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SCIENCE DIRECT
Carbohydrate Polymers 58 (2004) 215–219
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

MP-Chitosan protects Caco-2 cells from toxic gliadin peptides

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Received 2 April 2004; accepted 30 June 2004
Available online 31 July 2004

Abstract

Caco-2 human intestinal cell line was exposed, both separately and simultaneously, to gliadin pepsin-tryptic digest and MP-chitosan. MP-Chitosan was able to depress the gliadin toxicity, and to lower caspase-3 activation and apoptosis, nitric oxide production, and lactate dehydrogenase release by the cells, whilst improving the cell viability. The interaction between MP-chitosan and mannose-receptor might account for some of these effects.

Keywords: Celiac disease; Chitosan; Cell toxicity; Apoptosis; Gliadin

1. Introduction

The celiac disease is an enteropathy caused by permanent intolerance to gliadin, the alcohol-soluble protein fraction of wheat, in genetically predisposed individuals (Catassi et al., 1994; Goggings & Kelleher, 1994; Sollid, 2000). The celiac disease in his typical form is histologically characterised by villi atrophy, hyperplasia of the crypts, and lymphocytes infiltration of the lamina propria in the small bowel. These histological alterations lead to serious malabsorption of nutrients, vitamins and minerals (Abdulkarim & Murray, 2003). Various hypotheses have been proposed to explain the pathogenesis of the celiac disease. Although, it is largely accepted that an altered immune response in involved in pathogenesis, some reports suggest that gliadin could have a direct toxic effect on the intestinal mucosa (Maiuri et al., 1996; Pittschieler, Ladinser, & Petell, 1994). According to this theory, gliadin acts as a lectin able to bind to glycosylated residues, called 'lectin binding sites' expressed by enterocytes and some other cells, and initiates an inflammatory response (Damjanov, 1987; Kolberg & Sollid, 1985). In fact, it has been demonstrated that the incubation of Caco-2 with gliadin resulted in membrane damage, impairment of cell proliferation and induction of apoptosis (Giovannini et al., 2003).

Mannan and oligomers of *N*-acetyl-D-glucosamine exert protective effect on intestinal mucosa specimens of patients with active celiac disease, when they are cultured with wheat gliadin pepsin-tryptic digest (Auricchio et al., 1990). Therefore, we hypothesize that chitosan might protect the intestinal cells from gliadin toxicity in a manner similar to mannan and oligomers of *N*-acetyl-D-glucosamine.

The current literature provides information about the oral administration of chitosan for a number of purposes, ranging from the management of hypercholesterolemia (Muzzarelli, 2000) to drug delivery to the intestine (Muzzarelli, 1999), but no information is available on the consequences of the chitosan administration to celiac patients nor on the protecting effect of chitosan on the human intestinal cells exposed to gliadin. Previous work by DeVincenzi, Dessì and Muzzarelli (1993) was confined to the demonstration that 5-methylpyrrolidinone chitosan (MP-chitosan) reacts with the gliadin-derived peptide fraction responsible for the agglutination of K562(S) cells.

Chitosan, the copolymer of (1,4)-linked *N*-acetyl-β-D-glucosamine and glucosamine, is widely studied for its anti-inflammatory properties (Jollès & Muzzarelli, 1999;

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doi:10.1016/j.carbpol.2004.06.045

Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Tz-Chong, Fu, & Shen, 2003), and other aspects of interest in biomedical applications; one modified chitosan is MP-chitosan, widely assessed as a biocompatible polysaccharide (Muzzarelli, 1991, 1992, 1996; Muzzarelli, Ilari, & Tomasetti, 1993). The binding of chitosan to mannose receptor on cell membrane may be crucial for the regulation of certain biochemical activities of chitosan (Mori et al., 1997; Shibata, Metzeger, & Myrvik, 1997).

