD Biosciences, San Jose, CA) followed by a FITC-conjugated secondary antibody (DyLight® 488, Vector Laboratories, Burlingame, CA), and counter-stained with the nucleic acid stain Hoechst 33258 (Invitrogen, Carlsbad, CA). Soluble E-cadherin fragments released from epithelial junctions were quantified in tissue-conditioned media using an enzyme-linked immunosorbent assay and following the manufacturer's instructions (R&D Systems, Minneapolis, MN).

Evaluation of leukocyte trans-epithelial migration in mucosal constructs

To test whether 5-FU treatment facilitates neutrophil transmigration through epithelial layers we used HL-60 cells (ATCC) driven to granulocyte differentiation by DMSO treatment (1.25% DMSO in RPMI media) for 5 days, as we described previously.²³ After treatment with 5-FU (10 μ M) for 16h, tissues were washed three times and KSFM media supplemented with 50 ng/ml fMLP (Sigma-Aldrich, St. Louis, MO) as a chemoattractant were added below the culture inserts.²⁴ HL-60 cells were added apically to each 5-FU-treated mucosal construct (3×10^6 cells/construct in 20 μ l of RPMI) and were allowed to migrate through the epithelial layers for 6h. To visualize cells that had migrated through epithelial layers a portion of each tissue was fixed in 4% PFH and paraffin-embedded sections were stained for myeloperoxidase (MPO), as fully differentiated HL-60 cells express abundant amounts of this granulocytic enzyme.²⁵ MPO⁺ cells were detected using an anti-MPO monoclonal antibody (10 μ g/mL for 3 hours, R&D Systems, Minneapolis, MN), followed by NorthernLights™ 557-conjugated secondary antibody (R&D Systems,

Minneapolis, MN) and counterstained with Hoechst 33258. To confirm microscopic findings, MPO activity levels were also measured in the remaining portion of each tissue, using a colorimetric assay. Prior to enzymatic assay tissues were washed in PBS twice, weighed and stored in -80°C . To quantify MPO activity each tissue was placed in an Eppendorf tube with 200 μl of 50 mM potassium phosphate buffer containing 5 mg/ml hexadecyltrimethylammonium bromide (pH 6.0), sonicated for 5 min on ice and centrifuged at 10,000 rpm for 10 min. Supernatants (10 μl) were transferred into 96 well plates and tested for MPO activity with a colorimetric assay using freshly prepared o-dianisidine dihydrochloride and H_2O_2 as a substrate.²⁵ MPO activity was expressed in units (U) of MPO/mg tissue, where one unit of MPO is defined as the amount needed to degrade 1 μmol of H_2O_2 per minute at room temperature.

Microorganisms and infection

C. albicans strain SN425 (reference strain, derived from SC5314) has a normal hyphal phenotype and was provided by Dr. Clarissa J. Nobile (University of California, Merced). *C. glabrata* strain GDH2269 is a human oral commensal isolate obtained from ATCC. *Candida* strains were maintained in YPD agar (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) (Fisher Scientific, Pittsburgh, PA) and grown overnight in YPD broth, aerobically, at room temperature, on a shaker prior to experimentation. Because of the well-known anti-fungal activity of 5-FU, in preliminary experiments we tested the effect of 5-FU on fungal biofilms seeded in 96 well polystyrene plates (10^4 fungal cells/well). In these experiments biofilms were grown in RPMI-10% FBS or artificial saliva media²⁶ supplemented with increasing concentrations of 5-FU (0.08–8 μM) for 5h and fungal metabolic activity was measured using the XTT assay, as previously described.²³

S. oralis 34 (kindly provided by Dr. P. Kolenbrander) and *S. mitis* 49456 (ATCC) were grown in Brain Heart Infusion (BHI) medium (Oxoid, Thermo Scientific, Waltham, MA), under aerobic static conditions at 37°C , in a 5% CO_2 incubator, one day before each experiment. Prior to each experiment overnight stationary phase cultures of streptococci were inoculated in fresh BHI broth and then allowed to reach the late logarithmic phase for 3–4 h (optical density at 600 nm of 1.0, corresponding to 10^7 cells/ml). Constructs were switched to culture media without antibiotics for 16–24h prior to microbial inoculation. Each mucosal construct was inoculated with 20 μl media containing 10^6 fungal (*C. albicans* or *C. glabrata*) or 10^7 bacterial (*S. oralis*, or *S. mitis*) cells, and incubated at 37°C , in a 5% CO_2 incubator for 6–16 hours. 5-FU was washed out immediately prior to inoculation by transferring tissue inserts in wells containing fresh media three times, each for 1h.

Mucosal biofilm formation was assessed in 5 μm thick, paraformaldehyde-fixed, paraffin-embedded tissue sections as we previously described.^{20,21,27} Briefly sections were stained by immunofluorescence for *C. albicans* or *C. glabrata* with a FITC-labeled anti-Candida polyclonal antibody (Meridian Life Science, Saco, ME). For *S. oralis* and *S. mitis* biofilms, fluorescence *in situ* hybridization (FISH) with the Streptococcus-specific oligonucleotide probe STR405 was used as described previously.²¹ Tissues infected with *C. albicans* were counterstained for E-cadherin, since we have previously shown E-cadherin dissolution to be associated with greater mucosal invasion of this organism.^{28,29} In these tissues E-cadherin

was visualized using Alexa 568 conjugated secondary antibody (1:100 dilution, A11031, Invitrogen, Carlsbad, CA). Biofilms were quantified on each mucosal construct using Image J to measure the area occupied by microorganisms both above and below the epithelial layer in 4–8 microscopic fields per tissue sample. Images were obtained using a Zeiss Axio Imager M1 microscope and an EC Plan-Neofluar 20× NA 0.5 air objective and further analyzed using the AXIOVISION LE64 software.

