

Wheat gliadin induces apoptosis of intestinal cells via an autocrine mechanism involving Fas–Fas ligand pathway

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Abstract Wheat gliadin and other cereal prolamins have been said to be involved in the pathogenic damage of the small intestine in celiac disease via the apoptosis of epithelial cells. In the present work we investigated the mechanisms underlying the pro-apoptotic activity exerted by gliadin-derived peptides in Caco-2 intestinal cells, a cell line which retains many morphological and enzymatic features typical of normal human enterocytes. We found that digested peptides from wheat gliadins (i) induce apoptosis by the CD95/Fas apoptotic pathway, (ii) induce increased Fas and FasL mRNA levels, (iii) determine increased FasL release in the medium, and (iv) that gliadin digest-induced apoptosis can be blocked by Fas cascade blocking agents, i.e. targeted neutralizing antibodies. This favors the hypothesis that gliadin could activate an autocrine/paracrine Fas-mediated cell death pathway. Finally, we found that (v) a small peptide (1157 Da) from durum wheat, previously proposed for clinical practice, exerted a powerful protective activity against gliadin digest cytotoxicity.

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Key words: Gliadin-derived peptide; Apoptosis; CD95/Fas; Caco-2

1. Introduction

Apoptosis is central to the maintenance of the epithelial functions of the gut as it is involved in the normal enterocyte turnover. Under physiological conditions apoptotic cells are restricted to the tips of the villous in the small bowel and are replaced by an equal number of proliferating immature crypt cells [1]. In contrast, in immuno-mediated disorders such as inflammatory bowel disease or celiac disease (CD), an increased number of enterocytes undergo premature apoptosis all along the crypt–villous axis [2,3]. The combination of

markedly increased apoptosis and altered turnover rates results in architectural changes in the mucosa, characterized by villous atrophy and crypt hyperplasia. CD and its symptoms, observed in genetically predisposed individuals, are due to the ingestion of the bread wheat prolamins fraction gliadin, as well as the corresponding prolamins fractions of rye, barley and probably oats [4–6]. The mechanism by which these cereal prolamins fractions, whose toxicity is not destroyed by digestion with gastropancreatic enzymes, damage the celiac small intestine is not clear yet. Although it is largely accepted that an altered immune response to prolamins-derived peptides is involved in the pathogenesis and the progression of CD [7–9], a direct non-immune-mediated activity of gliadin-derived peptides on the jejunal/duodenal tract was also hypothesized. Several studies, in fact, support the hypothesis that wheat gliadin displays a direct cytotoxic activity against enterocytes and that this is inversely correlated with the differentiation state of the cells [10–12]. For instance, it was shown that gliadin was also capable of inducing cytotoxic effects on cultured intestinal biopsy specimens obtained from celiac patients with activation of lamina propria T lymphocytes and macrophages [13]. We have previously demonstrated that the treatment of human intestinal cells in culture (Caco-2) throughout their early phases of differentiation with gliadin-derived peptides from bread wheat resulted in cytotoxic effects, such as membrane damage, impairment of cell proliferation and cell differentiation, cell detachment and cell death by apoptosis [14–16]. On the other hand, we had shown that a small peptide with 1157 Da molecular weight, identified as the sequence H₂N-gln-gln-pro-gln-asp-ala-val-gln-pro-phe-COOH, not only was able to prevent the cytotoxicity [17,18] induced by peptic-tryptic (PT) digests of prolamins fractions from the cereals that are not tolerated by celiac patients, but also counteracts all the cytotoxic effects described above for the Caco-2 cell line [16]. As we described elsewhere, this peptide was characterized and identified from a gliadin component of durum wheat, and seems to be responsible for its lesser toxic activity, as resulted from studies performed in vitro [17]. We felt this peptide would eventually become the focus of interest in the clinical practice [16–18]. The aim of the present work was thus to investigate the mechanisms underlying the apoptosis induced by gliadin-derived peptides in Caco-2 cells, exposing the cells to the PT digest of the bread wheat gliadin (GI–PT), mimicking intestinal physiological conditions. Since the Caco-2 cell line retains many of the

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Abbreviations: CM-H₂DCFDA, 5-chloromethyl-2',7'-dichloro-dihydrofluoresceindiacetate; DEVD-CHO, Asp-Glu-Val-Asp-aldehyde; DHR 123, dihydrorhodamine 123; DMEM, Dulbecco's modified Eagle's medium; HE, hydroxyethyl; GI–PT, gliadin–peptic-tryptic; IETD-CHO, Ile-Glu-Thr-Asp-aldehyde; LEHD-CHO, Leu-Glu-His-Asp-aldehyde; PI, propidium iodide; PT, peptic-tryptic; ROS, reactive oxygen species

morphological and enzymatic features typical of normal human enterocytes [19,20], it is largely used as a model system for evaluating the effects of normal dietary constituents as well as additives, contaminants, toxicants, oxidants and drugs [21,22]. In the present work we found that (i) gliadin-derived peptides are potent and specific inducers of receptor-mediated apoptosis in human colon cells via a target activity on Fas/FasL pathway, and (ii) this apoptotic proneness can be significantly counteracted by the administration of the '1157 Da' peptide derived from durum wheat.

