

Surfactants Enhance the Tight-Junction Permeability of Food Allergens in Human Intestinal Epithelial Caco-2 Cells

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Key Words

Food allergen · Caco-2 cells · Intestinal epithelial cells · Ovomucoid · Surfactant · Tight junction · Allergen transportation

Abstract

Background: Food additives are responsible for certain allergic types of symptoms. Here Caco-2 cell monolayers were used as a model of the intestinal epithelium for the study of the effect of a food grade surfactant. We determined whether or not the presence of a surfactant enhances the transportation of food allergens across human intestinal epithelial Caco-2 cells. **Methods:** This study investigated sucrose monoester fatty acids, which are a food grade surfactant. As an in vitro model of human epithelial cells, Caco-2 cells were grown in monolayers and exposed to different doses of the surfactant in conjunction with ovomucoid, a major egg white allergen. The integrity of the monolayer was assessed by measuring transepithelial electrical resistance (TEER). The permeability of tight junctions and transport of the antigen were studied. **Results:** TEER correlated with the permeability of tight junctions. TEER significantly decreased upon exposure to a surfactant, indicating an increase in

ovomucoid permeability without degradation. The surfactant induced shortening in microvilli, actin disbandment and structural separation of tight junctions. The results indicate that food grade surfactants can increase the paracellular uptake of food allergens.

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Introduction

Surfactants are known to affect the absorption of substances from the gastrointestinal tract [1], but little is known about the mechanisms underlying the effects of surfactant on biological membranes such as those lining the epithelium of the gastrointestinal mucosa. Intestinal absorption is an important process which determines the bioavailability of nutrients and physiologically active substances in foods and ingredients [2], but is poorly understood. The paracellular pathway in the intestinal epithelial cell layer provides a highly dynamic transport route for certain molecules [3]. These pathways act as a molecular barrier and are dependent on intercellular tight junctions which are regulated by various substances including certain surfactants such as bile salts [4]. Food components that are ingested orally and are present in the intestinal

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lumen also participate in the regulation of tight-junction permeability. Food grade surfactants (another term for surface-active agents) play important roles in food processing such as starch complexing, protein interactions, aeration, emulsification and crystal modifications. Previous reports have shown that food-derived substances modulate tight junctions [5, 6]. Certain food additives, such as flavor enhancers (e.g. monosodium glutamate), preservatives (e.g. BHA-butylated hydroxyanisole), colorants (e.g. tartrazine yellow and erythrosine), texturing agents, emulsifiers, starches and gums, have been shown to be responsible for certain allergic symptoms, such as asthma, urticaria and anaphylaxis [7].

The effects of surfactants on intestinal permeability are usually studied either in situ or in excised intestinal segments [8]. These models provide important information on the interaction between surfactants and the intestinal epithelial barrier. High concentrations of surfactants cause cell death, and the results obtained in whole-tissue models are difficult to interpret. This has led to the use of simpler models based on human epithelial cell lines that differentiate enterocyte-like cells under conventional cell culture conditions [9, 10]. Caco-2 is a human colon carcinoma cell line which spontaneously exhibits various enterocytic characteristics such as brush-border enzymes [11], nutrient transporters [12, 13] and intracellular tight junctions [11]. Caco-2 cell lines can be used as a model for studying drug and peptide absorption, and the effects of various hormones and ionophores on epithelial integrity [14, 15]. These cell lines are morphologically and functionally similar to intestinal enterocytes. The effect of surfactants on the cell membranes and tight junctions (including their permeability) can be studied in Caco-2 monolayers. Caco-2 monolayers are used to study the transepithelial passage of antigens, intestinal transportation for various nutrients and the mechanisms involved in various infectious agents of the intestinal tract. There are many reports on the regulation of paracellular permeability by food-derived substances using Caco-2 monolayers [16].

In this study, we employed one of the most popular food grade surfactants (sucrose monoester fatty acids) which are used widely to improve food characteristics, especially their texture, and to stabilize emulsions in various food products such as baked goods and ice cream. The effects of this additive on the permeability of human epithelial cells were studied, and the transepithelial passage of ovomucoid – a major food allergen of eggs – was determined using a Caco-2 cell monolayer. The integrity of the monolayer was assessed by measuring transepithe-

lial electrical resistance (TEER) and the transport of the food allergen through the monolayer; the morphology was investigated microscopically.

