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Interaction of Tea Polyphenols and Food Constituents with Model Gut Epithelia: The Protective Role of the Mucus Gel Layer

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ABSTRACT: The luminal surface of the gastrointestinal tract is covered by a mucus gel layer that acts to protect gut epithelial cells from the harsh luminal environment. This study investigated the use of two human colonic adenocarcinoma cell lines, HT29-MTX-E12 and HT29, as a model to mimic gut epithelium with and without a mucus gel layer. The effect of adding the tea polyphenols epigallocatechin gallate (EGCG) and epicatechin (EC) to the cells with subsequent examination of cell morphology and viability was assessed. EGCG, at the concentrations tested, was very toxic to the HT29 cells, but less toxic to the HT29-MTX-E12 cells, suggesting that the mucus gel layer on the HT29-MTX-E12 cells can protect the cells against EGCG toxicity. In contrast, EC had no effect on the viability of either the HT29 or HT29-MTX-E12 cells, suggesting that proteins within the mucus gel layer on the apical surface of gut epithelial cells may bind to the galloyl ring of EGCG. The effect of adding food-related ingredients with the ability to complex with EGCG, β-casein and maltodextrin, on cell viability was also examined. The presence of β-casein was very effective in protecting the cells against the toxicity effect of EGCG, but maltodextrin, at the concentration tested, was less effective in protecting against this toxicity. In conclusion, the results demonstrate that the mucus gel layer on HT29 human colonic adenocarcinoma cells may protect these cells against EGCG toxicity. In addition, the data showing reduced toxicity of EC compared to that of EGCG suggest that the cytotoxic effects of high polyphenol levels may be associated with the ability of polyphenols to interact with cellular proteins and mucins.

KEYWORDS: *HT29, HT29-MTX, gut epithelial cells, EC, EGCG, β-casein, maltodextrin*

INTRODUCTION

In the human body, the apical epithelial surfaces of the respiratory, gastrointestinal (GI), and reproductive tracts are coated by mucus, the complex and viscoelastic secretion synthesized by specialized goblet cells in the columnar epithelium. Mucus is composed primarily of water but also contains ions, glycoproteins, proteins, and lipids. However, it is the high molecular weight mucin glycoproteins (mucins) that are responsible for the nonlinear viscoelastic and gel properties of mucus. Mucins are encoded by different mucin genes (MUC genes) and, to date, 20 genes have been identified.[1] The MUC genes display site-specific distribution; for example, MUC5AC and MUC6 are produced by the surface and glandular epithelia of gastric mucosa, respectively, whereas MUC2 is found in the small and large intestines.[2] Because of their large molecular weight, high carbohydrate content, and extended conformation in solution, mucins endow mucus secretions with the high viscosity and necessary chemical diversity to interact, entrap, and transport microorgansims, particles, and noxious substances. Collectively, the mucins are considered to be key factors in the physiological defense of the GI tract.

Within the food and pharma industries, there is a significant interest in the biological effects of plant-derived polyphenols. These compounds, which are characterized by containing several phenolic groups (often in the form of galloyl (3,4,5-trihydroxybenzoyl) groups), have been shown to have a variety of effects in animals and humans.[3,4] Specific plant polyphenols have been reported to exhibit health benefits; for example, it has been reported that tea polyphenols (which include catechin (C), epicatechin (EC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG)) show several beneficial effects including antioxidant, anti-inflammatory, and anticarcinogenic properties.[5] Indeed, EC has been shown to be one of the most potent antioxidants present in the human diet,[6] whereas EGCG, the most abundant catechin in green tea extract, has been reported to prevent cancer in a number of animal models.[7] However, in addition to these proposed health benefits, it has also been suggested that plant polyphenols of intermediate to high molecular weight (also known as tannins) may reduce the nutritional value of some foodstuffs.[8,9] Diets rich in tannins have been shown to decrease feed utilization efficiency and perturb mineral absorption. The observed reduced food efficiency is thought to be associated with the inhibition of digestive enzymes by polyphenols, and it has also been proposed that polyphenol metabolites, such as gallic acid, may have toxic effects on liver absorption.[4] In addition, polyphenol−metal chelation is considered to be the main mechanism leading to reduced nonheme iron absorption in groups susceptible to iron deficiency.[10] Very high levels of plant polyphenols can also cause GI tract irritation.[11] Although the full biological role of polyphenols is not clear, there is shared consensus that they benefit plants. Plant polyphenols are often astringent, and it is the astringency that polyphenols contribute

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to unripe fruits that results in their avoidance by herbivores until the fruit seeds are mature and ready for dispersal.[4]

Polyphenols of intermediate to large size have the ability to bind to proteins and precipitate them.[3,12] Therefore, a proposed defense mechanism against these potentially harmful compounds is interaction with salivary proteins, in particular the proline-rich proteins (PRPs), a class of proteins that account for approximately 70% of the human parotid saliva.[13,14] The basic PRP variant in saliva is able to form complexes with polyphenols through the hydrophobic interaction of exposed galloyl rings (if present) with the pyrrolidine ring of the proline residue.[8] The insoluble complexes formed prevent the absorption of the polyphenols from the intestinal canal and interaction with other biological compounds.[15] It is this capacity to precipitate proteins, in particular the salivary proteins, that is believed to give polyphenols their astringent character. Mucins have also been shown to bind polyphenols.[16,17] It is possible, therefore, that the GI tract mucins may also have a role in protecting the body against the potential harmful effects of these compounds, by binding to them and preventing absorption.

The purpose of this study was to determine if the presence of a mucus layer can indeed modulate the interaction of tea catechins with gut epithelia. In this study we used two cell lines derived from human colonic adenocarcinoma, HT29 and its subclone HT29-MTX-E12. In the study we examined the effects of adding high doses of tea catechins on cell viability. The HT29 and HT29-MTX-E12 cell lines are of interest as, in addition to being derived from the GI tract, the HT29-MTX-E12 subclone is mucin-producing. The parental cell line HT29 is heterogeneous, consisting of >95% of undifferentiated cells and a small proportion of differentiated mucus-secreting and columnar absorptive cells in the postconfluent state. However, if this cell line is treated with the anticancer drug methotrexate (MTX), differentiated populations of mucin-secreting goblet cells are obtained.[2] These cells (HT29-MTX) express several membrane and secretory MUC genes, with MUC5AC, normally expressed in the stomach, being the major gene expressed. It has previously been shown that postconfluent cultures of a HT29-MTX subclone (E12) form a mucus gel layer 100−160 μm thick.[18] This compares to mucus gel layer thicknesses of 107 ± 48 and 134 ± 68 μm for human right and left colon, respectively.[19]

Previous studies have indicated that the tea catechin EGCG is significantly less bioavailable than EC.[20,21] EC differs structurally from EGCG in that it lacks a galloyl ring. It is known that the galloyl ring of EGCG binds to salivary PRPs; however, it is less clear whether this chemical group binds to intestinal mucins and whether the reduced bioavailability of EGCG is due to increased binding of this polyphenol to the mucus gel layer. We therefore examined the effect of these two catechins on the viability of postconfluent HT29 and HT29-MTX cultures. We chose to study catechin concentrations that are typically found in the GI tract environment, namely, 400 and 4000 μM EGCG and comparable concentrations of EC. The lower concentration corresponds to the average amount of EGCG in a standard cup of green tea, whereas the higher concentration is equivalent to the average human daily dietary intake. The effect of adding two common food ingredients, β-casein and maltodextrin, known to bind to EGCG, on cell viability was also examined.

MATERIALS AND METHODS

Materials. HT29, a human colon adenocarcinoma cell line (passage 155), and its mucus-secreting subclone, HT29-MTX-E12 (passage 49), were obtained courtesy of Thomas Kissel (Department of Pharmaceutics and Biopharmacy, University of Marburg, Germany).

Dulbecco's modified Eagle's medium (DMEM) with Glutamax, 0.05% trypsin−EDTA solution, nonessential amino acids (NEAA), and EBSS (Earle's balanced salts solution, calcium and magnesium free) were obtained from Invitrogen, Paisley, U.K. Fetal bovine serum Gold (FBS), heat-inactivated. was obtained from PAA, Yeovil, U.K. Trypsin-neutralizing solution (TNS) was obtained from Lonza, Wokingham, U.K. A 0.4% Trypan blue solution and a 1% Alcian blue solution (in 3% acetic acid) were obtained from Sigma, Poole, U.K.