2. Materials and methods

2.1. Peptic-trypsin (PT) digest

The gliadin fraction was extracted from whole cereal flour of bread wheat (*Triticum aestivum*, S. Pastore variety) as previously described by Auricchio et al. [12]. The gliadin fraction or bovine serum albumin (BSA) (used as an inactive control protein), were subjected to sequential digestion according to de Ritis et al. [11], to obtain the corresponding PTs. At the end of the procedure, PTs were heated for 30 min at 100°C, lyophilized and stored at -20°C.

2.2. '1157 Da' peptide

The peptide with molecular weight 1157 Da was identified from gliadin of durum wheat (Adamello variety) as previously described [17]. The peptide was synthesized by Primm Company (Milan, Italy) with the solid phase method using the Applied Biosystems model 431 A, and was purified up to >99% by reverse-phase high-performance liquid chromatography (RT-HPLC) on a Varian 5020 system [17].

2.3. Cell cultures

The human colon cell line Caco-2 was obtained from the European Collection of Cell Cultures (Salisbury, UK). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Hyclone, Cramlington, UK) with 4.5 g glucose per liter, supplemented with 1% (v/v) non-essential amino acids (Flow Laboratories, Irvine, Scotland, UK), 0.2 mM L-glutamine (Flow Labs), 50 U/ml penicillin (Flow Labs), 50 µg/ml streptomycin (Flow Labs), and 10% (v/v) fetal calf serum (Flow Labs), at 37°C in a humidified atmosphere of 5% CO₂ in air.

2.4. Experimental procedures and treatments

For each experiment 5×10^5 cells were seeded in 25 cm² tissue culture flasks (Falcon, Becton Dickinson, Meylan, France) as previously described [17]. On culture day 5, differentiating Caco-2 cell monolayers were washed and treated as described below. Cell number was evaluated daily with a ZM Coulter Counter (Coulter Electronics, Luton, UK). *PT digest*: GI-PT digest or BSA-PT digest, dissolved in the medium, were sterilized by filtration through a 0.2 µm Millipore membrane (Millipore, Bedford, MA, USA) and added to 5-day cultures at a final concentration of 1 mg/ml as previously reported [16] for 6, 12, 18, 24 and 48 h. The PT digest from BSA was used as inactive control. *'1157 Da' peptide*: The '1157 Da' peptide was administered in the sterile medium 2 h before exposure to GI-PT digest, and maintained in the culture medium during treatments. The PT digests and '1157 Da' peptide concentrations used in the present investigation were chosen on the basis of experiments previously published by our group [14–16]. *Fas*: Cell cultures were incubated with (i) 15 ng/ml anti-Fas activating antibody (clone CH11); (ii) 1 µg/ml anti-Fas inhibiting antibody (clone ZB4) (Upstate Biotechnology, Lake Placid, NY, USA) 2 h before exposure to GI-PT digest; (iii) 2 µg/ml anti-FasL inhibiting antibody (clone NOK-1) (Pharmingen, San Diego, CA, USA) 2 h before exposure to GI-PT digest. As further control, either mouse IgM (control for Fas-triggering) or mouse IgG₁ (control for Fas- and for FasL-neutralizing) were also considered. *caspase inhibitors*: 2 h before the apoptotic stimuli described above, 100 µM DEVD-CHO (Asp-Glu-Val-Asp-aldehyde; cell permeant caspase-3 inhibitor), LEHD-CHO (Leu-Glu-His-Asp-aldehyde; cell permeant caspase-9 inhibitor), or IETD-CHO (Ile-Glu-Thr-Asp-aldehyde; cell permeant caspase-8 inhibitor) (Biosource International, Camarillo, CA, USA) were directly added to the culture medium. Cells treated with caspase inhibitors alone were used as controls.

2.5. Apoptosis evaluation

Apoptosis was quantitatively evaluated by the following flow and static cytometry methods: (i) double staining by using annexin V-FITC and propidium iodide (PI) (ApoAlert Annexin V apoptosis kit; Clontech Laboratories, Palo Alto, CA, USA) according to the manufacturer's instructions. Two-color cytometric analysis (fluorescence-activated cell sorting [FACS]) was performed on a Coulter Epics Elite ESP cell sorter (Miami, FL, USA) with an argon-ion laser tuned at 488 nm; (ii) staining with chromatin dye Hoechst (Molecular Probes) as previously described [23].

2.6. Intracellular redox state

Control and treated cells (5×10^5) were harvested and incubated in 495 µl of Hanks' balanced salt solution (pH 7.4) with hydroethidine (HE; Molecular Probes), dihydrorhodamine 123 (DHR 123; Molecular Probes) or 5-chloromethyl-2',7'-dichloro-dihydrofluorescein diacetate (CM-H₂DCFDA, Molecular Probes) for detection of superoxide anion, hydrogen peroxide and GSH, respectively. After 15 min at 37°C samples were analyzed on a cytometer as previously described [24].

2.7. Human lymphocyte experiments

Activated human lymphocytes (6000 IU IL2+0.5 µg/ml PHA for 48 h) were triggered by 500 ng/ml of α-Fas (clone CH11) in the presence or absence of two different concentrations of peptide 1157 (0.5 mg/ml or 1 mg/ml). Aliquots of cells were removed after 48 and 72 h and analyzed by flow cytometry for apoptosis after annexin V/PI double staining.