Assessment of cytokines

Cytokine production was assessed in organotypic culture conditioned media after 16h of 5-FU exposure. In infection experiments, cytokines were quantified after 16h of biofilm growth on tissues that had been exposed to 5-FU (10 μ M) for 16h prior to microbial inoculation, as described above. Tissue-conditioned media from 5-FU-untreated cultures inoculated with microorganisms for 16h served as controls in these experiments. Prior to cytokine assays, total protein concentration was quantified in each sample using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). Samples were diluted as needed in order to standardize the amount of protein analyzed for cytokine content, since the total epithelial cell numbers corresponding to each sample varies due to the fact that each organotypic culture has a slightly variable number of epithelial cell layers. Granulocyte-macrophage colony-stimulating factor (GM-CSF), Interleukin 1 beta (IL-1 β), IL-6, IL-8, IL-10, and Tumor Necrosis Factor-alpha (TNF α) were simultaneously quantified in each sample using the Luminex/MAGPIX system (HSTCMAG-28SK; Millipore, Billerica, MA).

Statistical Analyses

Data were analyzed using one-way ANOVA or the Kruskal-Wallis test, when data did not pass the normality test. Analyses were performed using the Graph-Pad Prism[®] software. Statistical significance for all tests was set at $P < 0.05$.

Results

5-FU inhibits epithelial cell proliferation in a time- and dose-dependent manner

We first used monolayer epithelial cultures to establish a dose range and duration of 5-FU treatment that inhibit cell proliferation without completely compromising cell culture viability (Fig. 1A, Supplemental). Cell proliferation, as reflected by BrdU incorporation levels, decreased with increasing exposure time and dose. A significant decrease in BrdU incorporation was noted after 24h with the higher concentrations tested (10 μ M and 100 μ M) and with longer exposure times. In a subsequent series of experiments we determined whether some cells would continue to incorporate BrdU for 24h or 48h after 5-FU was removed from culture media (Fig. 1B, supplemental). BrdU incorporation in cultures treated with 1 μ M 5-FU returned to untreated levels within 24h–48h, suggesting no significant long-term effect on DNA synthesis. Cultures treated with 10 μ M 5-FU retained their lower BrdU incorporation levels after removal of 5-FU, whereas cultures treated with the highest concentration of 5-FU (100 μ M) showed almost complete absence of incorporation 24h–48h after 5-FU removal, suggesting extensive cell damage. Thus this concentration was deemed too toxic for further use in the mucosal constructs.

5-FU-treated organotypic constructs exhibit histopathologic characteristics of oral mucositis

We next examined the effect of 5-FU treatment in organotypic oral mucosal constructs. Based on our preliminary observations above, lower concentrations of 5-FU (1–10 μM) were used with the intent to minimize toxicity and preserve the three-dimensional organization and structure of tissue constructs after treatment. A histologic examination of 5-FU-treated constructs using an H&E stain revealed no significant structural alterations on the multi-layer epithelium (Fig. 1A). However, at the higher dose tested (10 μM), more widening of epithelial intercellular spaces and cytoplasmic vacuolation were noted, consistent with histopathologic characteristics of oral mucositis.³⁰ These changes were accompanied by a statistically significant increase in LDH release compared to tissues treated with 1 μM 5-FU, which triggered similar LDH release levels with untreated tissues (Fig. 1B).

To reveal whether 5-FU exposure inhibited cell proliferation of epithelium in organotypic constructs, we performed BrdU incorporation assays following 5-FU treatment and stained tissue sections for intraepithelial BrdU. As seen in Figure 2A, untreated organotypic cultures contained multiple BrdU-positive cells (cells in S phase) in all epithelial layers, consistent with the squamous carcinoma phenotype of SCC15 cells. The number of BrdU positive cells diminished significantly in tissues treated with 1 μM 5-FU whereas there was an almost complete absence of BrdU staining in tissues treated with 10 μM 5-FU, indicating lack of DNA synthesis (Fig. 2A,C). To illustrate the effect of 5-FU exposure on mucosal cell apoptosis, we stained tissue sections for active caspase-3, an early stage apoptosis marker. Immunofluorescence staining for caspase-3 revealed a dose-dependent significant increase in the number of apoptotic cells in 5-FU-treated constructs, whereas minimal epithelial cell apoptosis was observed in untreated tissues (Fig. 2B,D). Active caspase 3-positive cells were seen in all epithelial layers, indicating that most cells were undergoing early apoptotic changes, especially at the higher 5-FU dose. The TUNEL[®] stain showed dose-dependent increase in late-stage apoptotic cells in tissues treated with 1 μM and 10 μM 5-FU, however TUNEL[®]-positive cells were seen mostly in the basal and parabasal layers (Fig. 2, supplemental). Since these layers were closer to the basally supplied media, it is reasonable to assume they were exposed to the drug earlier thus undergoing late stage apoptotic changes. Collectively these results showed that treatment of organotypic constructs for 16h with lower concentrations of 5-FU reproduced the major histopathologic characteristics of oral mucositis *in vivo*.^{8,10}

5-FU treatment decreases E-cadherin from adherens junctions and facilitates neutrophil trans-epithelial transport

Prompted by the observed increase in intercellular spaces in 5-FU-treated tissue constructs (Fig. 1A), we next examined the integrity of adherens junctions in the mucosal cell layers by assessing expression of E-cadherin. Immunofluorescence staining revealed a reduction of the E-cadherin fluorescence signal in tissues treated with 10 μM 5-FU (Fig. 3A). This finding was consistent with the significant increase in soluble E-cadherin protein fragments in conditioned media of tissues treated with 10 μM 5-FU (Fig. 3B).

