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Protective effects of mannan in Caco-2/TC7 cells treated with wheat-derived peptides

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Abstract

Celiac disease (CD) is characterized by a permanent intolerance to wheat gliadin and related proteins in genetically susceptible individuals. It is generally considered that CD is an immuno-mediated multifactorial disease, but a direct cytotoxic activity of gliadin-derived peptides (GL-PT) on intestinal mucosa cannot be excluded. Many efforts have been done to identify possible antagonists of this direct toxicity and several studies indicated that mannan and oligomers of N-acetylglucosamine, [*N*,*N*′-diacetylchitobiose (GLcNAc)2 and *N*,*N*′, *N*″;-triacetylchitotriose (GLcNAc)3], could be very promising candidates.

In the present study we investigated the ability of mannan, (GLcNAc)2 and (GLcNAc)3 to interfere with some toxic effects exerted by GL-PT, as cell growth and viability impairment, increased intestinal permeability and cellular inflammation, on a clone of the human intestinal Caco-2 cell line, Caco-2/TC7, expressing a more homogeneous population than the parental one.

Our present results demonstrate that mannan, among the three molecules investigated, is the most suitable to counteract the adverse effects induced by GL-PT on Caco-2/TC7 cells, for all the parameters considered in this study.

Keywords: Celiac disease; Caco-2/TC7 cells; Gliadin-derived peptides; Mannan

1. Introduction

Celiac disease (CD), or gluten sensitive entheropathy, is characterized by a permanent intolerance to wheat gliadin and related proteins in genetically susceptible individuals. Many theories have been formulated in the past years to explain CD pathogenesis, such as peptidase deficiency, lectin-like behaviour (Kolberg & Sollid, 1985) permeability defect, and immunological model. Various assays, reflecting the diverse set of hypothesis have been developed to obtain results supporting the different theories (McAdam & Sollid, 2000). Although it is generally accepted that CD is a multifactorial disease and that an altered immune response is involved in its progression (Sollid, 2000), a non-immuno-mediated, direct cytotoxic activity of gliadin-derived peptides on the intestinal mucosa cannot be excluded (Maiuri et al., 1996). According to this last hypothesis, gliadins interact with glycosidic residues lectin binding sites situated on the enterocytes membranes causing damage to the base of the villi which includes disarrangement of the cytoskeleton, increased endocytosis and a shortening of the microvilli (Pusztai et al., 1993). The structural changes induced by wheat-germ agglutinin on intestinal epithelial cells elicit functional changes, including increased permeability, which may facilitate the passage of dietary antigen causing, in genetically susceptible individuals, an antigenic stimulation and the breaking of the immunological tolerance (Cordain, Toohey, Smith, & Hickey, 2000).

In the past years, it was observed that bread wheat gliadin peptides exert a reversible agglutinating activity on undifferentiated human K 562 cells (Auricchio et al., 1984; Auricchio, De Ritis, De Vincenzi, & Silano, 1985;

Abbreviations CD, celiac disease; CP3, caspase 3; FCS, foetal calf serum; (GLcNAc)2, *N*,*N*′-diacetylchitobiose; (GLcNAc)3, *N*,*N*′,*N*″-triacetylchitotriose; GL-PT, gliadin peptic–triptic digest; HEPES, *N*-(2-hyroxyethyl)piperazine-*N*′-2-etane-sulfonic acid; TEER, trans epithelial electrical resistance.

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De Vincenzi, De Ritis, & Dessi, 1993; De Vincenzi, Dessi, Giovannini, Maialetti, & Mancini, 1995) and that mannan and oligomers of *N*-acetylglucosamine, *N*,*N*′-diacetylchitobiose (GLcNAc)2 and *N*,*N*′,*N*″-triacetylchitotriose (GLcNAc)3 were very active in preventing such effect (Auricchio et al., 1987)

Moreover, mannan and its oligomers exhibited a protective effect on intestinal mucosa specimens of patients with active celiac disease cultured in presence of wheat-derived peptides (Auricchio et al., 1990), suggesting that the agglutinating and toxic peptides are bound by carbohydrates, and that some sugars may exert a protective effect on the toxic activity of cereal prolamin peptides in human celiac intestine.