Caco-2 cells (human colon carcinoma) are, at present, one of the best in vitro systems to mimic the intestinal mucosa. Despite their neoplastic origin, these cells are able to differentiate spontaneously to mature enterocytes after 21 days of culture and express the brush border enzymes. Furthermore, when cultured under specific conditions, they display the properties of an epithelial barrier (De Angelis, Vincentini, Brambilla, Stammati, & Zucco, 1998; Pinto et al., 1983; Zucco et al., 1994).

The intestinal cells themselves are able to show an inflammatory response to an injury (Molmenti, Ziambaras, & Pelmutter, 1993). Evidence for an acute phase response in inducing apoptosis pathways (Giovannini et al., 2003) and increasing nitric oxide production by nitric oxide synthetase activation (De Ritis et al., 1979). These parameters along with cell viability and LDH production, an index of cell necrosis, would be convenient for the evaluation of the PT–GL damage and the protective effect of chitosan.

Aim of this work is to assess the protection Caco-2 cells from gliadin toxicity and define the mechanisms by which chitosan interferes with the gliadin noxious action on intestinal cells. In order to achieve this, the Caco-2 human intestinal cell line was exposed to gliadin; to PT-digest and to gliadin PT-digest admixed with chitosan. Then, the following determinations were carried out: (i) cell viability; (ii) LDH production by the cells as measure of cell necrosis; (iii) caspase-3 activity in the cells as an index of cell apoptosis; and (iv) NO release in the cell culture medium as a measure of cellular inflammation.

2. Materials and methods

2.1. Caco-2 cells

Human intestinal Caco-2 cells (TC7 clone) were kindly donated by Dr Stammati, Istituto Superiore Sanità, Rome. Cells were routinely grown and maintained in 25 cm^3 culture flasks at 37 °C in a humidified atmosphere of 5% CO_2 in Dulbecco Modified Eagle's Medium with glucose (4.5 g/l), supplemented with 2 mM L-glutamine, 1% non-essential amino-acids, 50 U/ml penicillin, 50 ug/ml streptomycin and 10% thermally inactivated fetal calf serum. All of the materials were obtained from Hyclone (Cramlington, UK). Cells were routinely passed every 7 days (Falcon, Free Lake, NJ) and the medium changed at least twice a week. Passages between 60 and 85 were used for the experiments. For the experiments described in this paper, Caco-2 cells were used after 5 days of culture, because they are more sensible to PT–GL toxicity when they still are in the undifferentiated stage (Pinto et al., 1983).

2.2. Chitosan

Methylpyrrolidone chitosan (MP-chitosan) (degree of acetylation = 23%, MW = 20,000 Da) was prepared from *Crangon crangon* chitin according to a proprietary protocol (Muzzarelli, 1991, 1992, 1996). A stock solution of MP-chitosan dissolved at 1 mg/ml was prepared in 0.01 N acetic acid. MP-Chitosan was used for the experiments at the concentration of 5 μg/ml. Preliminary experiments (data not shown) were performed to evaluate the optimal concentration, since it has been shown to be toxic for the cells at high concentrations.

2.3. Gliadin peptic-triptic digest

The gliadin fraction was extracted from whole cereal flour of bread wheat (*Triticum aestivum*, San Pastore variety obtained from Istituto per la Cerealicoltura, Rome). The gliadin fraction was subjected to digestion with pepsin and trypsin, according to De Ritis et al. (1979), to obtain the gliadin pepsin-triptic digest (GL–PT). The latter were heated for 30 min at 100 °C, lyophilized and stored at −20 °C. The PT digest was dissolved in DMEM at the concentration of 1 mg/ml and filtered through 0.45 nm filters.

2.4. Cell viability

For this determination, 5×10^3 cells per well were seeded in 96 multiwell plates. After 24 h, the medium was removed and cells were treated with chitosan, PT–GL, and chitosan in admixture with PT–GL, for 24, 48 and 72 h. The reduction of the cell growth rate was determined by uptake of the vital dye Neutral Red. The dye present in viable cells was retrieved by extraction and measured spectrophotometrically at the wavelength of 540 nm by a microplate reader (Novapath, Biorad, Hercules, CA).