2.8. Evaluation of cell death molecules

Surface and intracytoplasmic expression of CD95/Fas and Fas ligand were verified by flow cytometry using specific monoclonal antibody (MAB). For evaluation of surface antigens control and treated Caco-2 cells were incubated on ice with anti-CD95 (Becton Dickinson, Mountain View, CA, USA) or with anti-Fas ligand (Pharmingen). After 1 h at 4°C, samples and isotypic controls were washed and incubated for 30 min with FITC-labeled anti-mouse (Sigma). For intracellular evaluation, Caco-2 cells were pelleted and fixed in 70% ice-cold methanol. After washing, samples were stained with the same MAB for 1 h at 37°C and then with FITC-labeled anti-mouse (Sigma) for an additional 30 min. After washing, cells were analyzed with a FACScan flow cytometer (Becton Dickinson) equipped with a 488 argon laser. At least 20 000 events have been acquired. Data were recorded and statistically analyzed by a Macintosh computer using CellQuest Software.

2.9. Extraction of total RNA and semiquantitation by reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA from cultured Caco-2 was extracted by the Trizol (Gibco BRL, Grand Island, NY, USA) isolation method. 1 µg of total RNA was used for RT-PCR analysis. PCR was performed using the following couples of primers: 5'-CCC AAA TAG GAG TGT ATG CAG AGG-3' and 5'-GCC ATT AAG ATG ACC AAG G-3' for amplification of Fas; 5'-CCA CCG CCA CCA CCA CCA-3' and 5'-TCT TCC CCT CCA TCA TCA CC-3' for Fas ligand; 5'-CCA CCC ATG GCA AAT TCC ATG GCA-3' and 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3' for amplification of GAPDH as housekeeping gene. The samples were incubated in an automated heat-block Minicycler (MJ Research, Waltham, MA, USA) by using the following parameters: 95°C for 30 s, 68°C for 90 s and 72°C for 90 s using 42 cycles (Fas), and 94°C for 60 s, 59°C for 60 s and 72°C for 120 s using 34 cycles (FasL). The PCR products were electrophoresed on a 1.5% agarose gel containing ethidium bromide. Densitometric analysis was performed by a FX molecular imager (Bio-Rad, Hercules, CA, USA).

2.10. Assay of Fas ligand in Caco-2 cell supernatants

Fas ligand concentrations in the supernatants of Caco-2 cells were evaluated by sensitive and specific immunoassays, using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA). Caco-2 cells, seeded as described above, were exposed to GI-PT alone or GI-PT+1157 Da for different times. Supernatant aliquots were removed after 6, 18, 24 and 48 h of treatment and assayed for Fas ligand content according to the manufacturer's instructions.

2.11. Statistical analysis

The data are presented as the arithmetic mean for each experimental point \pm S.E.M. Statistical calculations were performed using a one-way ANOVA. Differences among groups were examined using the Bonferroni *t*-test when the *F* value was significant. A *P* value < 0.05 was considered significant.

3. Results

3.1. Gliadins impair cell homeostasis by inducing apoptosis in Caco-2 cells

We considered subcellular activity of GI-PT by studying cell death by apoptosis, evaluated by annexin-V/PI double staining to detect early apoptotic events clearly. Quantitative

analyses over six different experiments indicated a significant increase ($P < 0.01$) of the apoptotic phenomenon after 24 and 48 h of GI-PT exposure with respect to control cells (a 3.7- and 3.9-fold increase, respectively) (Fig. 1A). However, a lower apoptotic rate was detected after 48 h with respect to 24 h of GI-PT exposure, owing to the increased amount of necrotic cells, as reported in Fig. 1B, right panel, second quadrant. It is noteworthy to underline that the increase of apoptotic cells, observed in GI-PT-treated Caco-2 cultures, was accompanied by a significant reduction of counted cells. In fact, cell number per flask was $35 \times 10^5 \pm 44 \times 10^4$ and $66 \times 10^5 \pm 14 \times 10^4$ in treated cells, $43 \times 10^5 \pm 52 \times 10^4$ and $82 \times 10^5 \pm 17 \times 10^4$ in control cells at 24 and 48 h, respectively. The analysis of apopto-

