Available online at www.sciencedirect.com
ScienceDirect
Journal of Nutritional Biochemistry 17 (2006) 611–625
Journal of Nutritional Biochemistry

The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway

Mar Larrosa, Francisco A. Tomás-Barberán, Juan Carlos Espín*

Research Group on Quality, Safety and Bioactivity of Plant Foods, Department of Food Science and Technology, CEBAS-CSIC, P.O. Box 164, Campus de Espinardo, 30100 Espinardo, Murcia, Spain

Received 3 August 2005; received in revised form 6 September 2005; accepted 7 September 2005

Abstract

Polyphenol-rich dietary foodstuffs have attracted attention due to their cancer chemopreventive and chemotherapeutic properties. Ellagitannins (ETs) belong to the so-called hydrolysable tannins found in strawberries, raspberries, walnuts, pomegranate, oak-aged red wine, etc. Both ETs and their hydrolysis product, ellagic acid (EA), have been reported to induce apoptosis in tumour cells. Ellagitannins are not absorbed in vivo but reach the colon and release EA that is metabolised by the human microflora. Our aim was to investigate the effect of a dietary ET [pomegranate punicalagin (PUNI)] and EA on human colon cancer Caco-2 and colon normal CCD-112CoN cells. Both PUNI and EA provoked the same effects on Caco-2 cells: down-regulation of cyclins A and B1 and upregulation of cyclin E, cell-cycle arrest in S phase, induction of apoptosis via intrinsic pathway (FAS-independent, caspase 8-independent) through bcl-XL down-regulation with mitochondrial release of cytochrome *c* into the cytosol, activation of initiator caspase 9 and effector caspase 3. Neither EA nor PUNI induced apoptosis in normal colon CCD-112CoN cells (no chromatin condensation and no activation of caspases 3 and 9 were detected). In the case of Caco-2 cells, no specific effect can be attributed to PUNI since it was hydrolysed in the medium to yield EA, which entered into the cells and was metabolised to produce dimethyl-EA derivatives. Our study suggests that the anticarcinogenic effect of dietary ETs could be mainly due to their hydrolysis product, EA, which induced apoptosis via mitochondrial pathway in colon cancer Caco-2 cells but not in normal colon cells.

Keywords: Ellagitannin; Cytochrome *c*; Caspases; Bcl-XL; Colon cancer

1. Introduction

Polyphenols are plant secondary metabolites ubiquitously found in commonly consumed fruits, vegetables and derived products such as wine and tea. There is increasing evidence regarding the link between the intake of fruits and vegetables and the lower incidence of certain types of cancer, attributing this effect to dietary polyphenols [1–4].

Ellagic acid (EA; Fig. 1) is a polyphenol that has been reported to show antiproliferative activity, to cause cell-cycle arrest and to induce apoptosis in many human cancer cell lines such as bladder T24, cervical carcinoma (CaSki), leukaemia MOLT-4, breast MCF-7 and Hs 578T, and prostate DU 145 cells [5–8].

The occurrence of free EA in dietary foodstuffs is rather uncommon. Ellagic acid is usually conjugated with a glycoside moiety (glucose, arabinose, xilose, etc.) or, even more commonly, forms part of polymeric molecules called ellagitannins (ETs) (Fig. 1) [9]. Ellagitannins are included within the so-called hydrolysable tannins that can be hydrolysed producing EA via spontaneous lactonisation of hexahydroxydiphenic acid (Fig. 1). The occurrence of ETs has been reported, among others, in walnuts, pomegranates (fruit and juice), persimmon, oak-aged wines (leakage of ETs from oak barrel to wine), strawberries, raspberries, blackberries (and their derivatives such as juices, jams and jellies), peach, plum, muscadine grape and wine, etc. [9]. The induction of cell-cycle arrest and apoptosis in human leukaemia HL-60 and human non-small cell lung cancer A549 cells [10,11] as well as the antimetastatic and antiproliferative activities in HT1080 fibrosarcoma cells [12] has been reported for a number of ETs.

* Corresponding author. Tel.: +34 968 396344; fax: +34 968 396213.
E-mail address: jcespin@cebas.csic.es (J.C. Espín).