2.5. Lactate dehydrogenase release

The cells were seeded at the density of 50×10^3 in 6-well plates. Five days after seeding, at preconfluency, the cells were incubated for 48 h with GL–PT, MP-chitosan or both. The culture medium was collected and the LDH content was measured by a colorimetric assay. This assay is based on the reduction of NAD by the LDH. Lactate dehydrogenase release was expressed as the percentage of release in the medium above total LDH production by the cell. This was obtained by treating the cells with triton-x-100 (Sigma TOX-7). Finally, the LDH production was corrected for spontaneous release of LDH by the untreated cells.

2.6. Nitric oxide determination

Cells were seeded at the density of 30×10^3 in a 24-well plate, using DMEM without phenol red. On day 5 of culture, cells were exposed to GL–PT, interferon-gamma, MP-chitosan for 48 h. The NO production was measured in culture medium by a spectrophotometric assay based on the Griess reaction. The Griess reagents (1% sulphanilic acid in 0.5 N HCl, 0.1% naphthyletylenediamine) were added to an equal volume of cell culture medium and absorbance at 540 nm was measured after 1 h. The nitrite concentration was calculated with respect to a standard curve of sodium nitrite.

2.7. Caspase-3 activity

Each experiment was conducted by seeding 80×10^4 cells in 75 cm^3 flasks and grown in DMEM. On day 5, cells were treated with the different compounds for 24 h, then the medium was collected and the cells harvested. To evaluate quantitatively the apoptosis in treated Caco-2 cells, a colorimetric assay (Sigma CASP-3-C) has been used according to the manufacturer's instructions. The assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp *p*-nitroanilide by caspase-3, resulting in release of *p*-nitroaniline, that exhibits high absorbance at 405 nm. Measurements were standardized to cellular protein amount and expressed as percent of apoptotic cells above untreated control.

2.8. Statistical analysis

Each experiment was performed at least three times. 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 viability

The cell viability was measured as neutral red uptake. The incubation with PT diminished significantly the cell viability, expressed as OD. This finding is very evident after 48 h of treatment and it is still relevant after 72 h of incubation. Chitosan exposure at the concentration of 5 μg/ml did not reduce the cell viability compared to the control during the 72 h interval of the experiment. When the cells were exposed simultaneously to chitosan and PT, the cell viability curve is similar to that of untreated cells, resulting above 95% compared to the control (Fig. 1).

3.2. LDH release

LDH release in the culture medium was measured as an index of cell necrosis. The 48 h incubation of cells with PT determined a very slight increase of LDH release (about 15% compared to the control). No significant induction of LDH release was detected after treatment with chitosan, as well. The simultaneous exposure to chitosan and PT digest did not increase the LDH formation compared to the other treatment (Fig. 2).

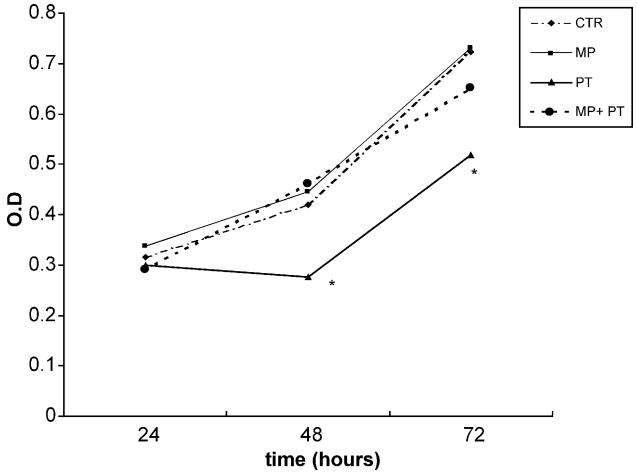

Fig. 1. Cell viability measured as Neutral Red uptake after 24, 48 and 72 h of incubation with gliadin peptic tryptic digest (GL–PT) (1 ug/ml), methylpyrrolidinone chitosan (MP) at 5 μg/ml, or both together. Asterisks indicate significant differences from control (*$P<0.001$).