EGCG was obtained from DSM (Switzerland) under the product name Teavigo (94% of EGCG). EC was sourced in-house and was derived by fractionation from green tea extract. The purity of EC used was 98.4% as determined by HPLC. Maltodextrin (Glucidex 20, dextrose equivalent 20, M_W = 10 kDa) was obtained from Roquette (U.K.). β-Casein (95% lyophilized powder) was obtained from Sigma (U.K.) and was used without further purification.

Cell Culture. HT29 and HT29-MTX-E12 cells were maintained in DMEM/Glutamax (with 110 mg/L pyruvate and 4.5 g/L glucose), containing 10% FBS and 1% NEAA. Cells were passaged twice a week. Cells were passaged by first incubating with EBSS buffer for 20 min and then with 0.05% trypsin−EDTA solution for 4−6 min at 37 °C. Once the cells were detached from the tissue culture plastic, the trypsin−EDTA solution in the cell suspension was neutralized by adding TNS. At each passage cells were seeded at 2×10^4 cells/cm^2. Cells were incubated at 37 °C and 5% CO_2.

Assessment of HT29 and HT29-MTX-E12 Cell Morphology and Mucin Production. HT29 and HT29-MTX-E12 cells were seeded into wells of 6-well plates at 2×10^4 cells/cm^2. Cells were maintained in DMEM/Glutamax/10% FBS/1% NEAA, without passaging, for various times up to 21 days. At later time points (day 7+) the cells required feeding with fresh DMEM/Glutamax/10% FBS/1% NEAA every day. At the selected time points cell morphology and mucin production by both cell types were examined. Cell morphology was examined by light microscopy using a Leica DFC320 microscope (with ×4, ×10, and ×20 objectives). Images were acquired using Jasc Paint Shop Pro 7.04 software. Mucin production was detected using Alcian blue stain: the cells were first fixed with chilled 95% ethanol/5% glacial acetic acid for 10 min and then incubated with 1% Alcian blue/3% acetic acid for 5 min at room temperature. The cells were subsequently washed three times with PBS to remove any residual stain, and mucin production (assessed by the level of blue staining) was examined by light microscopy. Paint Shop Pro 7.04 software was used to capture images.

Cell morphology was also assessed using transmission electron microscopy (TEM). Cells were cultured, without passaging, for up to 21 days. At days 7, 14, and 21 the cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 90 min. After washing, the cells were incubated with 1% osmium tetroxide for 30 min. The cells were then washed and incubated with 1% uranyl acetate overnight. The next morning, the samples were dehydrated by washing with ethanol and detached from the bottom of the plastic wells using acetone. Small pieces of the cell layer were removed and transferred to vials containing acetone. They were then washed several times with acetone to remove all residual plastic material. The cells were then resuspended into 50:50 TABB hard plus resin/acetone and left to mix for 24 h. The 50:50 resin/acetone was replaced with pure resin, and the samples were again mixed for 24 h. The samples were then placed in flat embedding molds containing pure resin and were left to cure for 48 h at 60 °C. Sections 130 nm thick were cut using a Leica UC6 microtome and collected on 200 mesh copper EM grids. Sections were examined using a JEOL JEM 2100 electron microscope operated at 200 kV, and images were obtained using a Gatan Ultrascan 4K camera and Digital Micrograph software (Gatan Inc.).

Effect of EGCG, EC, Maltodextrin, and β-Casein on Cell Morphology and Viability. The effects of selected compounds on

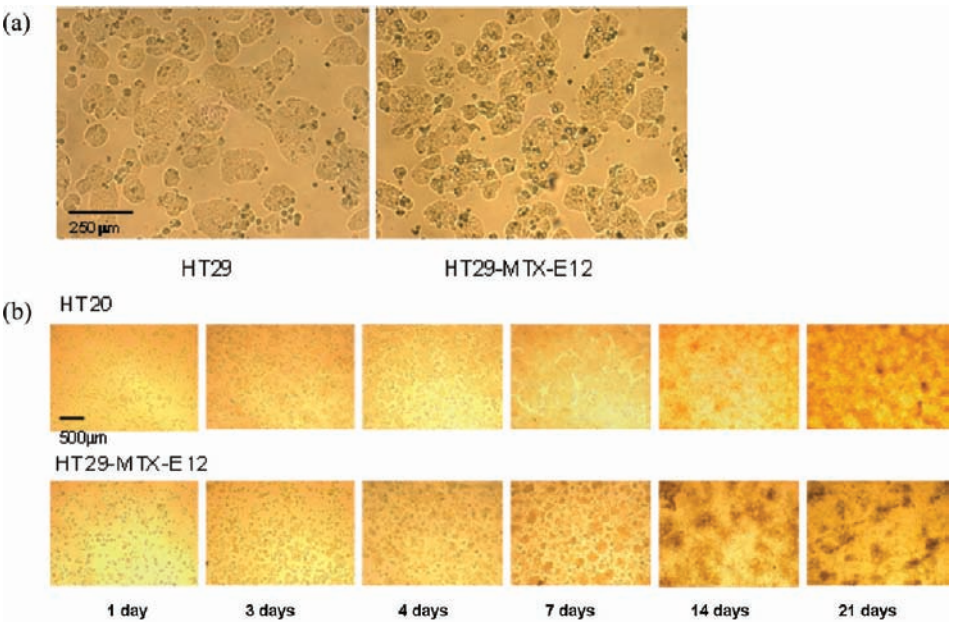

Figure 1. Representative images of the morphology of HT29 and HT29-MTX-E12 cell lines at (a) day 3 (×10 objective) and (b) days 1, 3, 4, 7, 14, and 21 (×4 objective).

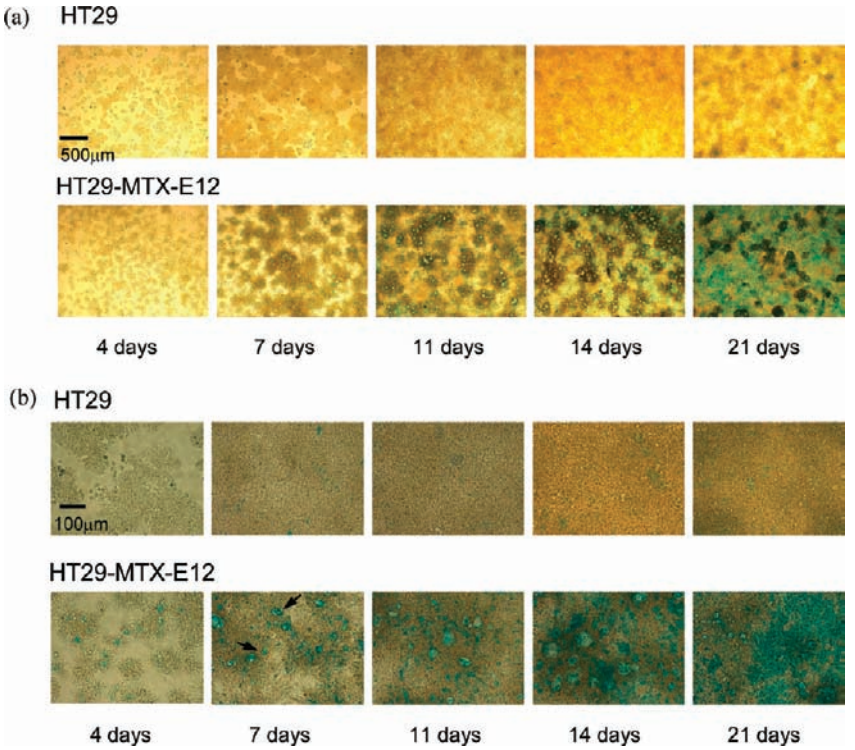

Figure 2. Representative images of mucin production by HT29 and HT29-MTX-E12 cell lines at days 4, 7, 11, 14, and 21: (a) ×4 objective; (b) ×20 objective. Mucin production was visualized by Alcian blue staining. Arrows show vesicles containing Alcian blue reactive substance.