Mannans are complex polymers containing mannose found in bacteria, molds, higher plants and yeast, where represent the major structural components (45% in *Saccharomyces cerevisiae*). Mannan has shown to possess a broad range of biological activities when administered to man and animals, thus is considered as potential therapeutic agent (Tizard, Carpenter, Mc Analley, & Kemp, 1989). It has been demonstrated that mannan oligosaccharides behave as prebiotics, due to their stimulating effect on villi development of growing turkeys and pigs (Hooge, 2004) and are able to decrease the colonization of enteric pathogens such as *Salmonella*, *E. coli* or *Campilobacter* and improve intestinal health in different species of animals (Spring, Wenk, Dawson, & Newman, 2000).

In the present study we investigated the ability of mannan, (GLcNAc)2 and (GLcNAc)3 to interfere with the toxic effects exerted by the gliadin-derived peptides (GL-PT) on a clone of the human intestinal Caco-2 cell line (Caco-2/TC7) (Chantret et al., 1994). In fact, despite their tumoral origin, Caco-2 cells are a widely used intestinal in vitro model for their ability to mimic the absorptive epithelium and to reach a good morphological and functional differentiation. In particular, Caco-2/TC7 clone has been chosen for its homogeneous population, together with a more closely resemblance of barrier properties to the small intestinal epithelium in vivo (Sambuy et al., 2005). In order to investigate if mannan and its oligomers can interact with gliadin adverse effects on intestinal epithelial cells we have utilized Caco2/TC7 cells, in undifferentiated and differentiated phases of growth, to simulate some features of coeliac disease. Cell growth and viability impairment, increased intestinal permeability and cellular inflammation have been considered for this purpose.

2. Materials and methods

2.1. Materials

All tissue culture media were obtained from Gibco BRL (Gaithersburg, MD, USA). Culture vessels were purchased by Falcon (Beckton-Dickinson, Labware, NJ). Mannan (from *S. cerevisiae*), (GLcNAc)2, (GLc-NAc)3, Actinomycin D were purchased from Sigma Chemical Co. (St Louis, MO, USA).

2.2. Cell cultures

Human intestinal Caco-2/TC7 clone was a kind gift of Dr Rousset at INSERM, Paris, France. Cells were grown and maintained as previously described (De Angelis, Vincentini, Brambilla, Stammati, & Zucco, 1998) in Dulbecco modified essential medium containing 4.5 g/l glucose, supplemented with 2 mM L-glutamine, 50 U/ml penicillin, 50 ug/ml streptomycin, 1% non-essential aminoacids, 1% HEPES and 10% heat inactivated FCS. Experiments were performed on passages from 80 to 95.

2.3. Preparation of gliadin peptic–triptic digest (GL-PT)

Pure exaploid bread wheat (*Triticum aestivum*, S. Pastore variety) was kindly supplied by the Istituto Sperimentale per la Cerealicoltura, Rome, Italy. Gliadin was extracted as previously described by Auricchio et al. (1984). The protein fraction was submitted to peptic–triptic sequential digestion according to De Ritis et al. (1979). At the end of the whole procedure, GL-PT digest was heated for 30 min at 100 °C, lyophilised, and stored at −20 °C until use. GL-PT digest was dissolved in the culture medium and sterilized by filtration through a 0.45 μm Millipore membrane. The peptic–triptic BSA digest was used as negative control. The protein content of GL-PT digest was determined according to Lowry, Rosebrough, Farr and Randall (1951).

2.4. Exposure conditions

Treatments have been performed with GL-PT (1 mg/ml), mannan (1.5 mg/ml), (GLcNAc)2 (1.5 mg/ml), (GLcNAc)3 (0.65 mg/ml) and GL-PT combined with each of the three sugars dissolved in culture medium.

2.5. Cell viability

Caco-2/TC7 cells were seeded at a density of 5×10^3 cells/well in a 96 multiwell plate. Twenty-four hours after seeding, culture medium was changed and cells were exposed for 48, 72 and 96 h as reported in exposure conditions. Cell viability was measured as Neutral Red uptake, according to Borenfreund and Puerner (1985) and spectrophotometrically detected by a microplate reader (Novapath, Biorad, Hercules, CA) at a wavelength of 540 nm.