Materials and Methods

Materials

Transwell polyester culture plates with a filter diameter of 12 mm and a pore size of 0.4 μm were obtained from Corning Costar (New York, N.Y., USA). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Gibco (Burlington, Ont., Canada). Sucrose monoester fatty acids (HLB-16) were obtained from San-Ei Gen FFI (Osaka, Japan). The flasks for growing cells were obtained from Corning Costar. A lactate dehydrogenase (LDH) assay kit was purchased from Sigma (Sigma-Aldrich Canada, Oakville, Ont., Canada).

Cell Culture

Caco-2 cell lines were used as a model of the intestinal epithelium, and this cell line was purchased from the American Type Culture Collection (Rockville, Md., USA). The cells were cultured in DMEM with 20% FCS, supplemented with 1% non-essential amino acids (Gibco) in the presence of penicillin and streptomycin (Gibco), and incubated at 37°C in 5% CO₂. Twenty-five to thirty passages were used in all monolayers employed in these transport experiments. The medium was changed three times a week. Two generations of cells were grown on flasks for 21–23 days after seeding, and then transferred to a transwell plate (1.0 $\times$ 10⁵ cells/ml). The cells were cultured for 14–21 days in DMEM with 20% FCS. The culture medium was 1.5 ml on the basolateral side and 0.5 ml on the apical side.

TEER Measurement

The tight-junction permeability of the Caco-2 monolayer was evaluated by measuring TEER. The monolayers were checked routinely on alternate days by using the Millicell-ERS system (Millipore, Bedford, Mass., USA). TEER values were found to be well correlated with the change in paracellular permeability of the cell monolayer [17]. TEER was derived from Ohm's law by measuring the potential difference change induced by an external current pulse. TEER was measured before and after adding an assay sample, and the effect of each assay sample was expressed as the TEER relative to that at zero time.

Transport Studies

The transport experiments were performed with the transwell filters. The initial TEERs were 350–400 $\Omega\text{ cm}^{-2}$, and the integrity of the monolayer was determined. The monolayers were set in a 12-well plate and washed with Hanks balanced salt solution (HBSS) twice after aspiration of the medium, and the wells were transferred to a new cluster plate containing HBSS buffer. The surfactant HLB-16 (sucrose monoester fatty acids) was added from a 1 mg/ml stock in a dose-dependent manner to the apical portion, and TEER was monitored every 15 min. A well to which no surfactant was added was used as the control. After incubation with the surfactant for 90 min, the antigen (ovomucoid) was added at a concentration of 200 μg /well and incubated for 60 min at 37°C. TEER values were monitored before and after the addition of the protein. After incubation, both the apical and basal samples were recovered for further analysis.

Determination of Protein Transport Using ELISA and SDS-PAGE

The transepithelial transportation rate of the surfactant (apical to basolateral flux) of ovomucoid in the Caco-2 monolayer was determined using ELISA and SDS-PAGE. The amounts of ovomucoid recovered from the basolateral side were quantified and determined as transport flux using ELISA (in nanograms per hour per square centimeter). In brief, the 96-well polystyrene plate (Corning) was coated with 100 μ l of the anti-ovomucoid IgG prepared in our laboratory using rabbits (0.5 μ g/ml) in 0.1 M carbonate buffer, pH 8.0, overnight and blocked with 150 μ l of 2% bovine serum albumin in the same buffer for 2 h at 37°C. The wells were washed twice with phosphate-buffered saline (PBS), pH 7.2, containing 0.05% (w/v) Tween-20 (PBST) and incubated with the samples from the transwell plate along with the standard (ovomucoid at 0–100 ng/ml) for 1 h at 37°C. After washing the plate with PBST, the plate was incubated with 100 μ l of biotin-conjugated anti-ovomucoid antibody (diluted 1:1,000 in PBS) for 1 h at 37°C. The plate was again washed twice and incubated with 100 μ l of Extravidin alkaline phosphatase conjugate (Sigma, diluted 1,000 times) for 1 h at 37°C. After washing six times, the plate was developed with 100 μ l of *p*-nitrophenyl phosphate disodium (Sigma, 1 mg/ml) in 0.1 M diethanolamine buffer, pH 9.8, for 1 h at room temperature, and the absorbance was measured at 405 nm using a microplate reader (Bio-Rad, Hercules, Calif., USA) after the enzyme reaction was stopped by the addition of 25 μ l of 3 N NaOH. A polyacrylamide gel (SDS-PAGE, 12.5%) was run and stained with 0.05% (w/v) Coomassie brilliant blue stain (Sigma) to visualize the protein in the apical, basal and the control sample after the serum sample was concentrated using a centrifugal filter with a molecular mass cutoff of 1 kDa (Centricon, Millipore).