E-cadherin dissolution from adherens junctions has been shown to facilitate localized neutrophil transmigration in mucosal epithelia³¹, we thus hypothesized that 5-FU treatment would facilitate neutrophil movement through epithelial layers in mucosal constructs. Indeed immunofluorescence staining in mucosal constructs treated with 10 μ M 5-FU showed a greater number of MPO⁺ HL-60 cells migrating to the basal mucosal layers, compared to untreated constructs (Fig. 4AB). In agreement with the histologic findings, MPO activity associated with 5-FU-treated tissues was significantly higher compared to untreated tissues (Fig. 4B). These results suggested that the mucosal neutrophilic response associated with oral mucositis *in vivo* may be facilitated by the negative effect of chemotherapy on epithelial E-cadherin expression.

5-FU treatment does not significantly alter mucosal biofilm growth

To test the hypothesis that mucosal damage inflicted by 5-FU promotes microbial growth, we exposed tissues to 5-FU for 16h and then inoculated them with microorganisms for an additional 16h incubation period. Preliminary experiments showed that 5-FU concentrations ranging between 0.08–8 μ M inhibited fungal biofilm metabolic activity (Fig. 3, supplemental), thus prior to microbial inoculation 5-FU was removed from tissue constructs and fresh culture media were added basolaterally, as described in methods. The biofilm surface area on 5-FU-treated mucosal constructs formed by *C. glabrata*, *S. oralis* and *S. mitis* increased by approximately two-fold compared to untreated constructs, however, with the exception of *S. oralis*, this increase was not statistically significant (Fig. 5A). In some 5-FU-treated tissues infected with *C. albicans*, areas of localized mucosal fungal invasion, corresponding to reduced E-cadherin signal, were observed in 6h biofilms (arrows, Fig. 5B). However, after 16h of growth, extensive tissue damage with hyphal organisms traversing through the entire submucosal compartment were noted, in both 5-FU-treated and untreated tissues (arrows, Fig. 5B). Along the same lines, in *C. glabrata* biofilms organisms could be seen frequently as clusters within epithelial layers in 5-FU-treated constructs (arrows, Fig 5C). Finally, both streptococcal species formed superficial, non-invasive biofilms regardless of 5-FU tissue treatment (Fig. 5C).

5-FU-treated organotypic constructs respond to commensal organisms with increased proinflammatory cytokines

In order to further validate this experimental model as a mucositis model we first examined the proinflammatory cytokine response of mucosal constructs to 5-FU, focusing on a panel of cytokines associated with oral mucositis in humans.¹ Treatment of organotypic constructs with 10 μ M 5-FU for 16h resulted in a significant increase of GM-CSF, IL-6, IL-8 and TNF α in tissue-conditioned media, cytokines with a central role in oral mucositis pathogenesis^{1,3} (Table 1).

We next tested the hypothesis that 5-FU treatment affects the epithelial proinflammatory response to microbial challenge by comparing 5-FU-treated and untreated mucosal tissues in their cytokine response to mucosal biofilm growth. As expected from our previous work in organotypic models^{32,33} in untreated tissues, *C. albicans* elicited a release of all proinflammatory cytokines tested, which was significantly higher compared to *C. glabrata* and *S. oralis*. In general, the cytokine response of untreated tissues to *C. glabrata* or *S. oralis*

biofilms was low and close to control (uninfected) tissue levels (Table 1 and Fig. 6). Importantly, *C. albicans* induced a significantly higher production of GM-CSF and TNF α in 5-FU-treated compared to untreated tissues. Similarly, 5-FU-treated tissues responded to *C. glabrata* with a higher IL-6 response, compared to untreated tissues (Fig. 6). Along the same lines, *S. oralis* triggered a significant IL-6 release above uninfected levels only in 5FU-treated tissues (Table 1 and Fig. 6). In summary, these results suggest that 5-FU treatment primes the oral mucosa for an exacerbated proinflammatory response to certain fungal and bacterial commensal organisms.

Discussion

Three dimensional organotypic constructs provide an organizational complexity that is between the culture of single cell types and *ex vivo* organ cultures. In this setting epithelial cells form multiple stratified layers resembling the upper alimentary tract mucosa both morphologically and functionally.¹⁹ Using an oral mucosal organotypic construct we developed a cancer chemotherapy-induced mucositis model where we can begin to ask mechanistic questions regarding the role of commensal mucosal organisms in the initiation or exacerbation of oral mucositis. In addition to cost and ethical considerations, an advantage of organotypic models over animal testing includes the ability to monitor dynamic and rapid mucosal biological responses to chemotherapy agents and microorganisms. Although we can use this model to ask focused, mechanistic questions regarding the role of commensals in pathogenesis, it should be acknowledged that oral mucositis is a condition with a complex multifactorial pathogenesis, including local and systemic factors (e.g. xerostomia, neutropenia), that cannot be entirely recapitulated *in vitro*.