HT29 and HT29-MTX-E12 cell morphology and viability were examined. Cells were seeded into wells of 6-well plates at 2×10^4 cells/cm^2 and maintained in DMEM/Glutamax/10% FBS/1% NEAA, without passaging, for 13 days. At day 13, all serum-containing medium was removed from the cells, and the cells were incubated twice with serum-free medium (DMEM/Glutamax/1% NEAA) for 15 min at 37 °C and 5% CO_2. Selected compounds at the appropriate concentrations, in serum-free medium, were then added to the cells. All treatments were in triplicate. Cells were incubated at 37 °C and 5% CO_2 for 10 min, 1 h, 4 h, and 24 h. At each time point cell viability was assessed using the Trypan blue exclusion assay: cells in each well were trypsinized, and 20 μL of each of the cell suspensions obtained was added to 20 μL of 0.4% Trypan blue solution and incubated at room temperature for approximately 10 min. Cell viability was then recorded by counting the number of total and blue (nonviable) cells using a hemocytometer. Percentage cell viability was calculated as follows:

$$\%\ \text{cell viability} = \frac{\text{no. of viable cells (unstained)}}{\text{no. of total cells}} \times 100$$

Statistical analysis was performed using Student's t test.

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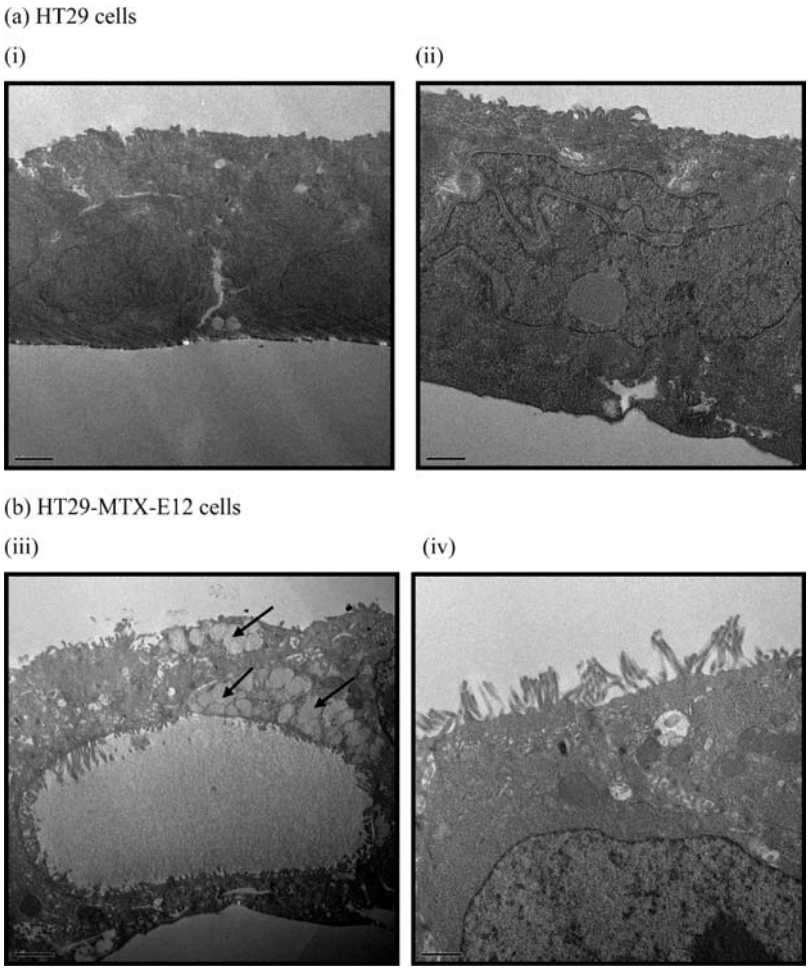

Figure 3. Representative TEM images of postconfluent cultures of (a) HT29 and (b) HT29-MTX-E12 cells (day 7). Sections are perpendicular to the bottom of the well. Note the lack of structure in the HT29 cells, with few microvilli and no mucin bodies visible. In contrast, the HT29-MTX-E12 cells show the presence of mucin droplets (arrowed) and more numerous microvilli. Scale bars: (i, iii) 2 μm; (ii, iv) 1 μm.

In some experiments light microscopic images of cell morphology were also taken.

RESULTS

Time Course Experiment To Assess Cell Morphology and Mucin Production. A time course experiment was carried out to examine the morphology of the HT29 and HT29-MTX-E12 cells over time and to assess mucin production by both cell types. The cells were seeded into 6-well plates at 2×10^4 cells/cm^2 and incubated, without passaging, at 37 °C and 5% CO_2 for a period of up to 21 days. At days 1, 2, 3, 4, 7, 11, 14, and 21 the morphology of both cell lines was examined by light microscopy. Mucin production was assessed at days 4, 11, 14, and 21 by staining with Alcian blue.

Figure 1 shows representative images of the morphology of both cell lines at selected time points. At early time points the morphology of the HT29-MTX-E12 cell line appeared to be very similar to that of the HT29 cell line: both cell lines exhibited epithelial morphology with a spherical appearance and extensive cell to cell contact (see Figure 1a). The HT29-MTX-E12 cells, however, appeared to be slightly more irregular than the HT29 cells. At higher magnification, dark granules were visible in both cell types, but more of these granules were visible in the HT29-MTX-E12 cells than in the HT29 cells. It has been proposed that these granules contain mucin.[22] At later time points, the cells became significantly more confluent and the appearance of the two cell lines began to differ (see Figure 1b). In the HT29 parent cell culture a uniform layer of cells was seen to develop, whereas in the HT29-MTX-E12 cell culture areas more densely populated with cells were evident, resulting in dark patches (see days 14 and 21). We can speculate that this alteration in cell morphology in the HT29-MTX-E12 cultures may have been due to the development of the mucus gel layer.

To examine mucin production the cells were fixed and stained with Alcian blue stain according to the protocol outlined under Materials and Methods. Alcian blue is a phthalocyanin dye commonly used to stain acid mucins.[23] Figure 2 shows representative images of mucin production by both cell types at days 4, 7, 11, 14, and 21. At day 4, the level of staining by the two cell types was very similar, with very few cells of either cell type staining blue (although some individual cells in the HT29-MTX-E12 culture were seen to stain positively with Alcian blue when observed using the ×20 objective). At day 7, however, the level of staining obtained was clearly greater in the HT29-MTX-E12 culture compared to the HT29 culture, with many individual cells staining positive for Alcian blue. Vesicles containing Alcian blue reactive substance were observed in the HT29-MTX-E12 culture (see arrows in Figure 2b). At days 11, 14, and 21 the level of staining observed in the HT29-MTX-E12 culture was significantly greater than in the HT29 control culture. A small number of selected cells did

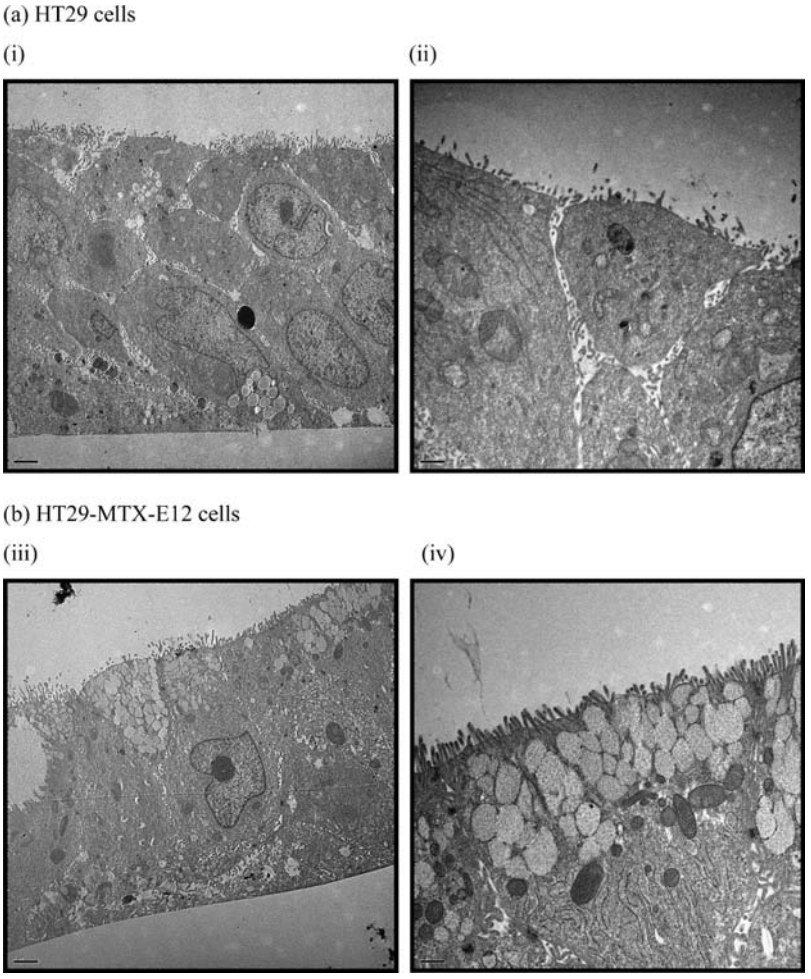

Figure 4. Representative TEM images of postconfluent cultures of (a) HT29 and (b) HT29-MTX-E12 cells (day 14). Sections are perpendicular to the bottom of the well. Microvilli are visible on the apical surface of the HT29 cells as are a small number of mucin droplets. In the HT29-MTX-E12 cultures mature goblet cells with numerous apical clustered mucin droplets are visible. Scale bars: (i, iii) 2 μm; (ii, iv) 1 μm.