LDH Assay

The cells were cultured for approximately 14 days, gently aspirated and washed twice with HBSS buffer. Surfactant (HLB-16) was added in various doses and incubated for 90 min at 37°C. Controls without the antigen samples were maintained. The TEER values were monitored every 15 min. After 90 min of incubation, 50 μ l of the buffer was carefully removed from the apical side and 50 μ l of the protein (containing 200 μ g of ovomucoid sample) was added and incubated for 60 min at 37°C. TEER values were measured before and after addition of the protein. The samples were recovered from both the apical and basolateral sides and measured for LDH release using an LDH diagnostic kit from Sigma. The activity was measured with standards using LDH (Sigma).

Fluorescence Microscopy

Caco-2 cells (1×10^5) were seeded to a transwell membrane (polyester, 0.4 μ m, Costar) and incubated for approximately 14 days when the monolayer had reached confluence. Caco-2 cell monolayers were rinsed three times with HBSS and then incubated for 10 min with or without surfactant at 50 μ g/well. After incubation, the membrane was cut from the unit and transferred onto a cover glass. The cell monolayers were fixed for 15 min with 4% paraformaldehyde (Sigma) in PBS and permeated with 10% Triton X-100 on ice for 5 min. The actin ring was stained for by incubating the cover glass with 500 μ l of FITC-labeled phalloidin (500 mM) (Sigma) in PBS for 60 min. The stained cells were observed with a fluorescence microscope (BX 60-FLA, Olympus, Tokyo, Japan).

Statistical Analysis

Data were analyzed by ANOVA (SPSS version 8.0 for Microsoft Windows; SPSS, Chicago, Ill., USA), and means were separated by Duncan's multiple range test. Significance was defined at $p < 0.05$.

Results

Cell Growth and Transportation

Caco-2 cells were constantly monitored for growth using TEER. There was no initial lag phase; the values increased until day 14 when a steady state was reached indicating the formation of a monolayer. The effect of the surfactant on the TEER value of the Caco-2 monolayer was measured in a dose-dependent way; the results are presented in figure 1 which plots concentration-dependent values as a function of incubation time. There were no significant changes in the TEER values of the monolayers for surfactant concentrations up to 20 μ g/well during a 90-min incubation. Surfactant concentrations greater than 50 μ g/well rapidly decreased TEER within 15 min of addition of surfactant, and continued to decrease during a 90-min incubation. No cell detachment was observed by optical microscopy during incubation with the surfactant, nor was a loss of monolayer integrity or disruption of cell membranes observed. The interaction of ovomucoid with the surfactant is illustrated in figure 2. TEER was measured with and without surfactant (5–180 μ g/well) in the presence of ovomucoid (200 μ g/well). Ovomucoid also significantly decreased TEER in the presence of surfactant (>50 μ g/well); however, the protein itself did not affect the TEER. The decrease in TEER was smaller than with surfactant only (fig. 1), so that a longer incubation time was required to produce the same TEER.

Transport Rate

The apical to basolateral flux of the antigen (ovomucoid) in the presence of surfactant across the Caco-2 monolayer was estimated using ELISA (fig. 3) and SDS-PAGE (fig. 4). Although the concentration of ovomucoid added to the apical side was constant (200 μ g/well), the transport rate across Caco-2 monolayer markedly increased in the presence of surfactant at concentrations higher than 50 μ g/well. The transport rate was observed to be constant at least for 60 min, and was dependent on the surfactant concentration. The protein recovered from the basolateral side exhibited an expected band on SDS-PAGE, thereby confirming the transport of the protein to the basal side. Ovomucoid antigen was resistant to hydrolysis by cellular peptidase, and was transported across Caco-2 without degradation (fig. 4).