Our model reproduced histopathologic changes of chemotherapy-induced oral mucositis in humans, such as cell vacuolation, induction of apoptosis and stimulation of key proinflammatory cytokines. Although these mucosal changes are relevant to 5-FU treatment, changes due to other classes of chemotherapeutic agents may not be as well represented by this model. Activation of NF- κ B and upregulation of proinflammatory cytokines and mediators has been associated with oral mucositis triggered by several classes of chemotherapeutic agents *in vivo*³⁴. In intestinal mucositis models, 5-FU activates the transcription factor NF- κ B in epithelial cells, whereas phagocytic cells respond with activation of NADPH oxidase and generation of reactive oxygen species which may play a role in further increasing inflammation and apoptotic cell death (reviewed in reference 35). The molecular mechanisms of cytokine responses to 5-FU in oral epithelium *in vitro* have not been reported. This novel organotypic model of the oral mucosa will thus allow further mechanistic studies into the regulatory networks of 5-FU-induced mucosal inflammation and apoptosis, as well as contributions by specific signaling pathways in each biological process. Although this model utilized a carcinoma cell line to expedite cell proliferation and three-dimensional tissue maturation, the model can be adapted to include any cell type including primary or hTERT-immortalized oral epithelial cells.^{19,22}

Several models were recently developed where the effect of either ionizing radiation or a chemotherapy agent (everolimus) on oral keratinocyte proliferation and inflammatory cytokine responses were examined, in order to recapitulate the mucositis-associated

epithelial changes observed *in vivo*.^{36–39} Most of these models used commercially available organotypic constructs and were characterized by a modest IL-6 and IL-8, but no detectable TNF α epithelial response.^{36–38} By comparison, in our model in addition to TNF α , 5-FU induced a GM-CSF, IL-6 and IL-8 response in oral epithelium, which resembles the inflammatory response of the oral mucosa in humans¹ and animals⁸. Differences in cytokine profiles among different organotypic models may be attributed to the different chemotherapeutic agents applied and/or differences in the origin, immortalized state or type of oncogenic transformation of the epithelial cells used.

It is impossible to correlate the 5-FU treatment of organotypic cultures to a clinical situation, because 5-FU regimens (dosage, continuous versus repeated infusions, duration) differ depending on the type of tumor, organ affected, presence of metastases and other clinical factors. Even within similar regimens, there is a wide range of concentrations reported in saliva, with large inter- and intra-individual differences (from 10,000–0.1 mg/L).⁴⁰ Interestingly, the optimal concentration of 5-FU in our organotypic model was similar to the effective initial blood concentration of 5-FU in a mouse model of oral mucositis⁸. In this recently published animal model, 5-FU chemotherapy reduced E-cadherin in oral and esophageal mucosal tissues.⁸ Thus the organotypic model reproduced the decrease of E-cadherin protein expression in oral epithelium by 5-FU *in vivo*. In mouse intestinal mucositis models others have found expression of occludin and claudin-1, to be significantly reduced by 5-FU treatment.⁴¹ Increase in intercellular spaces and dissolution of epithelial junction proteins may facilitate local neutrophilic transmigration, as shown in this study, but may also weaken the barrier function of the oral mucosa against chemicals or opportunistic pathogens.

Oral microbial community shifts during chemo- or radio-therapy have been studied in multiple cancer patient studies, with no emerging consistent patterns of change with mucositis development.^{16,42,43} In this study we tested the hypothesis that mucosal fungal or bacterial biofilm growth may be more robust on tissues treated with 5-FU *in vitro*. Increased adhesion and subsequent growth of oral microorganisms at mucositis sites may result from exposure of basement membrane extracellular matrix proteins such as collagen type IV, laminin, or fibronectin following epithelial damage.^{44,45} In our model, since epithelial layers were mostly preserved, we reasoned that nutrient diffusion through “open” epithelial junctions in our model could lead to more robust biofilm formation. Nutrients (mainly amino acids, or small protein fragments) could be originating from epithelial layers that eventually undergo cell lysis, or from the basally provided cell culture media. For example, L-glutamine which was added in our culture media promotes growth of fastidious streptococci, when added to a chemically defined bacterial growth medium.⁴⁶ This may explain the small but perhaps biologically significant increase in *S. oralis* and *S. mitis* biofilms in 5-FU-treated tissues. However, there was no detectable significant increase in fungal biofilm growth on 5-FU-treated mucosal surfaces, suggesting that epithelial damage alone may not be sufficient in promoting overgrowth of fungi on mucositis-affected tissues.