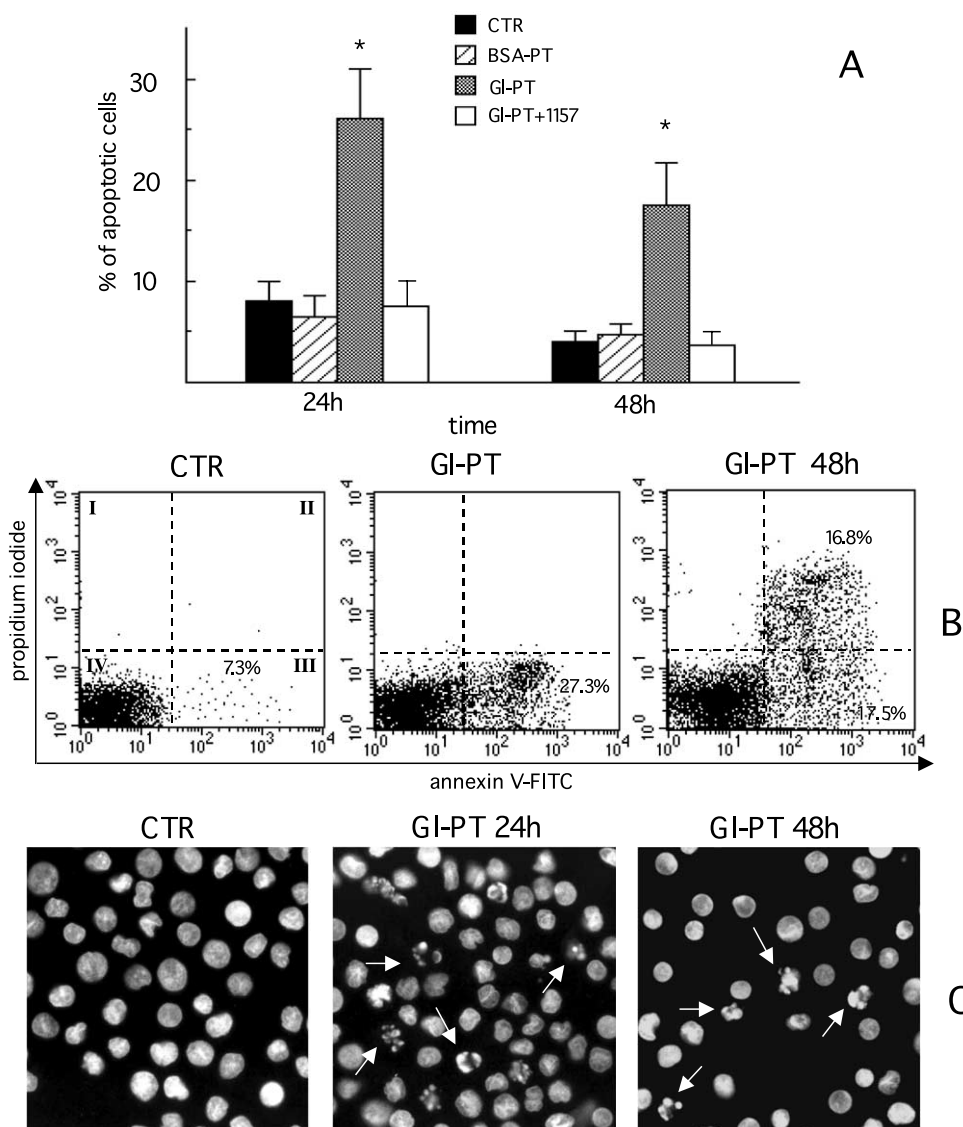


Fig. 1. Pro-apoptotic effects of GI-PT digest in intestinal cells. A: Quantitative analysis of apoptotic phenomenon. Values reported in the graph correspond to the percentage of single annexin V-positive cells (early apoptosis). $*P < 0.001$: GI-PT vs. control cells (CTR), BSA-PT-digest-exposed cells (BSA-PT), GI-PT-digest-treated cells (GI-PT) and GI-PT digest-treated cells preincubated with the '1157 Da' peptide (GI-PT+1157). Values are expressed as mean \pm S.E.M. of six independent experiments. B: Evaluation of apoptosis by FACS analysis of annexin V/PI double positive Caco-2 cells in: control cells (CTR, left panel), GI-PT for 24 h (middle panel) and 48 h (right panel). Note the increase in necrotic cells after 48 h of GI-PT treatment (second quadrant in the right panel). One experiment representative of four is shown. C: Fluorescence microscopy of adhering Caco-2 cell nuclei stained with Hoechst 33258. Left panel: control cells; middle panel: 24 h GI-PT treated cells; right panel: 48 h GI-PT treated cells. The arrows indicate chromatin condensation or clumping typical of apoptosis. Note the decreased cell density in 48 h GI-PT treated cells.

tic nuclei of adhering cells with Hoechst fluorescent dye confirmed these observations. Control cells (Fig. 1C, left panel) showed intact nuclei without any sign of chromatin condensation typical of apoptosis. After 24 h, GI-PT induced chromatin aggregation and/or clumping in some of cells (Fig. 1B, middle panel, arrows). Prolonging the treatment with GI-PT up to 48 h, we observed: (i) the presence of nuclei with clear signs of apoptosis (Fig. 1C, right panel, arrows), and (ii) the reduction of cell density caused by cell detachment from the substrate.

No significant difference in apoptosis rate was found using BSA-PT digest (BSA-PT) as further control: the percentage of apoptotic cells was similar to that of control samples (8.6%) (Fig. 1A). The effects of the *pre-treatment* with the '1157 Da', a small peptide from a gliadin component of durum wheat with 1157 Da molecular weight, was also taken into account. This agent fully counteracted the pro-apoptotic activity of GI-PT, being the percentage of apoptotic cells comparable to that of control samples (Fig. 1A).