Fig. 1. Effect of surfactant on the TEER of Caco-2 cell monolayers. TEER is expressed as the value relative to that at 0 min. Each value is the mean $\pm$ SD of four sets of experiments.

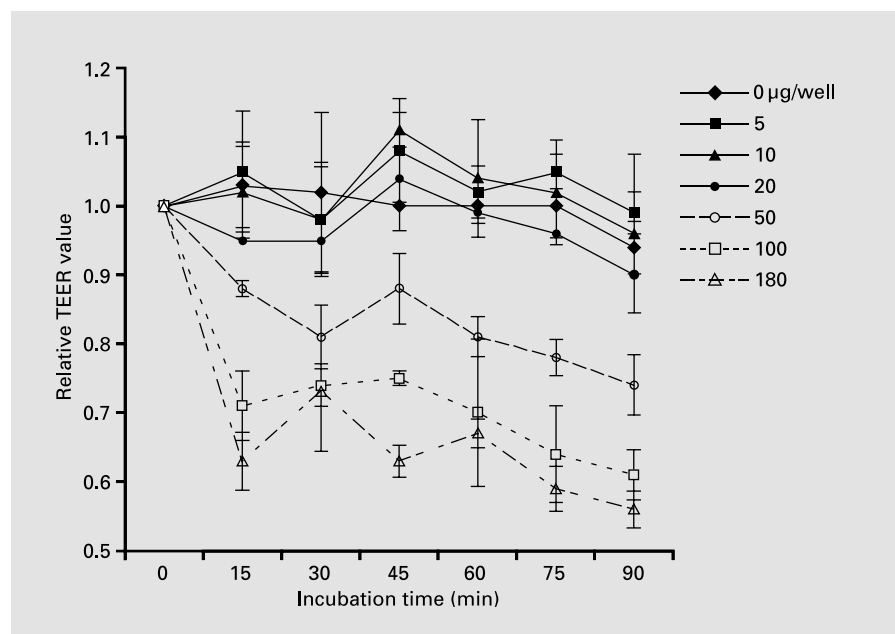
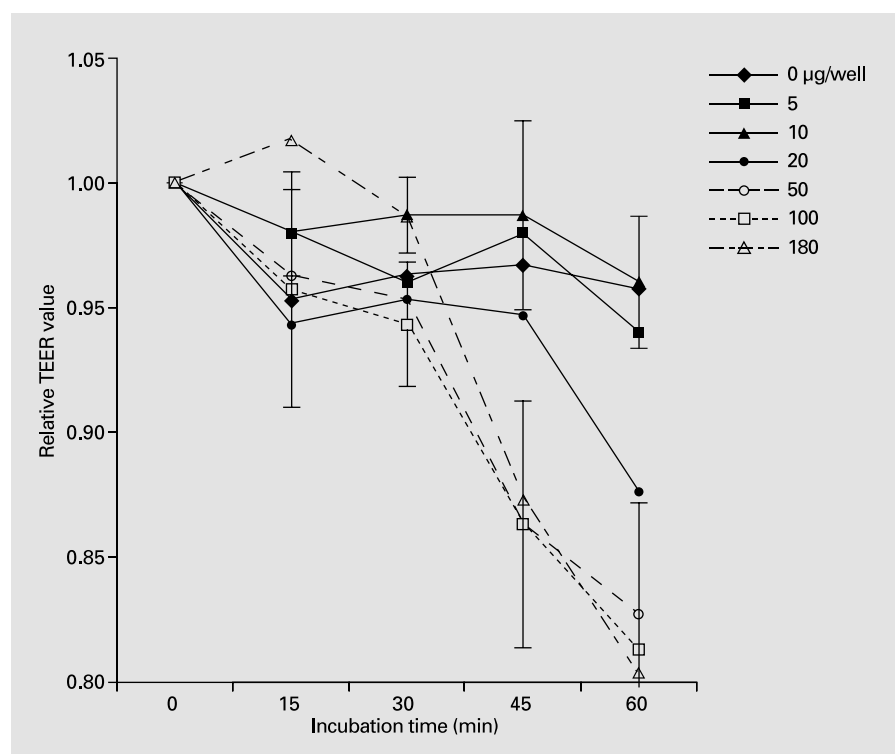


Fig. 2. Effect of ovomucoid on the TEER of Caco-2 cell monolayers as a function of surfactant concentration. TEER is expressed as the value relative to that at 0 min. Each value is the mean $\pm$ SD of four sets of experiments.