stain positively in the HT29 culture, but this was to be expected as the HT29 cell line is a heterogeneous cell line containing a small proportion of mucus-secreting cells. This trend agrees with previous work carried out in our laboratory, which demonstrated that, after 14 days of growth, approximately 74% of the HT29-MTX-E12 cell culture area stains with Alcian blue compared to approximately 16% of the HT29 cell culture.[24]

The morphology of both cell types was also assessed by TEM. Cells were seeded into 6-well plates at 2×10^4 cells/cm^2 and incubated, without passaging, for a period of up to 21 days. At days 7, 14, and 21 the cells were fixed and examined using traditional TEM methods. Representative images are shown in Figures 3−5. At day 7 the HT29 cells showed a general lack of structure, with no mucin droplets and few microvilli visible. In contrast, the structures of the HT29-MTX-E12 cells were more defined, with mucin droplets and a significant numbers of microvilli evident (see Figure 3). By day 14 more microvilli were visible on the apical surface of the HT29 cells, and a small number of mucin droplets could be seen. However, at this time point the HT29-MTX-E12 cells showed mature goblet cells with numerous apical clustered mucin droplets (see Figure 4). At day 21, slightly more mucin droplets were visible in the HT29 cultures. However, the small number seen was in contrast to the numerous mucin droplets and granules observed throughout the HT29-MTX-E12 cultures. Differences were also observed in the structure of the microvilli present on the two cell types at this time point. Although appearing quite dense on both cell types, the microvilli on the HT29-MTX-E12 cells appeared to be longer and showed light and dark patches. We can speculate that these light and dark patches may have been due to the undulating nature of the microvilli on these cells (see Figure 5).

Taken together, these preliminary analyses have demonstrated that 11−14 days of growth is required for the HT29-MTX-E12 cells to develop an observable mucus layer. Therefore, in subsequent experiments the cells were incubated for 13 days before they were treated with the tea polyphenols.

Effect of EGCG and EC on Cell Morphology and Viability. To investigate the interaction of cells with and without a mucus gel layer with tea polyphenols, experiments were carried out in which EGCG and EC were added to the HT29 and HT29-MTX-E12 cell lines and cell behavior was monitored (see Figure 6 for the chemical structures of these compounds).

The addition of 4000 and 400 μM EGCG was investigated (1830 and 183 mg/L, respectively; the lower concentration corresponds to the average amount of EGCG in a standard cup of green tea (180 mL)). These concentrations were previously studied by us in work investigating the physical complexation of salivary protein with EGCG and EC in relation to the

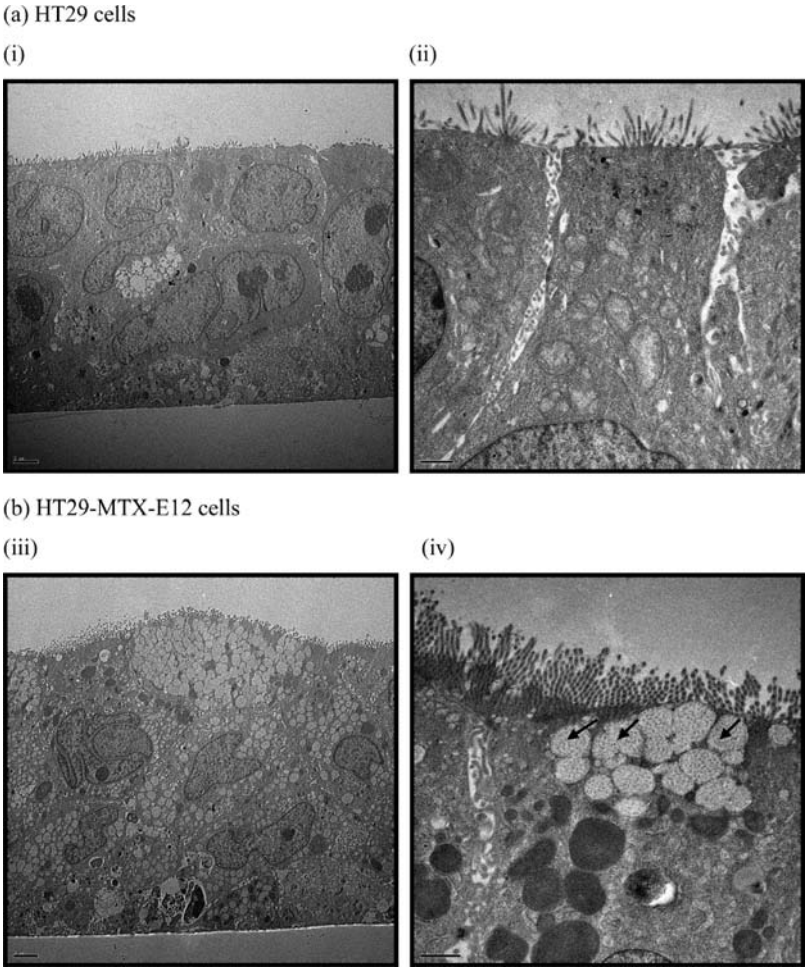

Figure 5. Representative TEM images of postconfluent cultures of (a) HT29 and (b) HT29-MTX-E12 cells (day 21). Sections are perpendicular to the bottom of the well. A small number of mucin droplets are visible in the HT29 cultures. In contrast, numerous mucin droplets and mucin granules (arrowed) are observed throughout the HT29-MTX-E12 culture. Note also the differences in the structure of the microvilli present on the two cell types (images ii and iv). Scale bars: (i, iii) 2 μm; (ii, iv) 1 μm.

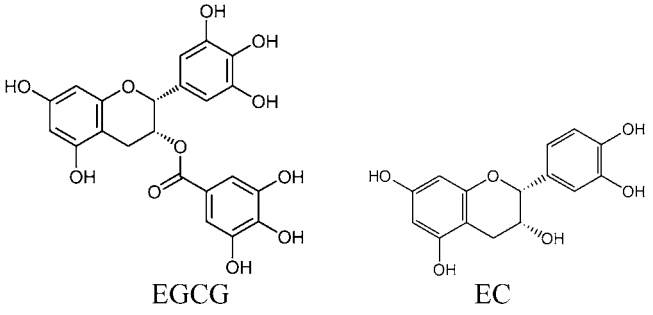

Figure 6. Chemical structures of EGCG and EC.

astringency perception.[25] We are aware that these concentrations are extremely high for cell culture studies, but in this work we were interested in assessing the toxicity effects of the polyphenols.

Cells were treated after 13 days in culture to ensure an observable mucus gel layer was present on the HT29-MTX-E12 cells. The cells were treated with the required concentrations of EGCG in serum-free medium and incubated for 10 min, 1 h, 4 h, or 24 h. Control cells were treated with serum-free medium only. Representative images of the two cell types after treatment with EGCG at 400 and 4000 μM are shown in Figure 7. At most time points the morphology of the cell cultures treated with both concentrations of EGCG appeared very similar to that of the controls. However, at 24 h, the HT29 cells treated with 4000 μM EGCG looked markedly different, with patches of damage being evident (interruption in the cell layer was visible as was irregularity of cell structure). Some damage was also evident in the HT29-MTX-E12 culture treated with 4000 μM EGCG after 24 h of incubation, but the level of damage appeared to be less than that observed in the HT29 culture.

