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Imaging analysis of the gliadin direct effect on tight junctions in an *in vitro* three-dimensional Lovo cell line culture system

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ABSTRACT

Tight junctions play a pivotal role in maintaining the integrity of the intestinal barrier. Their alteration is involved in the pathogenesis of celiac disease. Our aim was to investigate the gliadin effect on the tight junction proteins in an *in vitro* three-dimensional cell culture model through imaging analyses.

Lovo multicellular spheroids were treated with enzymatically digested (PT) gliadin 500 μg/mL and its effect on actin, occludin and zonula occludens-1, was evaluated by means of confocal laser microscopy, transmission electron microscopy and image capture analysis.

Compared to untreated spheroids, PT-gliadin-treated ones showed enlargement of the paracellular spaces (9.0 ± 6.9 vs. 6.2 ± 1.7 nm, $p < 0.05$) at transmission electron microscopy and tight junction protein alterations at confocal microscopy and image analyses. In untreated cell cultures thickness of the fluorescence contour of actin, zonula occludens-1 and occludin appeared significantly larger and more intense than in the treated ones. In occludin planimetric analysis the lengths of the integral uninterrupted cellular contour appeared longer in untreated than in PT-gliadin treated spheroids (71.8 ± 42.8 vs. 23.4 ± 25.9 μm, $p < 0.01$).

Our data demonstrated that tight junction proteins are directly damaged by gliadin as shown by means of quantitative imaging analysis.

1. Introduction

Celiac disease (CD) is a common chronic enteropathy with a prevalence of about 1/200 in Western countries; it occurs in genetically predisposed subjects and is triggered by the ingestion of prolaminic peptides (gliadin) derived from bread wheat, rye and barley. CD main lesion consists in an architectural rearrangement of the intestinal mucosa characterised by villous atrophy, crypt cell hyperplasia, and lymphocytic infiltration of the lamina propria and epithelium (Elli and Bardella, 2005; Elli et al., 2009; Freitag et al., 2004; Green and Cellier, 2007; Koning et al., 2005; Schuppan et al., 2005). Although the immunological response appears pivotal in the CD pathomechanism, the early steps of these processes are still unclear, in particular, how gluten peptides cross the intestinal barrier (IB) and reach the lamina propria where immunological reaction begins (Koning et al., 2005). IB is the interface, formed by the enterocyte layer, between the luminal and submucosal compartments of the gastrointestinal tract and its integrity is mainly maintained by the tight junctions (TJs) (Stevenson et al., 1986; Turner, 2006). The adjacent cells of the intestinal mucosa are bound together by a complex of specialized proteins. The first TJ-associated protein to be identified was zonula occludens-1 (ZO-1) whose C-terminal half contains an actin-binding site and mediates interactions between transmembrane proteins and cytoskeleton elements. Occludin, a 60 kD integral membrane protein in TJ strands, functions through its four transmembrane domains and claudin-1 and claudin-2 are 23 kD integral membrane proteins that function as major structural components of TJ strands (Schneeberger and Lynch, 2004; Sears, 2000; Stevenson et al., 1986; Tavelin et al., 2003).

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In vitro studies showed that integrity of the IB is disrupted in CD patients, mainly through a damage of the TJ specialized proteins (Clemente et al., 2003, 2000; Montalto et al., 2002; Pizzuti et al., 2004; Schulzke et al., 1998; Sjolander and Magnusson, 1988).

The aim of this study was to investigate and compare by means of imaging techniques the direct effect of gliadin on TJ proteins and cytoskeleton in an organ-like three-dimensional model of cell culture.

2. Material and methods

2.1. Gliadin digestion

Gliadin was purified from *Triticum aestivum* flour (Hereward Cultivar, UK) according to Capelli (Capelli et al., 1998). Digestion was performed as previously described by our group (Dolfini et al., 2002). Briefly, the gliadin was first incubated with pepsin at 37 °C for 24 h, and then with pancreatin at 37 °C for 3 h, adjusting to a pH of 8. The digested protein (PT-gliadin) was analytically controlled by means of RP-HPLC, SE-HPLC, and SDS–PAGE, freeze-dried and stored.

2.2. Multicellular three-dimensional cultures (MCSs) and gliadin treatment

Lovo cells from the human colon adenocarcinoma (ATCC, Rockville, USA) were grown in T25 flasks (PBI, Italy) at 37 °C in a water-saturated atmosphere of 5% CO_2 and 95% air until confluence and cultured in medium as previously described (Dolfini et al., 2003; Roncoroni et al., 2008). Cells were weekly removed using solution containing 0.25% (w/v) trypsin and 0.02% (w/v) EDTA (Sigma–Aldrich, Italy) and the cell suspensions reseeded. Mycoplasma contamination was regularly excluded using the Hoechst method (Dolfini et al., 2003).