LDH Assay

The effect of the surfactant on the human intestinal Caco-2 cell monolayer was examined by determining the LDH activity, which is shown in figure 5. A significant LDH release was observed upon the addition of the sur-

factant to the apical side of Caco-2 monolayer cells. No significant release of LDH was observed at low concentrations of surfactant (<50 $\mu\text{g/ml}$), but as the concentration increased, the release of LDH was increased. Ovomucoid itself did not affect LDH release.

3

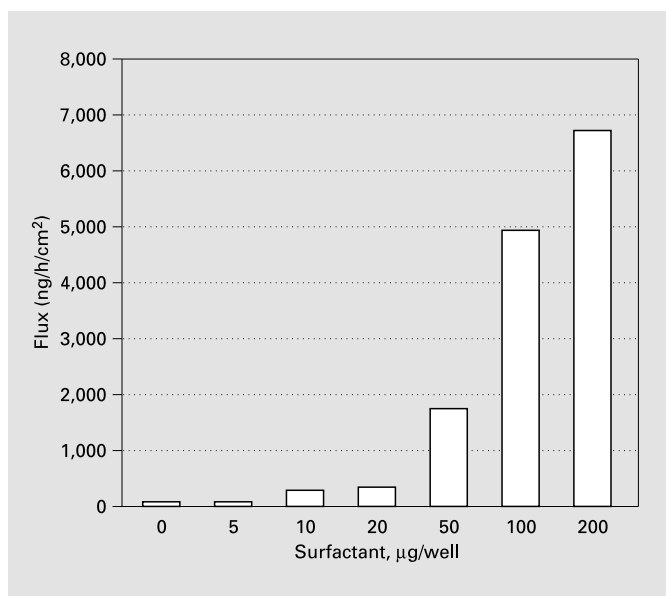
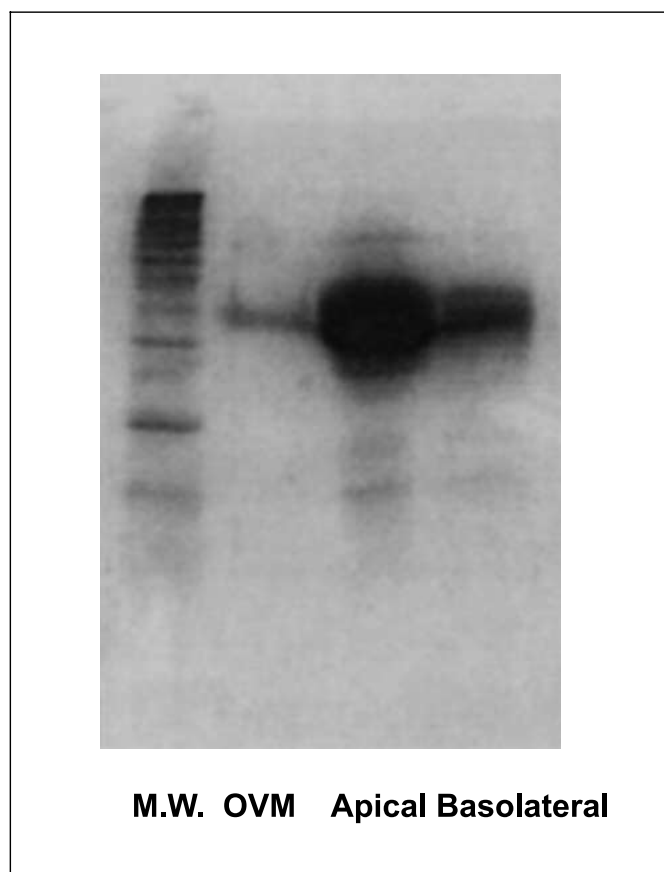


Fig. 3. Effect of surfactant on the transepithelial flux of ovomucoid across the Caco-2 cell monolayers. Each value is the average of three sets of experiments.