Cell viability was assessed by the Trypan blue exclusion method. Results from the cell viability test are shown in Figure 8. As expected, both concentrations of EGCG had a very detrimental effect on HT29 cell viability, with only 10% of the cells treated with 4000 μM EGCG remaining viable after 4 h; by 24 h, none of the cells treated with this concentration of EGCG remained viable. In addition, only 4.5% of the HT29 cells treated with 400 μM EGCG were viable after the 24 h incubation period (see Figure 8a). It was also observed that the cell medium became visibly brown after 4 h of incubation with EGCG, which was presumably due to oxidation of the EGCG. Indeed, previous studies have shown that the half-life of EGCG is approximately 130 min when used to treat HT29 cells.[26] The toxicity effects seen here are therefore due to the presence of EGCG and/or its metabolites. Cell viability of EGCG-treated

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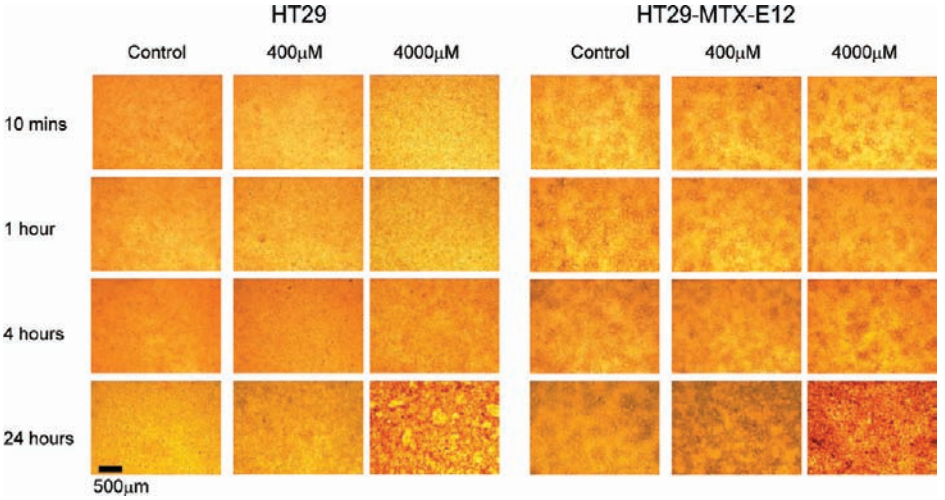

Figure 7. Representative images of the morphology of HT29 and HT29-MTX-E12 cell lines (day 13 cultures) after treatment with 400 and 4000 μM EGCG for 10 min, 1 h, 4 h, and 24 h (×4 objective).

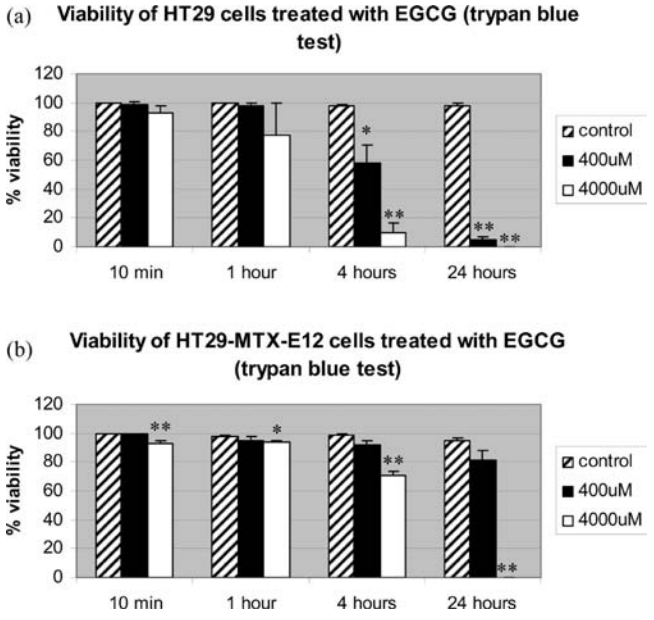

Figure 8. Viability of (a) HT29 cells and (b) HT29-MTX-E12 cells (day 13 cultures) after treatment with 400 and 4000 μM EGCG. Cell viability was measured using the Trypan blue exclusion assay. Symbols * and ** identify cases significantly different from control: *, $p < 0.05$; **, $p < 0.01$.

HT29-MTX-E12 cells was also assessed. Interestingly, in this culture the level of toxicity seen was much less. At 4 h, >70% of the cells treated with 4000 μM EGCG remained viable. At 24 h, none of the cells treated with this higher concentration of EGCG were viable, but >80% of cells treated with 400 μM remained viable (see Figure 8b). In a subsequent experiment (data not shown), approximately 50% of cells were found to be viable after treatment with 1000 μM EGCG for 24 h. It is possible that the HT29-MTX-E12 cells were simply more resistant to the toxicity effects of EGCG than their parental cell line HT29. However, these interesting results also suggest that the mucin gel layer of the HT29-MTX-E12 cells may offer some protection against the toxicity effects of EGCG and its metabolites.

The effect of adding EC to both cell lines was also investigated. EC differs structurally from EGCG in that it lacks the galloyl ring. This molecule also differs in the sensorial space regarding the concentration perception threshold for bitterness, which is significantly higher than that found for EGCG.[25]

In these experiments we decided to investigate the effect of adding to the cell cultures the same concentrations of EC as used in the EGCG study. However, due to an error made when the EC stock solutions were prepared, 200 and 2000 μM were used to treat the HT29 cells instead of 400 and 4000 μM. The HT29-MTX-E12 cells were treated with 4000 μM EC only. Examination of cell morphology by light microscopy demonstrated that none of the cultures treated with EC, at any time point, were different morphologically from the control cultures (data not shown). Results from the cell viability test are shown in Figure 9. As expected from other studies,[27,28] none of the concentrations tested had any effect on cell viability

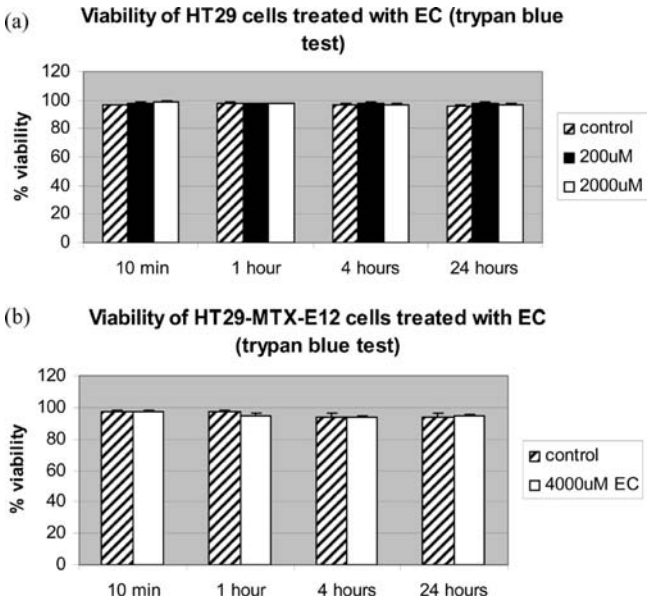

Figure 9. Viability of (a) HT29 cells and (b) HT29-MTX-E12 cells (day 13 cultures) after treatment with various concentrations of EC. Cell viability was measured using the Trypan blue exclusion assay.

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of either cell type, even after 24 h of incubation. These data suggest that these compounds interact differently with epithelial cells and that the galloylated polyphenols have the highest interaction with biological proteins. Because we did not observe any toxicity effects in EC-treated cells, this molecule was not investigated further.

Effect of Adding EGCG-Masking Ingredients on Cell Morphology and Viability. The effect of adding two common food ingredients, β-casein and maltodextrin, to the cells was then examined. The choice of these two ingredients was in part guided by our previous work investigating the astringency perception of EGCG, which was reduced when these two ingredients were separately added, either directly (maltodextrin) or as contained in a complex matrix (semi-skimmed milk), to the tested sample.[25]

The β-casein milk protein was chosen as a simple model to replicate the effect of salivary PRPs,[29] which are recognized as the main salivary components able to deactivate some of the antinutritional properties of polyphenols. Poncet-Legrand et al.[30] have shown that poly(L-proline), which is structurally similar to proline-rich proteins (PRPs), starts interacting with EGCG at a molar ratio (polyphenol/polyL-proline) of 0.33, signaled by the presence of aggregates. In milk, β-casein (MW ~ 24 kDa) is one of the major phosphoproteins, accounting for nearly 30% of total milk proteins. In this study we used 0.025 w/w% and 400 μM of β-casein and EGCG, respectively, which is equivalent to a molar ratio (polyphenol/protein) of 38.3.