2.6. Evaluation of apoptotic cells

Caco-2/TC7 cells were seeded at a density of 3.2×10^3 cells/cm^2 and grown in 100 mm Ø plates at 37 °C in an atmosphere of 5% CO_2.

On the fifth day after plating, cells were washed with serum free medium and treated, as reported in exposure conditions, for 24 h in complete culture medium with 5% FCS.

Actinomycin D (1 mg/ml) and untreated cells were used as positive and negative control respectively, to determine either the maximum level of apoptosis induction or the spontaneous apoptosis in Caco-2/TC7 cells.

Apoptosis was evaluated by the Caspase 3 Colorimetric Assay Kit (CASP-3-C, Sigma), based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide (Ac-DEVD-pNA) by caspase 3, resulting in the release of the *p*-nitroaniline (*p*NA) moiety, which has a high absorbance at 405 nm ($\varepsilon_{mM} = 10.5$). The concentration of the pNA released from the substrate is calculated from the absorbance values at 405 nm using a calibration curve of defined pNA solutions. Caspase 3 activity was expressed as μmol *p*NA released/min/ml of cell lysate.

2.7. Measurement of trans epithelial electrical resistance (TEER)

Trans epithelial electrical resistance (TEER) of Caco-2/TC7 cell monolayers was measured using a Millicell ERS device (Millipore Co., Bedford, MA, USA).

Cells were seeded at a density of 4.4×10^4 cells/cm^2 on polycarbonate inserts (0.45 μm pore diameter, 0.9 cm^2 area) and were left to differentiate for 19 days; medium was regularly changed three times a week. TEER was expressed as Ohms $\times$ cm^2, subtracting from each point the TEER value of the filter alone. TEER data were recorded at room temperature 4 and 24 h after treatment, performed as previously reported in the exposure conditions.

2.8. Nitric oxide release

Caco-2/TC7 cells were plated at a density of 2.5×10^4/well and grown on 24 multiwell plate for 7 days. Following 24 h of incubation at 37 °C in complete medium without phenol red, in the reported exposure conditions, 500 μl of culture medium were taken from the plates and mixed with an equal volume of Griess reagent (1% sulfanilic acid in 0.5N HCl and 0.1% *N*′-1-naphtyl-ethylendiamine-hydrochloride). Absorbance at 540 nm was measured after 30 min of incubation at room temperature in the dark. The combined concentrations of NO^{2-} and NO^{3-}, the degradation products of the nitric oxide in the culture medium, were determined by the Griess reaction (Green et al., 1982). The nitrite concentration was determined by reference to a standard curve of sodium nitrite and values were reported as μM/mg proteins/ml.

2.9. Statistical analysis

Each experiment was run in duplicate and repeated three times. The data are expressed as mean +/− SD. Data were analysed using the two sided Student test for unpaired variables ($P < 0.05$; $P < 0.01$).

3. Results and discussion

3.1. Cell growth

As reported in the literature, a reduction of cell viability has been demonstrated with digests from wheat gliadins (GL-PT) in different cell lines (Giovannini, Mancini, & De Vincenzi, 1996; Hudson, Cornell, Purdham, & Rolles, 1976; Rocca, Stammati, Zampaglioni, & Zucco, 1983; Stammati, Zucco, Cardelli, De Angelis, Mattei and Pino, 1985). These results are confirmed in the present paper, where a reduction in Neutral Red uptake, corresponding to a decrease of cell viability, was already evident after 48 h of GL-PT treatment, while mannan alone, as well as its oligomers, behaved as the control and did not alter cell growth during the whole treatment period.

Among the different sugars utilised in the present experiment, the most efficient in preventing GL-PT viability impairment on Caco-2/TC7 cells, was mannan followed by (GLcNAc)2 and (GLcNAc)3 (Fig. 1).