Fig. 4. Electrophoretic profiles of ovomucoid on the apical and basolateral sides of Caco-2 cell monolayers. Samples were recovered from each side and concentrated for electrophoresis. MW = Molecular marker; OVM = control ovomucoid; Apical = ovomucoid recovered from the apical side; Basolateral = ovomucoid recovered from the basolateral side.



4

Fig. 5. Release of LDH from the Caco-2 cell monolayers as a function of surfactant concentration. LDH activity was measured after incubating the cell with surfactant for 90 min. Each value is the average of three sets of experiments, and is expressed in Bergier-Broida (B-B) units per milliliter.

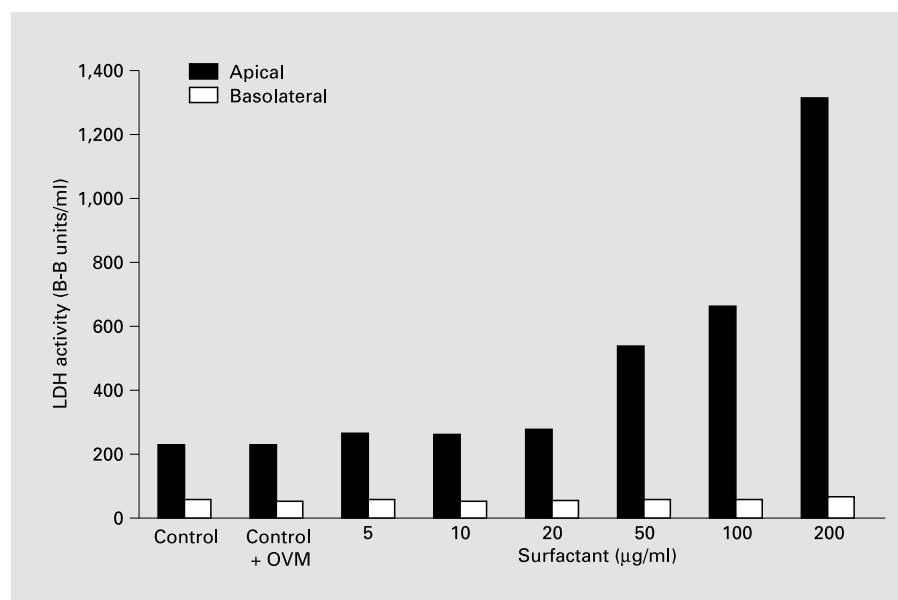
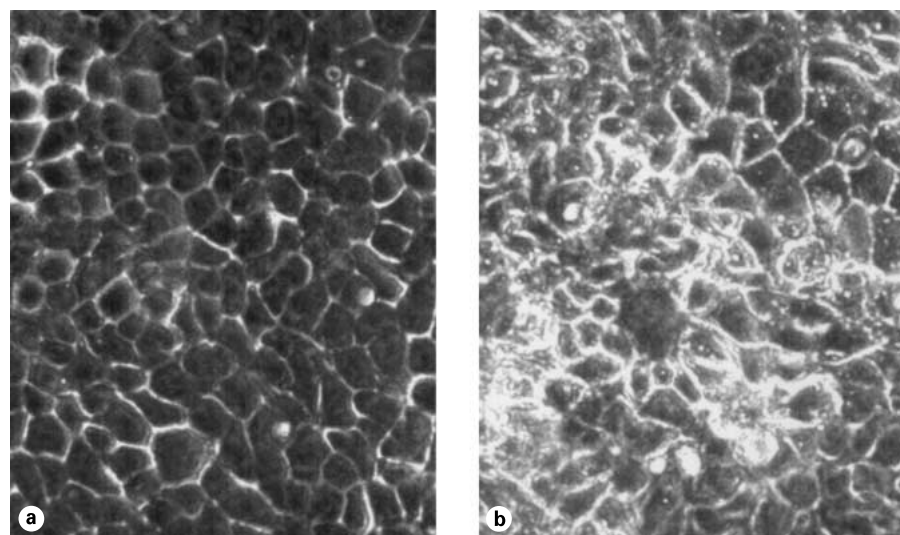


Fig. 6. Fluorescence photomicrograph of the actin filaments stained in Caco-2 monolayers. a Control. b Treated for 15 min with surfactant (50 μ g/well).