3.2. Gliadins induce redox imbalance in intestinal cells as a late event

Considering that redox imbalance can be an important event in apoptotic cell death, time course experiments were carried out by flow cytometry to evaluate the intracellular production of reactive oxygen species (ROS; specifically superoxide anions and hydrogen peroxide) and GSH content. For this purpose we used specific probes, namely HE (for detection of O_2^-), DHR 123 (for detection of H_2O_2) and CM- H_2 DCFDA (for GSH measurements). Soon after GI-PT treatment (6, 12 and 18 h), no changes in O_2^- , H_2O_2 production or GSH content were detected (data not shown). By contrast, as expected, once the apoptotic process was evident by flow cytometry analyses, i.e. as from 24 h after exposure to GI-PT, we observed a significant ($P < 0.01$) increase in O_2^- ($+98 \pm 9\%$) and in H_2O_2 ($+70 \pm 6\%$) production as well as a decrease (-82%) in GSH content with respect to untreated cells. These values were obtained by comparing the median values of the respective fluorescence intensity histograms and considering the median value of control untreated cells as 100%. These results suggest that redox imbalance is a tardive event, playing a secondary role in GI-PT-induced apoptosis.

3.3. Gliadin-induced apoptosis is mediated by the receptor-mediated pathway

To elucidate the subcellular pathway involved in GI-PT-induced apoptosis in Caco-2 cells, a series of experiments on the caspase cascade was carried out. We first considered the upstream caspases that are involved in the initiation phase of apoptosis, i.e. caspase-8 (mainly involved in receptor-mediated apoptosis) and caspase-9 (mainly involved in mitochondria-mediated apoptosis) [25]. In fact, experiments with caspase-8 and caspase-9 inhibitors clearly indicated that caspase-8 inhibitor IETD-CHO fully inhibited GI-PT-induced apoptosis, while LEHD-CHO, a caspase-9 inhibitor, did not exert any protective effect. The activity of caspase-3, an executioner downstream caspase, was also assessed by using the specific inhibitor DEVD-CHO. This compound reduced the GI-PT-induced apoptosis by 85% (Fig. 2A). These results clearly indicate that the apoptosis induced by GI-PT in Caco-2 intestinal cells may occur via the classical caspase cascade activation

and, more importantly, that the receptor-mediated apoptotic pathway involving caspase-8 activation plays a key role.

3.4. Gliadins induce apoptosis via the Fas/FasL pathway

Among cell death receptors, the Fas/APO-1/CD95 receptor was demonstrated to be widely expressed by many cell types, including enterocytes [26]. Its activation was recently reported to be responsible for enterocyte death by apoptosis in CD [27,28]. As verified by flow cytometry, Caco-2 intestinal cells do express the Fas molecule on their surface (median value of the fluorescence intensity histogram 27.4 ± 5). To evaluate the involvement of the Fas-mediated pathway in GI-PT-induced apoptosis, cultured Caco-2 cells were incubated with anti-Fas activating antibody (clone CH11) and, more importantly, with anti-Fas neutralizing antibody or anti-FasL neutralizing antibody in the presence and absence of the gliadin. The results obtained (Fig. 2B) indicated that anti-Fas antibody triggered apoptosis in Caco-2 cells, and that the percentage of apoptotic cells was significantly reduced by the presence of anti-Fas neutralizing antibodies (clone ZB4) in the medium. This clearly identified Caco-2 cells as typical Fas-susceptible cells. Interestingly, when the cells were induced to undergo apoptosis by GI-PT treatment in the presence of anti-Fas neutralizing antibodies, apoptosis was fully inhibited and normal apoptotic control values were restored. On the basis of these results, we focused further analyses on FasL apoptotic cascade. What we found was that anti-FasL neutralizing antibodies (clone NOK-1) were also able to significantly counteract GI-PT-induced apoptosis, clearly proving the involvement of the Fas/FasL pathway in the GI-PT-mediated apoptotic cascade. Interestingly, the presence of the '1157 Da' peptide in Caco-2 cell cultures, which was able to protect from GI-PT-induced apoptosis (see Fig. 1A), also hindered anti-Fas induced apoptosis, significantly reducing the percentage of apoptotic cells by $42 \pm 3\%$ ($P < 0.01$) (Fig. 2B). As a further control, in consideration of the plethora of data in the literature indicating human lymphocytes as the milestone of physiological Fas-induced apoptosis, the pro-apoptotic activity exerted by GI-PT and the anti-apoptotic activity of the '1157 Da' peptide were also evaluated in freshly isolated human activated lymphocytes. As with intestinal Caco-2 cells, a significant increase in the percentage of apoptotic cells was detected after treatment with GI-PT as well as with CH11 antibodies (Fig. 2C). Moreover, an anti-apoptotic activity of the '1157 Da' peptide versus CH11-mediated apoptosis was also detected (Fig. 2C). Altogether, these results seem to indicate that GI-PT and the small peptide from durum wheat can exert their activity, i.e. pro-apoptotic and anti-apoptotic, respectively in cells belonging to a different histotype.