Maltodextrin, produced from starch, is a polysaccharide that is commonly used as a food thickener. Polysaccharides can sterically stabilize phenolic compounds and hinder their natural aggregation, an observation commonly reported in the wine industry.[31,32] Depending on the type and molecular weight of adsorbing polysaccharides, bridging flocculation of polyphenols is also possible.[31] It is therefore possible that maltodextrin may bind with EGCG and reduce the toxicity effects seen in the adenocarcinoma cell lines. Little guidance was found in the literature to help us select the maltodextrin concentration, and therefore we set it at 1 w/w%, below the minimum level (5 w/w%) investigated in our previous study.[25]

HT29 and HT29-MTX-E12 cells (day 13 cultures) were treated with 400 μM EGCG with and without β-casein at 0.025%, or maltodextrin at 1%, in serum-free medium. Cells were also treated with β-casein and maltodextrin (in serum-free medium) alone. Control cells were treated with serum-free medium. The cells were then incubated at 37 °C and 5% CO_2 for 10 min, 1 h, 4 h, and 24 h, and cell viability was assessed using the Trypan blue exclusion assay. Figure 10 shows the cell viability for the HT29 and HT29-MTX-E12 cells treated with EGCG and β-casein. In the HT29 cell culture (see Figure 10a), no differences in cell viability after 10 min and 1 h for cells treated with EGCG, β-casein, or a combination of the two compounds were observed. However, at 4 h, cells treated with EGCG and β-casein were significantly more viable than cells treated with EGCG alone (p = 0.0003). This significant difference was also seen at 24 h, when approximately 7% of cells remained viable after treatment with EGCG alone, whereas >92% of cells treated with EGCG and β-casein remained viable (p < 0.0001). Treatment with β-casein alone did not affect cell viability. This result clearly indicates that β-casein can protect against the toxicity effects of EGCG on HT29 cells in this assay.

The results obtained for the HT29-MTX-E12 cells were similar, if less dramatic, as the overall toxicity caused by EGCG treatment was less (see Figure 10b). In this experiment, no

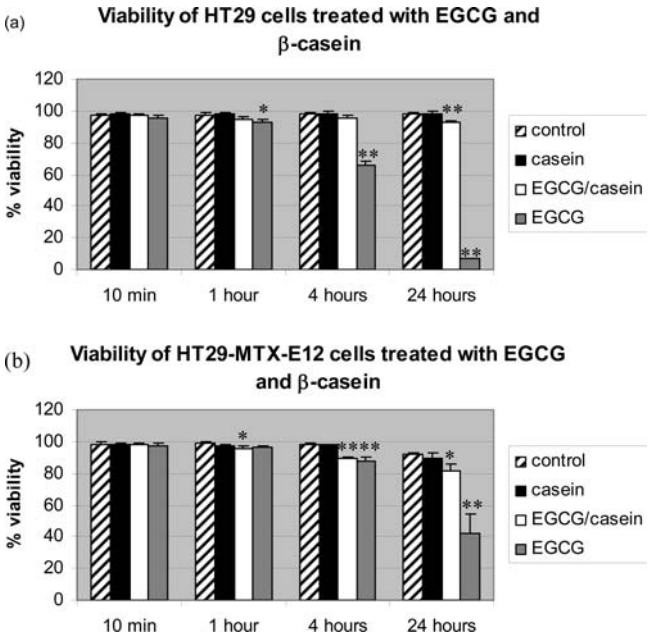

Figure 10. Viability of (a) HT29 cells and (b) HT29-MTX-E12 cells (day 13 cultures) after treatment with 400 μM EGCG and/or 0.025% β-casein. Cell viability was measured using the Trypan blue exclusion assay. Symbols * and ** identify cases significantly different from control: *, p < 0.05; **, p < 0.01.

differences in viability of cells treated with EGCG alone or EGCG and β-casein were detected at 10 min, 1 h, and 4 h. However, a significant difference (p = 0.014) was seen at 24 h (cells treated with EGCG alone showed a viability of approximately 42%, whereas cells treated with EGCG and β-casein showed an approximate viability of 81%), indicating that β-casein can also protect against the toxicity effects of EGCG in the HT29-MTX-E12 cell line. It will be noted that the viability of the EGCG-treated cells after 24 h in this experiment was lower than in our previous experiment (see Figure 8b). This difference may be explained by the scatter of data in both experiments and also by the observation that the control cells in the β-casein experiment were less viable overall, suggesting that the cells in this later experiment may have been more susceptible to the toxicity effects of EGCG.

Figure 11 shows the cell viability for the HT29 and HT29-MTX-E12 cells treated with EGCG and maltodextrin. For the HT29 culture, the results show that maltodextrin, at the concentration tested, protects against EGCG toxicity to some extent, but that the protection is not as complete as that shown by β-casein. At 4 h, cells treated with maltodextrin and EGCG were significantly more viable than cells treated with EGCG alone (91% viable compared to 66% viable, respectively; p = 0.0003). However, by 24 h this significant difference had been lost. With the HT29-MTX-E12 cells, however, no significant differences in viability for the EGCG and EGCG/maltodextrin-treated cells were seen at any time point. Clearly, this result differs from that obtained for the HT29 cells and may in part be due to the overall higher viability of the EGCG-treated HT29-MTX-E12 cells. However, it was also observed in this experiment that the viability of the control cells was lower than in previous experiments and, for the EGCG- and EGCG/maltodextrin-treated cells, there was significant scatter of data, leading to large error bars.

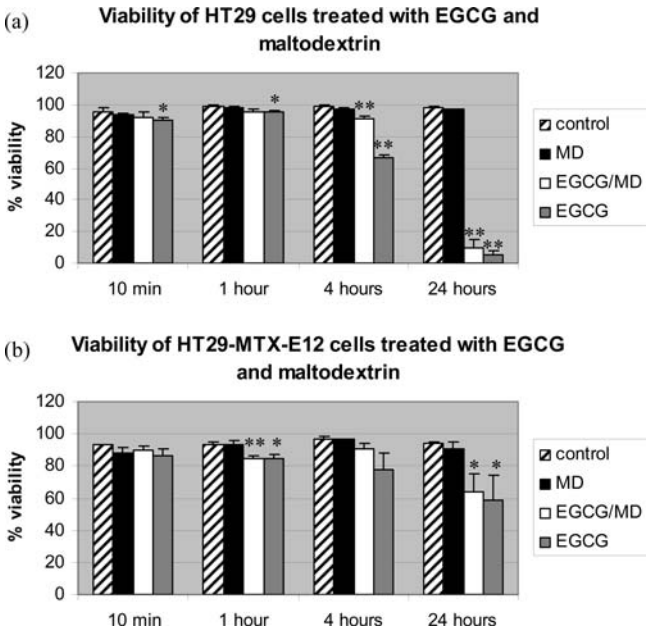

Figure 11. Viability of (a) HT29 cells and (b) HT29-MTX-E12 cells (day 13 cultures) after treatment with 400 μM EGCG and/or 1% maltodextrin. Cell viability was measured using the Trypan blue exclusion assay. Symbols $*$ and $**$ identify cases significantly different from control: $*$, $p < 0.05$; $**$, $p < 0.01$.

DISCUSSION

The luminal surface of the GI tract is covered by a mucus gel layer that acts to protect gut epithelial cells from the harsh luminal environment. In this study our aim was to determine if we could use two human colonic adenocarcinoma cell lines, HT29-MTX-E12 and HT29, as a model to mimic gut epithelium with and without a mucus gel layer. These cells are of interest as HT29-MTX-E12, a subclone of HT29, is mucus-producing, whereas HT29 is not. In our initial experiments we examined cell morphology and mucin production by both cell lines for a period of up to 21 days. Examination by light microscopy revealed that the morphology of the two cell cultures differed at later time points. At day 14, a uniform layer of cells was seen to develop in the HT29 cell culture, whereas the HT29-MTX-E12 cell culture showed areas more densely populated with cells, resulting in dark patches. We can speculate that the alteration in cell morphology in the HT29-MTX-E12 culture may have been due to the development of a mucus gel layer.