3.3. Cell apoptosis

To evaluate quantitatively the apoptosis in Caco-2 cells, the caspase-3 activity, a crucial mediator of programmed cell death, was measured. After 24 h treatment, the caspase-3 activity significantly increased with respect to the control, showing that GL–PT induced the apoptosis pathway in the Caco-2. The 24-h incubation with chitosan produced a 25% increment of caspase activity compared to the untreated cells, whereas after the incubation with staurosporine, used as positive control since it is an apoptosis inducer,

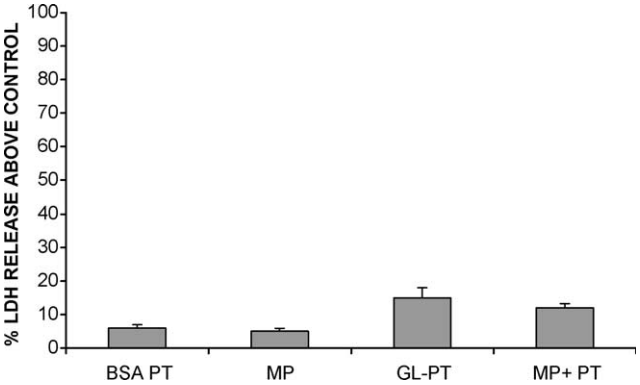

Fig. 2. LDH release in the culture medium expressed as % above control after 48 h of treatment. Values represent the result of three independent experiments, each performed in duplicate, and values are expressed as the mean OD±SD) in terms of percentage of controls.

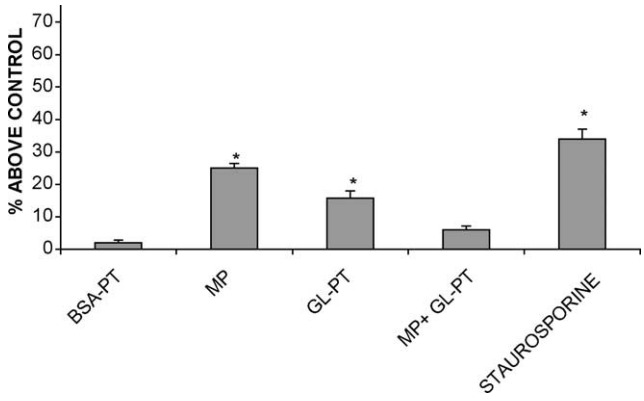

Fig. 3. Caspase activity after 24 h of incubation. Each column represents the mean (+SD) of three different experiments performed in duplicate. Asterisks indicate significant differences from control ($^{*}P<0.001$). Staurosporine of 1.5 μM was used as an apoptosis-inducing agent.

an increment of caspase-3 activity of 30% was noticed. On the other hand, bovine serum albumin (BSA) PT-digest, used as negative control, did not show a significant induction of caspase-3 activity (Fig. 3).

3.4. Nitric oxide production

Nitric oxide has been demonstrated to have an important role in the pathogenesis of the histological features of the celiac disease. It has been shown that Caco-2 are able to produce NO in the presence of a noxious stimulus (Vignoli et al., 2001) and furthermore, some cells produce NO after exposure to GL–PT-digest (Maiuri et al., 2003). Therefore, we carried out the measurement of NO release by the cells in the culture medium after exposure to PT–GL and chitosan as index of cell inflammation, using a spectrophotometric assay based on Griess reaction. As shown in Fig. 4, 48-h incubation with GL–PT induced NO release compared to the control. Chitosan increased the NO formation, as well. The simultaneous treatment of the cells with chitosan and PT resulted in a significant reduction of NO production, even less than that of the untreated cells. The treatment with interferon-gamma, a NO inducer used as a positive control, showed an important production of NO.