3.2. Apoptosis

Formation of apoptotic cells in the intestine in vivo is physiological and restricted to the tips of the villous, but in

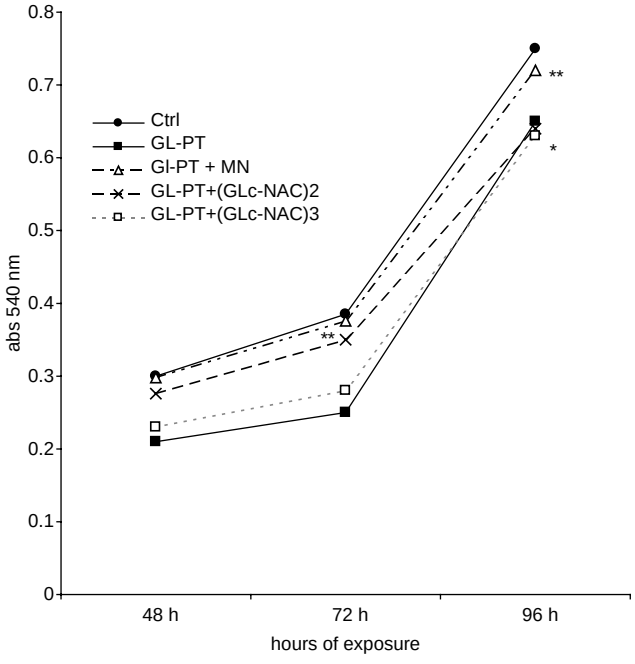

Fig. 1. Viability effects of GL-PT (1 mg/ml), and GL-PT combined with each of the three sugars (MN 1.5 mg/ml; $(GLc\text{-}NAC)_2$ 1.5 mg/ml; and $(GLc\text{-}NAC)_3$ 0.65 mg/ml) on Caco-2/TC7 cells, determined by NRU assay. Data are the mean of three separate experiments performed in duplicate. *$P < 0.01$ in respect to the control. **$P < 0.01$ in respect to the GL-PT treated cells.

Table 1
Caspase-3 activity of Caco-2/TC7 cells after 24 h of treatment

	CP3 activity (nmol/min/ml)
Ctrl	0.093 ± 0.007
GL-PT	0.116 ± 0.009*
Mannan	0.095 ± 0.012
(GLcNAc)2	0.094 ± 0.011
(GLcNAc)3	0.093 ± 0.007
GL-PT + mannan	0.087 ± 0.005
GL-PT + (GLcNAC)2	0.078 ± 0.006**
GL-PT + (GLcNAC)3	0.074 ± 0.005**

All values are average from three separate experiments performed in duplicate ± S.D. *$P < 0.01$ in respect to the control. **$P < 0.01$ in respect to the treated GL-PT cells.

pathological conditions, as celiac disease, this phenomenon is enhanced (Iwamoto, Koji, Makkiyama, Kobayashi, & Natane, 1996; Moss, Attia, Scholes, Walters, & Holt, 1996.). An increase of apoptotic cell formation was detected also in vitro, in Caco-2 cells treated with GL-PT and the CD95/Fas apoptotic pathway has shown to be involved (Giovannini, Matarrese, Scazzocchio, Varì, D'Archivio and Straface, 2003). Caspase-3 is a member of the CED-3 subfamily of caspase enzymes, that plays a pivotal role in the apoptotic processes as chromatin condensation and DNA fragmentation, as well as cellular blebbing (Porter & Janicke, 1999). In the present study, only GL-PT significantly increased caspase-3 activity in respect to the control after 24 hours exposure When Caco-2/TC7 cells were co-exposed to (GLcNAc)2 and (GLc-NAc)3 plus GL-PT, this combination was able to significantly revert the GL-PT apoptotic effect. Co-treatment with GL-PT and mannan gave a similar result, even if to a lesser extent Table 1).

3.3. Trans epithelial electrical resistance

Permeability plays a key role in the intestinal function, allowing selective processes of absorption and secretion of orally ingested molecules. TEER determination is routinely used to monitor the permeability of intestinal epithelial cell monolayers, because it has shown to be a simple and rapid indicator of barrier integrity (Artursson, 1990).

In our experiments, Caco-2/TC7 cells treated with GL-PT for 4 h showed a significant TEER decrease, indicating an impairment of the epithelial barrier, which remained constant for the following 24 h. That is in agreement with the results of El Asmar et al. (2003), who observed that GL-PT caused a decrease of TEER in Caco-2 cells after 3 hours exposure

In this study, the GL-PT adverse effect was partially reverted by GL-PT/mannan co-exposure, while (GLcNAc)2 and (GLcNAc)3, plus GL-PT, seemed to be less active on this respect (Table 2).