Since the oral microbiota is in constant cross-talk with the oral mucosa, it is plausible that microbial challenge of mucosal cells may contribute to inflammatory events that aggravate oral mucositis. Although very few studies have analyzed the relationship between mucositis

severity and changes in the fungal and bacterial microbiome longitudinally in humans, a recent study¹⁶ showed that as mucositis severity increased bacterial communities became more distinct from controls, suggesting an association with severity. Interestingly, in this study mucosae harboring a high abundance of *Streptococcus* species were correlated with the subsequent development of more severe mucositis. Commensals that colonize established mucositis lesions have been described as possible local “amplifiers” of the inflammatory response¹ however this had never been tested experimentally. Our study for the first time has shown that streptococcal and fungal species that are ubiquitous commensals of oral mucosal surfaces in humans, can amplify the proinflammatory epithelial response in tissues treated with chemotherapy agents. One possible explanation for this is that inflammatory activation of epithelial cells which is initiated by 5-FU may induce de novo expression or upregulation of microbial innate pattern recognition receptors, such as Toll-like or Nod receptors, that may amplify inflammatory cytokine signaling.⁴⁷

Oral *viridans* streptococci, particularly *mitis* group species identified as *S. oralis/mitis* are frequently associated with septicemia in oncology patients receiving high dose chemotherapy. Other than the type or dose of the cytotoxic therapy, neutropenia and oropharyngeal mucositis have been identified as the main risk factors.^{11,48} Like streptococci, commensal fungi such as *C. albicans* or *C. glabrata*, commonly cause septicemia associated with oral mucosal injury.^{10,12,13} The oral mucosa has been suggested to be a portal of entry for *viridans* streptococci in the bloodstream of neutropenic patients, especially after oral mucosal damage triggered by chemotherapy.⁴⁸ In our study, neither streptococcal species invaded the 5-FU-treated mucosal surfaces, whereas mucosal tissue invasion of *Candida* species was not significantly altered by chemotherapy treatment. These findings suggest that systemic factors such as prolonged neutropenia are required for mucosal barrier breach and systemic dissemination by these commensals, in addition to mucosal damage. Alternatively, synergistic action of bacterial and/or fungal commensal species may be needed for breach of the mucosal barrier, as we have previously shown that streptococci of the *mitis* group can synergize with *C. albicans* to activate epithelial calpains leading to E-cadherin dissolution.²⁹

In summary we have developed an organotypic model of chemotherapy-induced oral mucositis that reproduces histopathologic characteristics of this condition *in vivo*. Importantly, for the first time we have shown experimentally that certain oral commensal bacterial and fungal species can contribute to the mucosal inflammatory response when they colonize mucositis sites. This model provides unique opportunities for further mechanistic dissection of epithelial changes triggered by chemotherapy as well as for further investigations on the role of oral microorganisms as secondary inflammatory stimuli that can aggravate oral mucositis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Source: Public Health Service grants DE013986 and DE023632 from the National Institute of Dental and Craniofacial Research, NIH.

References

1. Sonis ST. Mucositis: The impact, biology and therapeutic opportunities of oral mucositis. *Oral Oncol.* 2009; 45:1015–20. DOI: 10.1016/j.oraloncology.2009.08.006 [PubMed: 19828360]
2. Lalla RV, Bowen J, Barasch A, et al. Mucositis Guidelines Leadership Group of the Multinational Association of Supportive Care in Cancer and International Society of Oral Oncology (MASCC/ISOO). MASCC/ISOO clinical practice guidelines for the management of mucositis secondary to cancer therapy. *Cancer.* 2014; 120:1453–61. [PubMed: 24615748]
3. Campos MI, Campos CN, Aarestrup FM, Aarestrup BJ. Oral mucositis in cancer treatment: Natural history, prevention and treatment. *Mol Clin Oncol.* 2014; 2:337–340. [PubMed: 24772297]
4. Peterson DE, O'Shaughnessy JA, Rugo HS, et al. Oral mucosal injury caused by mammalian target of rapamycin inhibitors: emerging perspectives on pathobiology and impact on clinical practice. *Cancer Med.* 2016; 5:1897–907. DOI: 10.1002/cam4.761 [PubMed: 27334013]
5. Murphy BA, Gilbert J. Dysphagia in head and neck cancer patients treated with radiation: assessment, sequelae, and rehabilitation. *Semin Radiat Oncol.* 2009; 19:35–42. DOI: 10.1016/j.semradonc.2008.09.007 [PubMed: 19028344]
6. Rosenthal DI. Consequences of mucositis-induced treatment breaks and dose reductions on head and neck cancer treatment outcomes. *J Support Oncol.* 2007; 5:23–31. [PubMed: 18046995]
7. Di Gennaro E, Bruzzese F, Pepe S, et al. Modulation of thymidilate synthase and p53 expression by HDAC inhibitor vorinostat resulted in synergistic antitumor effect in combination with 5-FU or raltitrexed. *Cancer Biol Ther.* 2009; 8:782–91. [PubMed: 19270508]
8. Bertolini M, Sobue T, Thompson A, Dongari-Bagtzoglou A. Chemotherapy induces oral mucositis in mice without additional noxious stimuli. *Transl Oncol.* 2017; 10:612–620. DOI: 10.1016/j.tranon.2017.05.001 [PubMed: 28666190]
9. Lee HR, Yoo N, Kim JH, et al. The therapeutic effect of PLAG against oral mucositis in hamster and mouse model. *Front Oncol.* 2016; 6:209.doi: 10.3389/fonc.2016.00209 [PubMed: 27800302]
10. Khan SA, Wingard JR. Infection and mucosal injury in cancer treatment. *J Natl Cancer Inst Monogr.* 2001; 29:31–36.
11. Bochud PY, Eggiman P, Calandra T, Van Melle G, Saghafi L, Francioli P. Bacteraemia due to viridans streptococcus in neutropenic patients with cancer: Clinical spectrum of risk factors. *Clin Infect Dis.* 1994; 18:25–31. [PubMed: 8054434]
12. Redding SW, Dahiya MC, Kirkpatrick WR, et al. *Candida glabrata* oropharyngeal candidiasis in patients receiving radiation treatment for head and neck cancer. *J Clin Microbiol.* 2002; 40:1879–81. [PubMed: 11980984]
13. Redding SW, Zellars RC, Kirkpatrick WR, et al. Epidemiology of oropharyngeal *Candida* colonization and infection in patients receiving radiation for head and neck cancer. *J Clin Microbiol.* 1999; 37:3896–900. [PubMed: 10565903]
14. Dupuy AK, David MS, Li L, et al. Redefining the human oral mycobiome with improved practices in amplicon-based taxonomy: discovery of *Malassezia* as a prominent commensal. *PloS One.* 2014; 9:e90899.doi: 10.1371/journal.pone.0090899 [PubMed: 24614173]
15. Ramla S, Sharma V, Patel M. Influence of cancer treatment on the *Candida albicans* isolated from the oral cavities of cancer patients. *Support Care Cancer.* 2016; 24:2429–36. [PubMed: 26638003]
16. Zhu XX, Yang XJ, Chao YL, et al. The potential effect of oral microbiota in the prediction of mucositis during radiotherapy for nasopharyngeal carcinoma. *EBioMedicine.* 2017; 18:23–31. DOI: 10.1016/j.ebiom.2017.02.002 [PubMed: 28216066]
17. Pedroso SH, Vieira AT, Bastos RW, et al. Evaluation of mucositis induced by irinotecan after microbial colonization in germ-free mice. *Microbiology.* 2015; 161:1950–60. [PubMed: 26224606]
18. Villa A, Sonis ST. Pharmacotherapy for the management of cancer regimen-related oral mucositis. *Expert Opin Pharmacother.* 2016; 17:1801–7. [PubMed: 27477002]
19. Dongari-Bagtzoglou A, Kashleva H. Development of a highly reproducible three-dimensional organotypic model of the oral mucosa. *Nat Protoc.* 2006; 1:2012–2018. [PubMed: 17487190]