3.5. Target activity of gliadin on the Fas-mediated pathway

To understand how GI-PT could activate the Fas/FasL apoptotic pathway in Caco-2 cells, we conducted a series of time course experiments focused on the Fas and FasL gene expression. As reported in Fig. 3A,B, the results obtained by RT-PCR showed a significant overtranscription of both genes, Fas (agarose gel electrophoresis in Fig. 3A) and FasL (agarose gel electrophoresis in Fig. 3B). In fact, densitometric analyses of cells exposed to GI-PT showed a highly significant ($P < 0.001$) increase in Fas (450%) and FasL (170%) mRNA expression with respect to control values at every time point

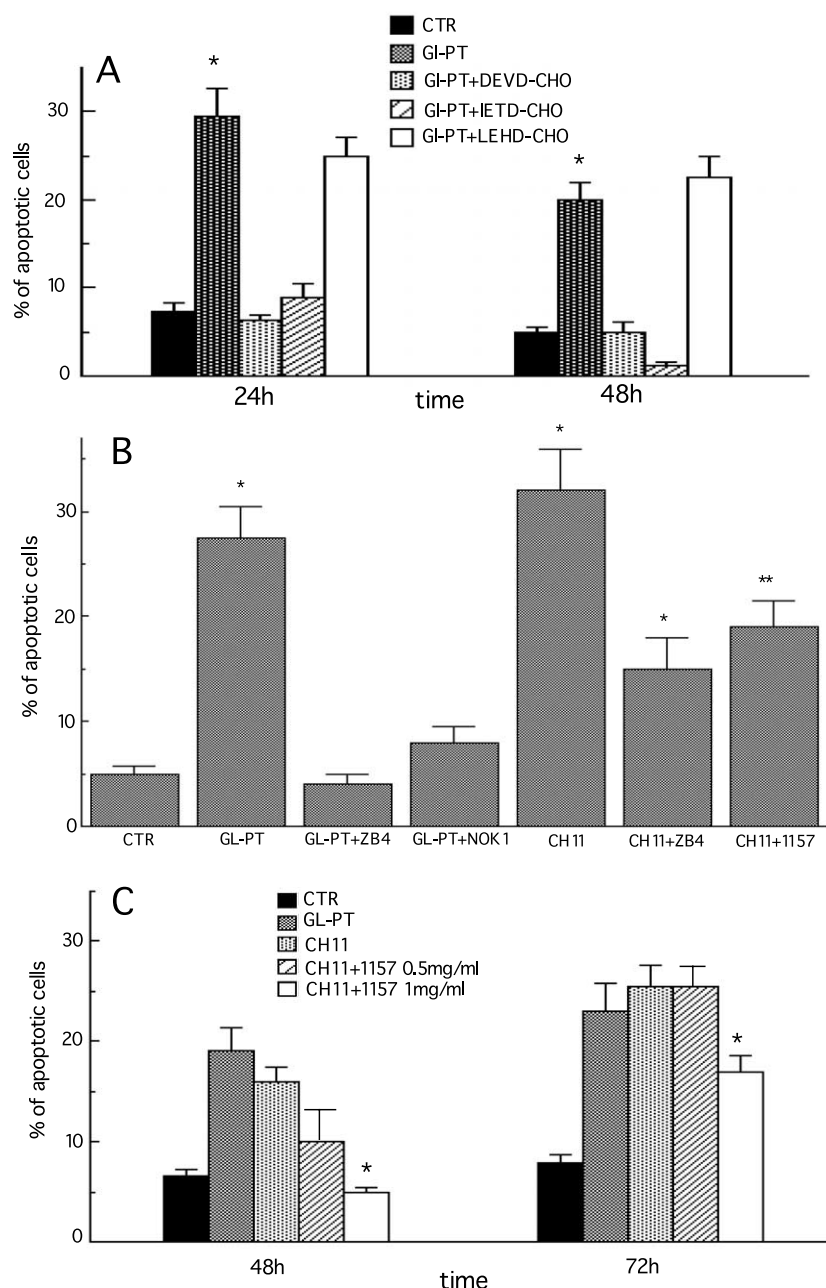


Fig. 2. Gliadins induce apoptosis mediated by caspase-8 and -3 via Fas/FasL pathway. A: Quantification of GI-PT-digest-induced apoptosis in the absence and presence of DEVD-CHO (caspase-3 inhibitor) (GI-PT+DEVD-CHO); LEHD-CHO (caspase-9 inhibitor) (GI-PT+LEHD-CHO); or IETD-CHO (caspase-8 inhibitor) (GI-PT+IETD-CHO). Values are expressed as mean \pm S.E.M. of four independent experiments. * P < 0.001: GI-PT vs. GI-PT+DEVD-CHO; GI-PT vs. GI-PT+IETD-CHO. B: Quantification of apoptosis in Caco-2 cells incubated with: (i) anti-Fas inhibiting antibody, clone ZB4, 2 h before GI-PT digest exposure (GI-PT+ZB4); (ii) anti-FasL inhibiting antibody, clone NOK-1, 2 h before GI-PT digest exposure (GI-PT+NOK-1); (iii) anti-Fas activating antibody, clone CH11, (CH11); (iv) anti-Fas activating antibody, clone CH11, + anti-Fas inhibiting antibody, clone ZB4, (CH11+ZB4); (v) anti-Fas activating antibody, clone CH11, + the '1157 Da' peptide (CH11+1157). The percentages of annexin-V positive cells 24 h after apoptotic triggering are reported as mean \pm S.E.M. of four independent experiments. * P < 0.001: GI-PT+ZB4 vs. GI-PT; GI-PT+NOK-1 vs. GI-PT; CH11+ZB4 vs. CH11. ** P < 0.01: CH11+1157 vs. CH11. C: Evaluation of apoptosis induced in human activated lymphocytes by GI-PT at the same concentration used in Caco-2 cells and by anti-Fas activating antibodies (CH11) in the presence/absence of two concentrations of the '1157 Da' peptide for 48 and 72 h. Note the significant reduction of apoptotic rate induced by the 1157 peptide. * P < 0.01: CH11+1157 (1 mg/ml) vs. CH11.