The morphology of both cell types was also assessed by TEM. This revealed significant differences in the ultrastructure of the two cell types. At day 14 a small number of mucin droplets were observed in the HT29 cell culture. However, at this time point the HT29-MTX-E12 culture showed mature goblet cells with numerous apical clustered mucin droplets. The differences observed were even more striking after 21 days in culture: although slightly more mucin droplets were visible in the HT29 culture, the small number seen was in contrast to the numerous mucin droplets and granules observed throughout the HT29-MTX-E12 culture at this time point.

Studies using Alcian blue staining also confirmed that an observable mucus gel layer was formed on the HT29-MTX-E12 cells by about 11 days in culture, so in subsequent experiments we treated cells that had been cultured, without passaging, for 13 days.

In the next set of experiments we investigated the effect of adding the tea polyphenols, EGCG and EC, to the cells by examining cell morphology and viability. It is difficult to estimate the polyphenol intake in the human diet, which may vary with dietary habit as well as geographically. This lack of knowledge is partly due to the shortage of reliable data for polyphenol content in foods. Santos-Buelga and Scalbert in their review[10] report the work from Deprez, who calculated that the human diet contains ~0.1% of polyphenols, corresponding to a concentration in the gut lumen of 1.2 g/L (or 4 mM catechin unit equivalent). On the basis of these data we used 4000 and 400 μM as the two concentrations of EGCG investigated in this work (1830 and 183 mg/L, respectively). The lower concentration corresponds to the average amount of EGCG in a standard cup of green tea (180 mL). EGCG accounts for between ~33 and 50%[33] of the total amount of polyphenols in a standard cup of green tea (~65 mg, internal communication), which equates to an average concentration of ~180 mg/L. Incidentally, these concentrations were already studied in previous work from our group investigating the physical complexation of salivary protein with EGCG and EC in relation to the astringency perception.[25] We are aware that these concentrations are extremely high for cell culture studies, but in this study we were interested in assessing toxicity effects of the polyphenols.

We found that EGCG, at the concentrations tested, was very toxic to the HT29 cells, but less toxic to the HT29-MTX-E12 cells. Although it is possible that the HT29-MTX-E12 cell line was simply more resistant to the toxicity effects of EGCG than its parental cell line HT29, these results also suggest that it is the mucus layer on the HT29-MTX-E12 cells that is able to offer some protection against EGCG toxicity. This agrees with work carried out by Keely et al.,[18] who, when examining the effects of adding poly(methacylate) polymer to the cell lines, found that the polymer was relatively less toxic to the HT29-MTX-E12 cells. EC was also tested in our model; this compound differs from EGCG in that it lacks a galloyl ring. As expected from the work of others,[27,28] EC had no effect on the viability of either the HT29 or HT29-MTX-E12 cells. These results suggest that proteins within the mucus gel layer on the apical surface of gut epithelial cells may bind to the galloyl ring of EGCG, thus preventing absorption and reducing the bioavailability of this potentially harmful compound. The lack of toxicity shown by EC, which does not have a galloyl ring, suggests that the cytotoxic effects of high polyphenol levels may be associated with the ability of polyphenols to interact with proteins and mucins. The effect of adding EC to the cell cultures was not investigated further.

The observation that proteins within the mucus gel layer may bind EGCG but not EC mirrors qualitatively previous investigations by our group.[34] In this work we examined the interfacial shear rheology of human whole saliva mixed with different tea catechins and showed that EGCG promoted the formation of a strong intermolecular network between salivary proteins, whereas EC did not. The strong network formation is the result of complexation of salivary PRPs with the galloyl group of the EGCG molecule. This interesting analogy seems to suggest that protein binding of polyphenols may be a first line of defense against these compounds and that complexation with the galloyl ring may be a specialized method to deactivate their potentially detrimental effects.

Various studies investigating absorption of flavan-3-ols after ingestion of green tea extracts have shown that substantial

quantities of these compounds are absorbed in the small intestine.[35,36] However, these studies have also revealed that EC and epigallocatechin (EGC) are absorbed more efficiently than their 3-*O*-galloylated analogues. It has been suggested that this difference in absorption may be due in part to transporter competition;[37] however, our findings suggest that binding of EGCG to the mucus gel layer may also reduce uptake. It is also interesting to note that in studies investigating the phenolic content of human fecal water, catechin and EC were detected, whereas EGCG was not.[38] It is known that gut flora catabolism of EGCG is quite extensive.[36] However, we can also speculate that the absence of EGCG may have been due to a proportion of EGCG being bound to mucins, preventing complete extraction of this compound in these studies.

The effects of adding ingredients with the ability to complex with EGCG, β-casein and maltodextrin, were then examined. The efficacy of β-casein stems from its proline-rich nature and its ability to complex with EGCG. Maltodextrin, a polysaccharide, binds to EGCG and can partially deactivate its functionality. We found in our assay that the presence of β-casein was very effective in protecting the cells against the toxicity effect of EGCG in both cell lines, but that maltodextrin, at the concentration tested, was less effective in protecting against this toxicity. The interactions of β-casein and EGCG have been investigated using a variety of biophysical techniques,[39,40] and these studies have shown that the β-casein molecule wraps itself around EGCG, supporting the view that the binding of β-casein reduces the availability of EGCG. About 25−30% of protein in cows' milk is β-casein. The authors therefore suggest that the addition of milk to tea is likely to have a significant impact on the physiological effects of EGCG. However, there are conflicting reports on whether binding of EGCG to milk (by adding milk to tea) reduces the bioavailability of EGCG in vivo.[41,42]

In conclusion, in this study we examined if the presence of a mucus gel layer can modulate the interaction of tea catechins with gut epithelia. Our results suggest that the mucus gel layer on HT29 human colonic adenocarcinoma cells is able to offer some protection against EGCG toxicity. In addition, our data showing reduced toxicity of EC compared to EGCG suggests that the cytotoxic effects of high polyphenol levels may be associated with the ability of polyphenols to interact with biological proteins.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Kim, H.; Park, S.; Park, S.; Moon, U.; Lee, B.; Yoon, S. Epigallocatechin-3-gallate inhibits interleukin-1β-induced MUC5AC gene expression and MUC5AC secretion in normal human nasal epithelial cells. *J. Nutr. Biochem.* **2008**, *19* (8), 536−544.

(2) Gouyer, V.; Wiede, A.; Buisine, M.-P.; Dekeyser, S.; Moreau, O.; Lesuffleur, T.; Hoffmann, W.; Huet, G. Specific secretion of gel-forming mucins and TFF peptides in HT-29 cells of mucin-secreting phenotype. *Biochim. Biophys. Acta* **2001**, *1539*, 71−84.

(3) Bennick, A. Interaction of plant polyphenols with salivary proteins. *Crit. Rev. Oral Biol. Med.* **2002**, *13* (2), 184−196.

(4) Mehansho, H.; Butler, L. G.; Carlson, D. M. Dietary tannins and salivary proline-rich proline: interaction, induction and defence mechanism. *Annu. Rev. Sci.* **1987**, *7*, 423−440.

(5) Koo, M. W. L.; Cho, C. H. Pharmacological effects of green tea on the gastrointestinal system. *Eur. J. Pharmacol.* **2004**, *500* (1−3), 177−185.

(6) Nair, C. K. K.; Salvi, V. P. Protection of DNA from γ-radiation induced strand breaks by epicatechin. *Mutat. Res.* **2008**, *650* (1), 48−54.

(7) Lambert, J. D.; Yang, C. S. Mechanisms of cancer prevention by tea constituents. *J. Nutr.* **2003**, *133*, 3262S−3267S.

(8) Charlton, A. J.; Baxter, N. J.; Khan, M. L.; Moir, A. J. G.; Haslam, E.; Davies, A .P.; Williamson, M. P. Polyphenol/peptide binding and precipitation. *J. Agric. Food Chem.* **2002**, *50*, 1593−1601.

(9) Frejnagel, S.; Wroblewska, M. Comparative effect of green tea, chokeberry and honeysuckle polyphenols on nutrients and mineral absorption and digestibility in rats. *Ann. Nutr. Metab.* **2010**, *56*, 163−169.