20. Sobue, T., Diaz, P., Xu, H., Bertolini, M., Dongari-Bagtzoglou, A. Methods Mol Biol. Vol. 1356. New York, NY: Humana Press; 2016. Experimental Models of *C. albicans* Streptococcal Co-infection; p. 137-52.
21. Diaz PI, Xie Z, Sobue T, et al. Synergistic Interaction between *Candida albicans* and Commensal Oral Streptococci in a novel *in vitro* mucosal model. Infect Immun. 2012; 80:620–32. [PubMed: 22104105]
22. Dongari-Bagtzoglou A, Kashleva H. Development of a novel three-dimensional *in vitro* model of oral Candida infection. Microb Pathog. 2006; 40:271–278. [PubMed: 16626928]
23. Xie Z, Thompson A, Sobue T, et al. *Candida albicans* biofilms do not trigger reactive oxygen species and evade neutrophil killing. J Infect Dis. 2012; 206(12):1936–45. [PubMed: 23033146]
24. Sachs UJ, Andrei-Selmer CL, Maniar A, et al. The neutrophil-specific antigen CD177 is a counter-receptor for platelet endothelial cell adhesion molecule-1(CD31). J Biol Chem. 2007; 282:23603–12. [PubMed: 17580308]
25. Cotter TG, Henson PM. A cell surface differentiation antigen involved in human neutrophil chemotaxis and degranulation. Clin Exp Immunol. 1983; 53:249–53. [PubMed: 6307561]
26. Xu H, Sobue T, Bertolini M, et al. *S. oralis* activates the Efg1 filamentation pathway in *C. albicans* to promote cross-kingdom interactions and mucosal biofilms. Virulence. 2017; 8:1–16. [PubMed: 27410461]
27. Bertolini M, Xu H, Sobue T, et al. Candida-streptococcal mucosal biofilms display distinct structural and virulence characteristics depending on growth conditions and hyphal morphotypes. Mol Oral Microbiol. 2015; 30:307–22. [PubMed: 25754666]
28. Villar CC, Kashleva H, Nobile CJ, Mitchell AP, Dongari-Bagtzoglou AI. Mucosal tissue invasion by *Candida albicans* is associated with E-cadherin degradation, mediated by transcription factor Rim101 and protease Sap5. Infect Immun. 2007; 75:2126–35. [PubMed: 17339363]
29. Xu H, Sobue T, Bertolini M, Thompson A, Dongari-Bagtzoglou A. *Streptococcus oralis* and *Candida albicans* synergistically activate μ -Calpain to degrade E-cadherin from oral epithelial junctions. J Infect Dis. 2016; 214:925–34. [PubMed: 27190184]
30. von Bültzingslöwen I, Jontell M, Hurst P, Nannmark U, Kardos T. 5-Fluorouracil induces autophagic degeneration in rat oral keratinocytes. Oral Oncol. 2001; 37:537–544. [PubMed: 11435181]
31. Chun J, Prince A. TLR2-induced calpain cleavage of epithelial junctional proteins facilitates leukocyte transmigration. Cell Host Microbe. 2009; 5:47–58. [PubMed: 19154987]
32. Villar CC, Kashleva H, Mitchell AP, Dongari-Bagtzoglou AI. Invasive phenotype of *Candida albicans* affects the host proinflammatory response to infection. Infect Immun. 2005; 73:4588–4595. [PubMed: 16040970]
33. Li L, Kashleva H, Dongari-Bagtzoglou A. Cytotoxic and cytokine-inducing properties of *Candida glabrata* in single and mixed oral infection models. Microb Pathog. 2007; 42:138–47. [PubMed: 17306958]
34. Logan RM, Gibson RJ, Sonis ST, Keefe DM. Nuclear factor-kappa B and cyclooxygenase-2 expression in the oral mucosa following cancer chemotherapy. Oral Oncol. 2007; 43(4):395–401. [PubMed: 16979925]
35. Ribeiro RA, Wanderley CW, Wong DV, et al. Irinotecan- and 5-fluorouracil-induced intestinal mucositis: insights into pathogenesis and therapeutic perspectives. Cancer Chemother Pharmacol. 2016; 78:881–893. [PubMed: 27590709]
36. Sonis S, Andreotta PW, Lyng G. On the pathogenesis of mTOR inhibitor-associated stomatitis (mIAS)-studies using an organotypic model of the oral mucosa. Oral Dis. 2017; 23:347–352. [PubMed: 27896917]
37. Tschachojan V, Schroer H, Aeverbeck N, Mueller-Klieser W. Carbon ions and X-rays induce pro-inflammatory effects in 3D oral mucosa models with and without PBMCs. Oncol Rep. 2014; 32:1820–8. [PubMed: 25174410]
38. Tobita T, Izumi K, Feinberg SE. Development of an *in vitro* model for radiation-induced effects on oral keratinocytes. Int J Oral Maxillofac Surg. 2010; 39:364–70. [PubMed: 20080035]