(6, 18, 24 h). Strikingly, but in agreement with the results reported above, the presence of the '1157 Da' peptide in the medium of Caco-2 cell cultures restored the control mRNA values for both Fas and FasL, wholly counteracting the effects of GI-PT (Fig. 3A,B).

3.6. Gliadins induced secretion of soluble FasL

On the basis of the above results, we investigated whether the increase in FasL mRNA detected after GI-PT exposure (Fig. 3B) also resulted in the production and release of the FasL molecule in the culture medium. Interestingly, an in-

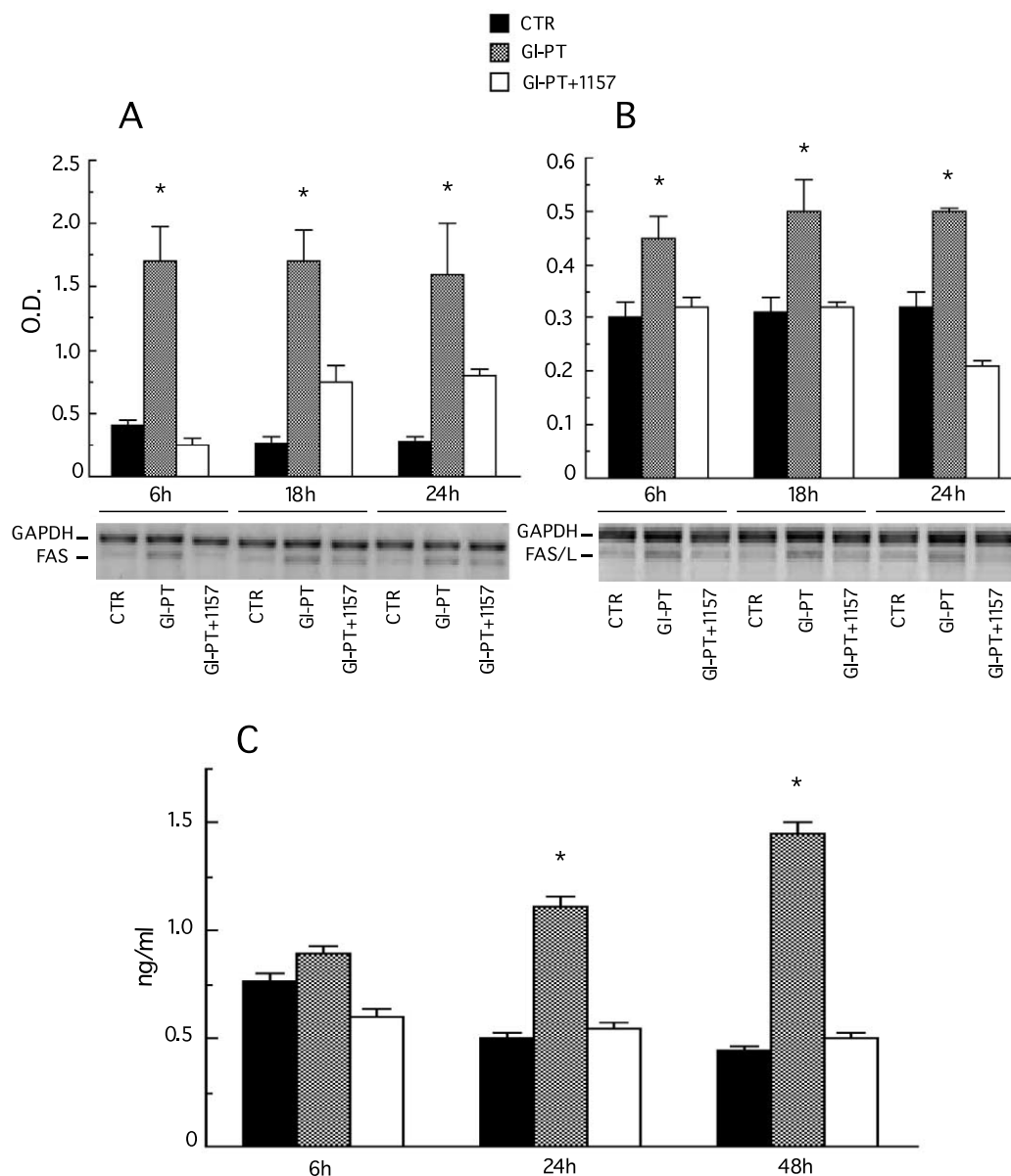


Fig. 3. Gliadins induce upregulation of the Fas/FasL system by increasing mRNA transcription and secretion of soluble FasL. Quantitative evaluation of Fas (panel A) and Fas/L (panel B) mRNA transcription by RT-PCR, reported as optical density (OD). Values are the mean \pm S.E.M. of four independent time-course experiments in duplicate, observed in untreated control cells (CTR), in GI-PT and in GI-PT-treated cells preincubated with the '1157 Da' peptide (GI-PT+1157) at different exposure times. * $P < 0.001$: GI-PT vs. CTR; GI-PT vs. GI-PT+1157. A representative agarose gel obtained by RT-PCR from four independent experiments is shown, for Fas (panel A) and Fas/L (panel B). C: Release of soluble FasL in the culture medium appears significantly increased after 24 and 48 h of cell exposure to GI-PT. The small peptide 1157 significantly impaired GI-PT-induced FasL secretion. * $P < 0.01$: GI-PT vs. CTR.

creased secretion of FasL was found starting within 6 h after GI-PT exposure ($P < 0.05$). FasL production increased further after 24 and 48 h of GI-PT treatment ($P < 0.01$). When the effects of the protecting '1157 Da' peptide were assessed, a complete inhibition of GI-PT-mediated FasL release was found, the values detected being quite similar to those found in control samples (Fig. 3C).

4. Discussion

The importance of apoptosis in tissue homeostasis has been demonstrated in numerous diseases, including intestinal dis-

metabolic degenerative diseases. Among these, increased apoptosis was related to mucosal flattening occurring in CD. Namely, an increased apoptosis rate has been hypothesized as the major factor responsible for villous atrophy in CD. The novelty of the present work is that after PT digestion gliadin bread wheat peptides induced apoptosis by directly acting via the 'activation' of an autocrine/paracrine mechanism involving FasL production. As a general rule, apoptosis has been described as mediated by two pathways that, although strictly intertwined, represent different ways to reach the same result, i.e. the death of a cell [29,30]. One pathway (receptor-mediated) mainly involves caspase-8 activation; the

other was shown to be primarily linked to mitochondrial changes and the activation of caspase-9, with release of cytochrome c into the cytosol and apoptosome complex formation [31–33]. Both caspase-8 and caspase-9 finally activate caspase-3 by proteolytic cleavage, and, in turn, caspase-3, one of the key executioners of apoptotic cell death, cleaves vital cellular proteins [25,34]. Our results, obtained with specific caspase inhibitors and neutralizing antibodies, appear to indicate that