(10) Santos-Buelga, C.; Scalbert, A. Proanthocyanidins and tannin-like compounds − nature, occurrence, dietary intake, and effect on nutrition and health. *J. Sci. Food Agric.* **2000**, *80*, 1094−1117.

(11) Isbrucker, R.; Edwards, J.; Wolz, E.; Davidovich, A.; Bausch, J. Safety studies on epigallocatechin gallate (EGCG) preparations. Part 2: Dermal, acute and short-term toxicity studies. *Food Chem. Toxicol.* **2006**, *44* (5), 636−650.

(12) Bravo, L. Polyphenols: chemistry, dietary sources, metabolism and nutritional significance. *Nutr. Rev.* **1998**, *56* (11), 317−333.

(13) Kauffman, D. L.; Keller, P. J. The basic proline-rich proteins in human parotid saliva from a single subject. *Arch. Oral Biol.* **1979**, *24*, 249−256.

(14) Bacon, J. R.; Rhodes, M. J. C. Binding affinity of hydrolyzable tannins to parotid saliva and to proline-rich proteins derived from it. *J. Agric. Food Chem.* **2000**, *48*, 838−843.

(15) Lu, Y.; Bennick, A. Interaction of tannin with human salivary proline-rich proteins. *Arch. Oral Biol.* **1998**, *43*, 717−728.

(16) Monteleone, E.; Condelli, N.; Dinnella, C.; Bertuccioli, M. Prediction of perceived astringency induced by phenolic compounds. *Food Qual. Pref.* **2004**, *15* (7−8), 761−769.

(17) Condelli, N.; Dinnella, C.; Cerone, A.; Monteleone, E.; Bertuccioli, M. Prediction of perceived astringency induced by phenolic compounds II: Criteria for panel selection and preliminary application on wine samples. *Food Qual. Pref.* **2006**, *17* (1−2), 96−107.

(18) Keely, S.; Rullay, A.; Wilson, C.; Carmichael, A.; Carrington, S.; Corfield, A.; Haddleton, D. M.; Brayden, D. J. *In vitro* and *ex vivo* intestinal tissue models to measure mucoadhesion of poly(methacrylate) and *N*-trimethylated chitosan polymers. *Pharm. Res.* **2005**, *22* (1), 38−49.

(19) Pullan, R. D.; Thomas, G. A.; Rhodes, M.; Newcombe, R. G.; Williams, G. T.; Allen, A.; Rhodes, J. Thickness of adherent mucus gel on colonic mucosa in humans and its relevance to colitis. *Gut* **1994**, *35* (3), 353−359.

(20) Lu, H.; Meng, X.; Lee, M.; Li, C.; Maliakal, P.; Yang, C. Bioavailability and biological activity of tea polyphenols. In *Food Factors in Health Promotion and Disease Prevention*; ACS Symposium Series; American Chemical Society: Washington, DC, 2003; pp 9−15.

(21) Henning, S.; Choo, J.; Heber, D. Nongallated compared with gallated flavan-3-ols in green and black tea are more bioavailable. *J. Nutr.* **2008**, *138* (8), 1529−1534.

(22) Behrens, I.; Stenberg, P.; Artursson, P.; Kissel, T. Transport of lipophilic drug molecules in a new mucus-secreting cell culture model based on HT29-MTX cells. *Pharm. Res.* **2001**, *18* (8), 1138−1145.

(23) Steedman, H. F. Alcian blue 8GS: a new stain for mucin. *Q. J. Microsc. Sci.* **1950**, *91*, 477−479.

(24) Singh, B. *Internal communication*, 2009.

(25) Rossetti, D.; Bongaerts, J. H. H.; Wantling, E.; Stokes, J. R.; Williamson, A.-M. Astringency of tea catechins: more than an oral lubrication tactile percept. *Food Hydrocolloids* **2009**, *23*, 1984−1992.
(26) Hong, J.; Lu, H.; Meng, X.; Ryu, J.-H.; Hara, Y.; Yang, C. S. Stability, cellular uptake, biotransformation, and efflux of tea polyphenol epigallocatechin-3-gallate in HT-29 human colon adenocarcinoma cells. *Cancer Res.* **2002**, *62*, 7241−7246.
(27) Adachi, S.; Nagao, T.; Ingolfsson, H. I.; Maxfield, F. R.; Andersen, O. S.; Kopelovich, L.; Weinstein, I. B. The inhibitory effect of (−)-epigallocatechin gallate on activation of the epidermal growth factor receptor is associated with altered lipid order in HT29 colon cancer cells. *Cancer Res.* **2007**, *67*, 6493−6501.
(28) Jung, Y. D.; Kim, M. S.; Shin, B. A.; Chay, K. O.; Ahn, B. W. EGCG, a major component of green tea, inhibits tumour growth by inhibiting VEGF induction in human colon carcinoma cells. *Br. J. Cancer* **2001**, *84*, 844−850.
(29) Hagerman, A. E.; Butler, L. G. The specificity of proanthocyanidin-protein interaction. *J. Biol. Chem.* **1981**, *256*, 4444−4497.
(30) Poncet-Legrand, C.; Edelmann, A.; Putaux, J. L.; Cartalade, D.; Sarni-Manchado, P.; Vernhet, A. Poly(L-proline) interactions with flavan-3-ols units: influence of the molecular structure and the polyphenol/protein ratio. *Food Hydrocolloids* **2006**, *20*, 687−697.
(31) Poncet-Legrand, C.; Doco, T.; Williams, P.; Vernhet, A. Inhibition of grape seed tannin aggregation by wine mannoproteins: effect of polysaccharide molecular weight. *Am. J. Enol. Vitic.* **2007**, *58*, 87−91.
(32) Riou, V.; Vernhet, A.; Doco, T.; Moutounet, M. Aggregation of grape seed tannins in model wine − effect of wine polysaccharides. *Food Hydrocolloids* **2002**, *16*, 17−23.
(33) Astill, C.; Birch, M. R.; Dacombe, C.; Humphrey, P. G.; Martin, P. T. Factors affecting the caffeine and polyphenol contents of black and green tea infusions. *J. Agric. Food Chem.* **2001**, *49*, 5340−5347.
(34) Rossetti, D.; Yakubov, G. E.; Stokes, J. R.; Williamson, A.-M.; Fuller, G. G. The interaction of human whole saliva (hws) and astringent dietary compounds investigated by interfacial shear rheology. *Food Hydrocolloids* **2008**, *22*, 1068−1078.
(35) Stalmach, A.; Troufflard, S.; Serafini, M.; Crozier, A. Absorption, metabolism and excretion of Choladi green tea flavan-3-ols by humans. *Mol. Nutr. Food Res.* **2009**, *53*, S44−S53.
(36) Auger, C.; Mullen, W.; Hara, Y.; Crozier, A. Bioavailability of polyphenon E flavan-3-ols in humans with an ileostomy. *J. Nutr.* **2008**, *138*, 1535S−1542S.
(37) Zhang, L.; Chow, M. S. S.; Zuo, Z. Effect of the co-occurring components from green tea on the intestinal absorption and disposition of green tea polyphenols in Caco-2 monolayer model. *J. Pharm. Pharmacol.* **2006**, *58*, 37−44.
(38) Jenner, A. M.; Rafter, J.; Halliwell, B. Human fecal water content of phenolics: the extent of colonic exposure to aromatic compounds. *Free Radical Biol. Med.* **2005**, *38*, 763−772.
(39) Jobstl, W.; O'Connell, J.; Fairclough, J. P. A.; Williamson, M. P. Molecular model for astringency produced by polyphenol/protein interactions. *Biomacromolecules* **2004**, *5* (3), 942−949.
(40) Jobstl, W.; Howse, J. R.; Fairclough, J. P. A.; Williamson, M. P. Noncovalent cross-linking of casein by epigallocatechin gallate characterized by single molecule force microscopy. *J. Agric. Food Chem.* **2006**, *54*, 4077−4081.
(41) Serafini, M.; Ghiselli, A.; Ferro-Luzzi, A. *In vivo* antioxidant effect of green and black tea in man. *Eur. J. Clin. Nutr.* **1996**, *50* (1), 28−32.
(42) van het Hof, K. H.; Kivits, G. A.; Weststrate, J. A.; Tijburg, L. B. Bioavailability of catechins from tea: the effect of milk. *Eur. J. Clin. Nutr.* **1998**, *52* (5), 356−359.