39. Lambros MP, Parsa C, Mulamalla H, et al. Identifying cell and molecular stress after radiation in a three-dimensional (3-D) model of oral mucositis. *Biochem Biophys Res Commun*. 2011; 405:102–6. [PubMed: 21216227]
40. Joulia JM, Pinquet F, Ychou M, et al. Plasma and salivary pharmacokinetics of 5-fluorouracil (5-FU) in patients with metastatic colorectal cancer receiving 5-FU bolus plus continuous infusion with high dose folinic acid. *Eur J Cancer*. 1999; 35(2):296–301. [PubMed: 10448274]
41. Song MK, Park MY, Sung MK. 5-Fluorouracil-induced changes of intestinal integrity biomarkers in BALB/c mice. *J Cancer Prev*. 2013; 18:322–9. [PubMed: 25337561]
42. Napeñas JJ, Brennan MT, Bahrani-Mougeot FK, Fox PC, Lockhart PB. Relationship between mucositis and changes in oral microflora during cancer chemotherapy. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. 2007; 103:48–59. [PubMed: 17178494]
43. Bergmann OJ. Alterations in oral microflora and pathogenesis of acute oral infections during remission-induction therapy in patients with acute myeloid leukaemia. *Scand J Infect Dis*. 1991; 23:355–366. [PubMed: 1909053]
44. Busscher HJ, van de Belt-Gritter B, Dijkstra RJ, et al. Intermolecular forces and enthalpies in the adhesion of *Streptococcus mutans* and an antigen I/II-deficient mutant to laminin films. *J Bacteriol*. 2007; 189:2988–95. [PubMed: 17277062]
45. Tomita H, Ike Y. Tissue-specific adherent *Enterococcus faecalis* strains that show highly efficient adhesion to human bladder carcinoma T24 cells also adhere to extracellular matrix proteins. *Infect Immun*. 2004; 72:5877–85. [PubMed: 15385489]
46. Mickelson MN. Chemically defined medium for growth of *Streptococcus pyogenes*. *J Bacteriol*. 1964; 88:158–64. [PubMed: 14197881]
47. Frank M, Hennenberg EM, Eyking A, et al. TLR signaling modulates side effects of anticancer therapy in the small intestine. *J Immunol*. 2015; 194:1983–95. [PubMed: 25589072]
48. Richard P, Amador Del Valle G, Moreau P, et al. Viridans streptococcal bacteraemia in patients with neutropenia. *Lancet*. 1995; 345:1607–9. [PubMed: 7783538]

Figure 1. Effect of 5-FU treatment on organotypic mucosal constructs

A: Histologic analysis by H&E staining of untreated tissues (CTR), compared to tissues treated with 1μM or 10μM 5-FU for 16h. A representative tissue section is shown from each condition tested in triplicate, in one of two independent experiments. Bars=50μm. **B:** Lactate dehydrogenase (LDH) released by untreated tissues (CTR) or tissues treated with 1μM or 10μM 5-FU for 16h. Results represent the average OD490 of tissue-conditioned media from tissues set up in triplicate, in one of two independent experiments. *P< 0.01 for a comparison with control (CTR) untreated tissues.

Figure 2. Epithelial cell DNA synthesis and apoptosis in organotypic mucosal constructs treated with 5-FU

A: Immunofluorescence staining for BrdU incorporation. Cells that have incorporated BrdU appear pink. **B:** Immunofluorescence staining for active caspase 3 (red) in epithelial cells.