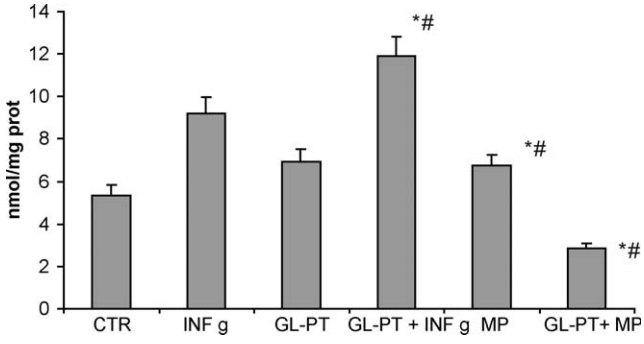

Fig. 4. Nitric oxide production after 48 h of incubation. Each bar represents the mean (+SD) of three different experiments performed in duplicate. Asterisks indicate significant differences vs. control ($^{*}P<0.001$) or vs. GL–PT ($^{\#}P<0.001$). Interferon-gamma, a NO-inducing agent, was used at the concentration of 25 μIU.

4. Conclusions

In this paper, we demonstrate that chitosan protects Caco-2 cells from gliadin peptide toxicity. Our results show that caspase-3 activity, a crucial mediator of programmed cell death, is enhanced in Caco-2 cells by both chitosan and PT, when incubated separately. On the other hand, when the cells are exposed to the mixture of MP-chitosan and PT, a significant decrease of caspase-3 activity is detected.

These data are in agreement with the previous finding that GL–PT induces apoptosis in intestinal cells via caspase-3 (Hasegawa, Yagi, Iwakawa, & Hirai, 2001).

Apoptosis of enterocytes is a major mechanism responsible for the damage of grains on intestinal mucosa of celiac subjects and it is pitoval in the resulting villous atrophy and hyperplasia of the crypt (Giovannini et al., 2003). Up to now, data about the effects of chitosan on intestinal cells are mostly about the tight junction opening and permeability to drugs (Holme, Hagen, & Dornish, 2000) whereas there are several experimental evidences that it induces apoptosis in bladder cancer cells and activation of arginine metabolic pathways in macrophages (Porporatto, Bianco, Riera, & Correa, 2003). The effects of MP-chitosan in promoting apoptosis in a human intestinal cell line is noteworthy.

The cell growth curves also underline that chitosan has very low toxicity, since the viability of chitosan-treated cells after 48 h is similar to that of the controls.

The LDH release, a marker of cell necrosis, is a further confirmation that chitosan is harmless to the cells. The fact that LDH content in the medium does not increase when the cells are exposed to PT digest shows that its toxic effect on the intestinal cells is mediated by apoptosis, and no necrotic event occurs.

Whereas the mechanism of the protective activity of chitosan is still unclear, an interference on the interaction between GL–PT and cell membrane is likely to occur: this hypothesis is supported by studies showing that a column of mannan is able to bind the peptides of GL–PT, that damage celiac disease in vitro models. On the other hand, chitosan can form protein complexes. Indeed, the interaction with the membrane receptor might account for the induction of caspase-3 activity promoted by MP-chitosan. The protective action of chitosan is likely to be due to the capability of this polysaccharide to bind to the gliadin peptides.

In conclusion, MP-chitosan prevents GL–PT toxicity in Caco-2 cells. If similar effect can be obtained in vivo is yet to be demonstrated, as well as the similarity of action of other modified and plain chitosans; however these results throw light on the mechanism of action of MP-chitosan and on some aspects of the pathogenesis of the celiac disease.

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