Three-dimensional cell cultures were initiated by seeding 4×10^5 cells/mL in 25 mL of complete medium and incubated in a gyratory rotation incubator (60 rev/min) at 37 °C in air (Colaver, Italy) as previously described (Dolfini et al., 2003). Cell clusters were visible after 4 days of culture, and the MCSs were usually complete within the seventh day (average diameter ± SD, 380 ± 49 μm). On the seventh day, the MCSs were treated with PT-digested gliadin (500 μg/mL) in a renewed medium containing PT-gliadin for further 4 days and subsequently taken for the evaluation of the endpoint parameters. The dose and the timing used were selected from four different concentrations (125, 500, 750, 1000 μg/mL) and on the basis of previous time course experiments conducted in our laboratory (Dolfini et al., 2002). The final dose (500 μg/mL) was chosen on the basis of its toxic effect without interfering in the formation of the MCS three-dimensional structure for a long exposure time. Seven independent series of experiments has been performed.

2.3. Transmission electron microscopy

The Lovo cell line spheroid samples were fixed in 2.5% glutaraldehyde in phosphate buffer (0.13 mol/L, pH range 7.2–7.4) for 1 h, and then washed in the same buffer. In order to avoid damaging or losing the MCSs, they were encapsulated in a liquid 2% agar solution solidifying at room temperature. The small MCS-containing cubes (about 1–3 mm) were normally handled and routinely processed for electron microscopy.

2.4. Intracellular F-actin analysis

Lovo MCSs were washed twice in PBS, fixed in 4% paraformaldehyde for 1 h, permeabilized with 0.4% Triton X-100 (Sigma–Aldrich, Italy) for 20 min, washed thrice for 5 min in PBS and stained for immunocytochemistry by means of incubation with TRITC-phalloidin (Sigma–Aldrich, Italy) (1:200 PBS) in a humid chamber at room temperature for 6 h. After washing thrice in PBS each for 5 min, 10 spheroids were transferred onto slides, and each slide was mounted with 90% glycerol in PBS. The excitation wavelength was 568 nm and emission 590 nm. The results were analyzed using a confocal laser scanning microscope (Leica TCSNT, Germany) LP590 filter.

2.5. Occludin and zonula occludens-1 analysis

Lovo MCSs were washed twice in PBS and fixed in ethanol for 30 min at 4 °C. After the first incubation, the samples were incubated with acetone (previously stored at −20 °C) for an additional 3 min at room temperature. They were then blocked and incubated for immunocytochemistry overnight with anti-occludin-FITC or anti-ZO-1-FITC (Zymed, CA, USA) (excitation wavelength was 488 nm, emission 530 nm), before being analyzed by means of confocal laser scanning microscopy (Leica TCSNT, Germany) BP530/30 filter.

2.6. Image capture and analysis

TEM images were randomly and blindly acquired by an expert pathologist (PB). Twenty paracellular spaces were measured evaluating the orthogonal distances between the external surfaces of the lateral plasmatic membranes of two adjacent cells, taken every 20 nm.

For confocal microscopy, after localization of fluorescence signals in the cell cultures, multiple adjacent high-power fields in each section corresponding to the MCSs were selected, acquired, and stored on a personal computer (Power Mac G4, 867 MHz, 640 MB RAM, Apple, Cupertino, CA) in JPEG format (5:1) at 32 bits/pixel on a 764–560 pixel matrix. Stored images were analyzed to determine the differences between PT-gliadin treated vs. untreated MCSs.

The intensity and fluorescence patterns of the PT-gliadin treated vs. untreated MCSs were evaluated. Before the analysis at least seven cells were identified in each power field by the experienced pathologist. Intensities based on a scale of 256 levels (0–255) were assessed along a linear sample perpendicular to the fluorescent contour staining of the cell for a total 100 measures.

Since in occludin analysis, differently from actin and ZO-1, several discontinuities in the pattern of the fluorescent contour staining were present, we measured, in 10 randomly selected images, the lengths in μm of the continuities by planimetry including once each fluorescence contour representative of a cellular interplay.

2.7. Statistical analysis

The data were analyzed using the paired Student *t*-test. The results are expressed as mean values ± SD, and a p-value of <0.05 was considered significant.

3. Results

At phase-contrast microscopy untreated and 4 days PT-gliadin (500 μg/mL) treated MCSs were similar in shape (bright-round) and size (371 ± 46.5 vs. 376 ± 22.2 μm, respectively).

With TEM, untreated MCSs showed a free external cell surface with short microvilli and adjacent cells were joined by complexes of TJs. In the cytoplasm there were pseudolumina with microvilli, frequent bundles of cytokeratin tonofilaments, and organelles (mitochondria, rough endoplasmic reticulum, Golgi complex and