C: Quantification of epithelial DNA synthesis. Results represent percentage of BrdU(+) cells (pink) as percent of the total number of epithelial cell nuclei (blue) quantified in the same image. **D:** Quantification of epithelial cell apoptosis. Results represent percentage of active caspase-3 (+) cells (red) as percent of the total number of epithelial cell nuclei (blue) quantified in the same image. Tissue constructs treated with 1μM or 10μM 5-FU are compared to control (CTR), untreated constructs. Mucosal cell nuclei were counterstained with the nucleic acid stain Hoechst 33258 (blue). Results are shown from each condition tested in triplicate, in one of two representative experiments. Bars=50μm.

Figure 3. Effect of 5-FU treatment on epithelial E-cadherin

A: Immunofluorescence staining of E-cadherin (green). Cell nuclei were counterstained with the nucleic acid stain Hoechst 33258 (blue). A representative immunofluorescence image is shown, from each condition tested in triplicate, in one of two independent experiments. Bars=20μm. **B:** Release of soluble E-cadherin fragments in organotypic tissue-conditioned media, as determined by ELISA. Error bars represent one standard deviation of the mean of triplicate wells in one of two independent experiments. Tissue constructs treated with 1μM or 10μM 5-FU for 16h are compared to control (CTR), untreated constructs. * $p < 0.01$ for a comparison with control (CTR) untreated tissues.

Figure 4. Effect of 5-FU treatment on neutrophil transmucosal migration

A: Immunofluorescence staining of MPO+ differentiated HL-60 cells (red) in untreated tissues (CTR) or tissues treated with 5-FU (10 μ M) for 16h. Cell nuclei were counterstained with the nucleic acid stain Hoechst 33258 (blue). A representative immunofluorescence image is shown, from each condition tested in triplicate, in one of two independent experiments. Bars=20 μ m. **B:** Quantitative assessment of HL-60 cell transmigration through epithelial cell layers in untreated (5-FU(-)) versus 5-FU-treated tissues ((5-FU(+)) shown above. Bars corresponding to the left Y-axis represent MPO activity in tissue lysates as assessed by a colorimetric assay. Error bars represent one standard deviation of the mean of

triplicate tissues in two independent experiments. MPO⁺ cell numbers, as assessed by immunofluorescence in 6 microscopic fields per condition, are plotted against the Y-axis to the right, with error bars indicating one SEM. *p<0.001

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Figure 5. Effect of 5-FU treatment on biofilm growth

A: *C. albicans* SN425, *C. glabrata* GDH2269, *S. oralis* 34 and *S. mitis* 49456 were inoculated on mucosal constructs that had been pre-treated with 10 μ M 5-FU for 16h. Biofilms were allowed to grow for 16h after treatment. Untreated mucosal constructs served as control. Biofilm surface area was quantified microscopically using Image J software, in 4–8 microscopic fields per biofilm, tested in triplicate, in two independent experiments. Results are expressed as mean fold biofilm surface area in 5-FU-treated over untreated tissues. * $p < 0.005$. **B:** *C. albicans* biofilms growing on untreated (CTR) or 5-FU-treated mucosal constructs for 6h or 16h. Immunofluorescence images are showing *C. albicans*

biofilms (green), epithelial cell E-cadherin (red) and nuclei (blue). Arrows indicate tissue invasion by fungal organisms. **C:** Immunofluorescence images are showing *C. glabrata* (green), *S. oralis* (red) and *S. mitis* (red) biofilms growing on untreated (CTR) or 5-FU-treated mucosal constructs for 16h. Cell nuclei are shown in blue. Arrow points to an intraepithelial cluster of *C. glabrata*. A representative immunofluorescence image is shown, from each condition tested in triplicate, in one of two independent experiments. Bars=50µm.

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Figure 6. Effect of microbial biofilms on cytokine synthesis in 5-FU-treated and untreated mucosal constructs

A panel of pro-inflammatory (GM-CSF, IL-1 β , IL-6, IL-8, TNF α) and anti-inflammatory (IL-10) cytokines were simultaneously analyzed in tissue-conditioned media after 16h of *C. albicans* (Ca), *C. glabrata* (Cg), or *S. oralis* (So) growth. Results represent mean cytokine levels from two independent experiments with conditions set up in triplicate. Error bars indicate one SD of the mean. *p<0.05 for a comparison between 5-FU-treated and untreated tissues. **p<0.01 for a comparison between untreated infected (this figure) versus untreated uninfected tissues (Table 1).

Table 1Cytokine response of organotypic oral mucosal cultures to 5-FU (10 μ M) treatment

	GM-CSF (pg/ml)	IL- β (pg/ml)	IL-6 (pg/ml)	IL-8 (ng/ml)	IL-10 (pg/ml)	TNF α (pg/ml)
untreated	86.7 $\pm$ 3.0	2.5 $\pm$ 0.7	70.6 $\pm$ 4.3	11.9 $\pm$ 0.4	1.2 $\pm$ 0.1	19.7 $\pm$ 1.2
5-FU treated	100.2 $\pm$ 1.4 *	2.2 $\pm$ 0.2	138.6 $\pm$ 10.0 **	17.7 $\pm$ 1.1 *	1.2 $\pm$ 0.2	28.8 $\pm$ 2.2 *

Results represent mean cytokine levels of the two conditions tested in triplicate in two independent experiments

* p<0.05,

** p<0.01