Table 2
TEER values (ohms cm^2) of Caco-2/TC7 cells after 24 and 48 h of exposure

	4 h	24 h
Ctrl	343.6 ± 43.2	389.1 ± 43.3
GL-PT	268.7 ± 65.5*	270.1 ± 77.5*
Mannan	373.0 ± 21.3	417.1 ± 34.5
(GlcNAc)2	351.3 ± 34.2	349.9 ± 32.1
(GlcNAc)3	332.4 ± 37.2	380.0 ± 35.4
GL-PT + mannan	331.0 ± 41.6**	35.2 ± 45.1**
GL-PT + (GlcNAc)2	275.0 ± 45.3	280.0 ± 45.7
GL-PT + (GlcNAc)3	259.6 ± 56.1	250.0 ± 58.9

Data are the the mean of three separate experiments performed in duplicate ± S.D. *$P < 0.01$ in respect to the control. **$P < 0.01$ in respect to the GL-PT treated cells.

3.4. Nitric oxide release

In enterocytes isolated from biopsies of celiac patients, an increase in activity and protein expression of the inducible isoform of nitric oxide synthase (iNOS) was detected (Murray, Daniels, Coupland, Smith, & Long, 2002; ter Steege, Burman, Arends, & Forget, 1997).

It has also been demonstrated that the constitutive isoform of nitric oxide synthase (cNOS) and iNOS are expressed in several intestinal cell lines, including Caco-2 cells. Recent studies have confirmed and further characterized iNOS expression in Caco-2 cells during the differentiation process and following treatment with inflammatory molecules, such as IFN-γ and PMA, natural toxins (Vignoli et al., 2001), or Cholera toxin (Berni Canani et al., 2003).

In this work, a significant increase of nitric oxide release in the culture medium of Caco-2/TC7 cells exposed for 24 h to GL-PT has been detected (Table 3). Cells co-treatment with GL-PT and mannan restored the basal levels of nitric oxide production, while the two oligomers did not interact in a significant manner

Table 3
Nitric oxide release in the medium of Caco-2TC/7 after 24 h of treatment

	μM/mgr/prot/ml
Ctrl	4.2 ± 0.5
GL-PT	6.0 ± 0.6*
Mannan	4.0 ± 0.3
(GLcNac)2	4.3 ± 0.6
(GLcNac)3	4.8 ± 0.8
GL-PT + mannan	4.8 ± 0.6**
GL-PT + (GLcNac)2	5.7 ± 0.9
GL-PT + (GLcNac)3	5.6 ± 0.9

Data are the the mean of three separate experiments performed in duplicate ± S.D. *$P < 0.01$ in respect to the control. **$P < 0.01$ in respect to the GL-PT treated cells.

4. Conclusions

In this study it was investigated if mannan and *N*-acetyl glucosamine oligomers [(GLcNA)2 and (GLcNA)3], were able to contrast GL-PT adverse effects on some crucial parameters of intestinal impairment in Caco-2/TC7 cells. The following conclusions can be drawn:

(i) GL-PT alters cell growth, nitric oxide production and caspase-3 activity of Caco-2/TC7 cells, according to what previously reported (Silano, Vincentini, Muzzarelli, Muzzarelli, & De Vincenzi, 2004). Moreover, GL-PT causes an alteration of the barrier integrity of differentiated cells grown on semipermeable filters, in particular of the ionic conductance of the paracellular pathway indicated by TEER reduction.

(ii) Mannan is able to counteract the GL-PT adverse effects observed in Caco-2/TC7 cells, displaying its effectiveness for all the parameters studied. These results are in agreement with the protective properties reported on intestinal mucosa specimens of patients with active celiac disease, cultured in presence of wheat GL-PT (Auricchio et al., 1990) and its ability to prevent the agglutinating activity of GL-PT in K562 cells (De Vincenzi et al., 1995).

(iii) Differently from what reported by Auricchio et al. (1990), in intestinal mucosa of celiac patients cultured with GL-PT, in the present experimental condition (GLcNAc)2 and (GLcNAc)3 are less effective than mannan in preventing GL-PT adverse effects in Caco-2/TC7 cells.

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