Fluorescence Microscopy

Fluorescence microscopy showed that the surfactant affected the structure of the actin filaments (fig. 6). After 15 min of exposure to the surfactant (50 μ g/well), the integrity of the actin was reduced and in some cells the perijunctional rings were partly disbanded. Actin appeared more flocculated than in the controls. These results suggest that the presence of the surfactant changed the tight-junction structure and permeability.

Discussion

The existence of various pathways for intestinal absorption has been reported previously, but the effect of food-derived substances on intestinal absorption is not well understood. The permeability of tight junctions could be physiologically regulated by intracellular messengers or external factors including food-derived substances [5, 6]. In this study, we have used a monolayer of human intestinal epithelial Caco-2 cells – cultured on a semipermeable filter – as a model for studying the effect of a food additive on intestinal epithelial permeability. There have been reports of apical to basolateral transport of oligopeptides in an intact form via the paracellular pathway [18–20]. We have used ovomucoid, a major egg allergenic protein which causes IgE-mediated food allergy in children under 3 years of age [21]. More recently, an intact ovomucoid antigen was detected in 12 of 37 human breast milk samples as an immune complex with IgA, sug-

gesting an uptake of the antigen without degradation across the human intestinal epithelial pathway [22]. The results obtained in the present study revealed that ovomucoid was involved in the transepithelial passage through the Caco-2 monolayer, and showed that tight-junction permeability was enhanced in the presence of a food grade surfactant. TEER is a highly sensitive parameter for membrane permeability, particularly that related to membrane-perturbing toxicants [23, 24]. The effect was concentration dependent: the TEER decreased with increasing dosage of surfactant increasing the tight-junction permeability. Upon addition of the food allergen ovomucoid into monolayers previously challenged by surfactant, TEER dropped considerably further indicating transepithelial passage of the intact antigen from the apical to the basolateral region which was further confirmed by the transport flux and SDS-PAGE gel. The intestinal absorption of proteins is limited by a variety of factors, including gastrointestinal secretions (e.g. proteolytic enzymes), the peristaltic activity of the intestine, the properties of the epithelial membrane and the mucosal immune system [2]. It is also well known that food allergies are more prevalent in children due to an immature gastrointestinal epithelial membrane barrier, allowing more protein through the barrier and into circulation [25]. The uptake of protein by the epithelial barrier occurs via a number of pathways, including endocytosis and exocytosis, via specific receptors, and across the tight junctions of epithelial cells; however, these may vary depending on the specific protein and on the state of intestinal maturity [25]. The present

study clearly demonstrates that a food grade surfactant, such as sucrose monoester fatty acids, can increase the paracellular uptake of ovomucoid antigens. Caco-2 cell lines express at least eight aminopeptidases [26], but in the present study no remarkable hydrolysis of protein during incubation of ovomucoid with Caco-2 cells was observed, which indicates that ovomucoid is resistant to cellular peptidases.

LDH exists in the cytosol of intact cells, so that when the plasma membrane of a cell is damaged it is released to the outside of the cells. Therefore LDH activity is a good index for assessing the cytotoxic effect of food substances on intestinal cell lines, including Caco-2 cells [27]. The LDH assay is a simple method for assessing cytotoxicity or cell viability by detecting toxicants which affect membrane permeability. TEER and LDH activity were found to be negatively correlated. There have been previous reports that TEER is highly sensitive to cytotoxicity, particularly to that caused by membrane-perturbing toxicants since a decrease in TEER is a clear indication of increases in cell permeability caused by toxins enhancing tight-junction permeability [23]. Increased absorption of food allergens also dynamically regulates tight-junction

permeability. The surfactant induced shortening in microvilli, actin disbandment and structural separation of tight junctions, as observed microscopically. A number of studies have documented that surfactants increase the epithelial transport of drugs [28, 29]; however, in most studies relatively high surfactant concentrations (i.e. concentrations at which the surfactant solubilizes the cell membrane, causing cell death, cell extraction and increased permeability) have been used [8]. Our present study is the first to demonstrate that a relatively mild food grade surfactant which is widely used in the food industry and is present in various food products can increase the paracellular uptake of food allergens, which is relevant to normal human uptake mechanisms. One possible explanation is that the toxicity of such surfactants increases the uptake of food allergens in infants whose intestinal barrier is too immature to prevent the invasion of antigens.

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