GI-PT-induced apoptosis is specifically mediated by CD95/Fas and it is under the control of caspase-8. We also found that Caco-2 intestinal cells, in the absence of any treatment, were able to express the CD95/Fas receptor and produce per se its ligand, FasL. However, these high basal levels did not result in high apoptotic rates. We can hypothesize that a sort of threshold should be surpassed for Caco-2 cells to undergo apoptosis. In fact, when exposed to gliadin, Fas receptor expression increased significantly as well as FasL production, though to a lesser extent. As a result, high apoptotic rates were detected. Hence, the hypothesis of a gliadin-mediated autocrine mechanism leading to cell degeneration can be reasonably considered. In the same vein, Ciccocioppo et al. [27] suggested that Fas/Fas ligand-mediated enterocyte apoptosis was involved in mucosal flattening in CD, as resulting from the overexpression of the Fas receptors on enterocytes and Fas ligand on lymphocytes in celiac mucosa. It was also suggested [28] that gliadin might be directly responsible for Fas upregulation, playing a pivotal role in epithelial damage. Hence, we can hypothesize that gliadin might function as an apoptosis-sensitizing compound, boosting specific intracellular processes [27,28,35] and increasing intestinal cell apoptotic proneness. Our results were obtained with the Caco-2 cell line widely used as a suitable in vitro model for pharmacotoxicologic studies regarding the intestine [36–38] and, more recently, to address the dysregulated intestinal function specifically related to CD [39–41]. Hence, our results, albeit in vitro, seem to provide helpful suggestions regarding the pathogenic mechanisms underlying CD.

In the present work we also show that a small peptide from a gliadin component of durum wheat can exert powerful protective effects. This compound was actually capable of protecting intestinal cells from both GI-PT- and Fas antibodies-induced apoptosis, probably by impairing Fas/FasL triggering. In addition, it inhibited Fas-mediated apoptosis in freshly isolated human activated T lymphocytes. This is an interesting finding in view of future studies aimed at the understanding of the immune modulating activities exerted by GI-PT. As reported by several in vitro and in vivo studies [42–45], the sequences -Gln-Gln-Gln-Pro- and/or -Pro-Ser-Gln-Gln- are known to be a common feature of toxic gliadin-derived peptides. Interestingly, remarkable homologies were identified between these toxic sequences and the protective amino acid sequence within the '1157 Da' peptide (H_2N -gln-gln-pro-gln-) [17,18,40]. This could suggest that this peptide could be recognized by the same (or structurally-related) binding sites on the cell surface. It is thus likely to interfere with the apoptotic switch by inhibiting the sequence of biological events leading to Fas-FasL-mediated cell death. Further investigations are under way to elucidate the mechanism by which this peptide interferes with intestinal cell fate. If the protective activity exerted by the '1157 Da' peptide is confirmed by in vivo studies, new clinical perspectives will be opened in the management of CD.

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