0955-2863/$ – see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.jnutbio.2005.09.004

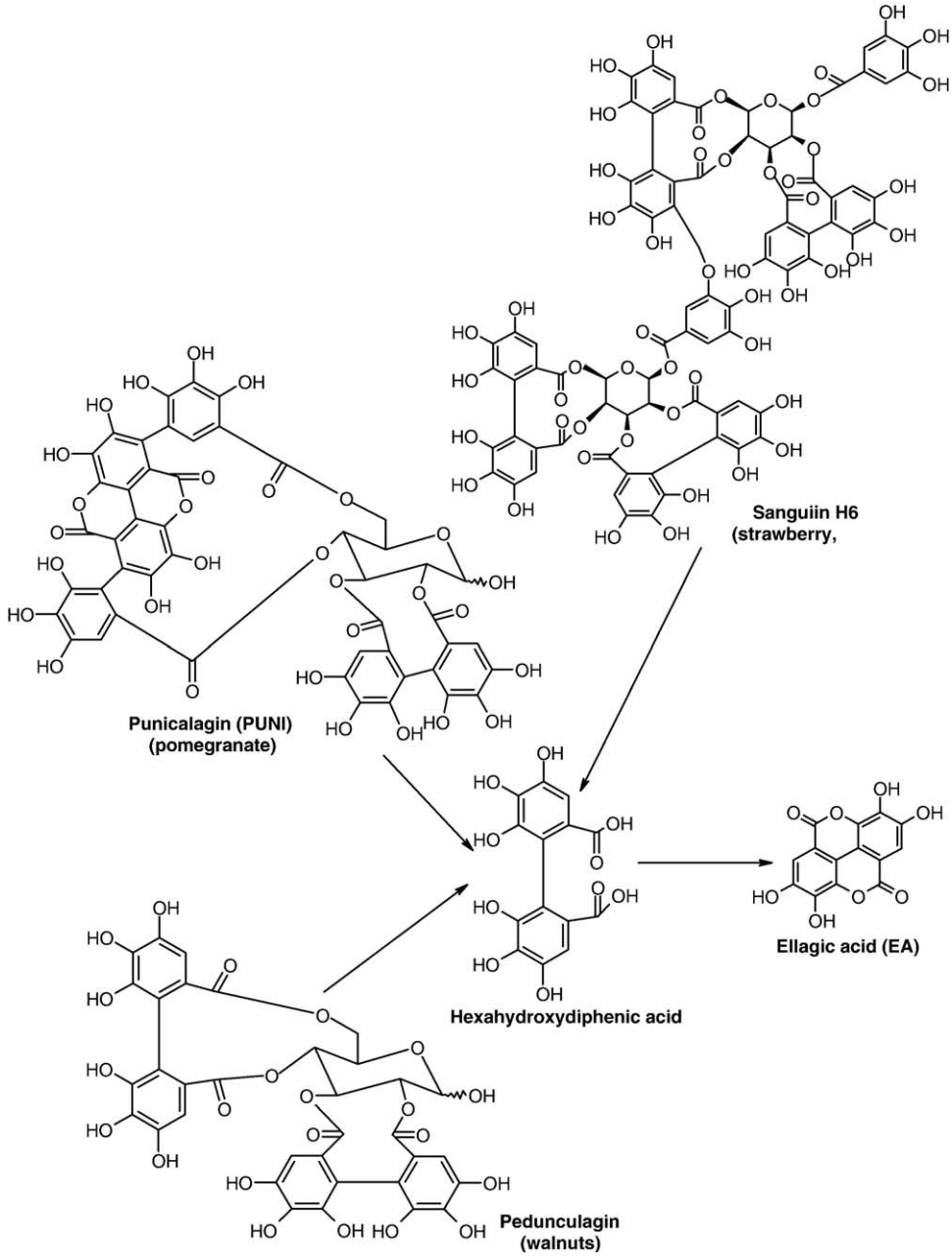

Fig. 1. Hydrolysis of representative ETs from dietary foodstuffs to release EA.

Most of the above assayed cell models (bladder, breast, lung, etc.), describing the biological effects of both ETs and EA (such as molecular regulation of apoptotic pathways), must assume that these polyphenols are absorbed to reach these target tissues in order to exert the potential anti-carcinogenic effect. However, these studies did not take into account the bioavailability of both ETs and EA.

A very important issue when assaying the biological activity of dietary molecules is their bioavailability and metabolism. In fact, molecules appearing in blood or excreted in urine can be very different from those ingested [13]. This is the case of ETs and EA. Recently, we have demonstrated that complex dietary ETs from different sources (walnuts, strawberry, pomegranate, oak-aged wine and raspberry) are not absorbed in humans but hydrolysed to yield EA, which is further metabolised by the human colonic microflora to yield bioavailable 3,8-dihydroxy-6H-dibenzo[*b,d*]pyran-6-one ('urolithin A') derivatives [14,15]. These metabolites appear in blood, urine and faeces of human volunteers almost 1 day after consumption of a single dose of ET-enriched meal, and complete clearance is not achieved after 56 h of the intake [15]. Therefore, a more plausible context to assess the potential biological effects of EA should include the assay of both ETs (as the main dietary EA source) and EA (as the in vivo hydrolysis ET product) on colon cancer lines since these molecules do reach the colon, and their complete metabolism

can take various days [15]. However, detailed reports regarding the effects of EA on colon cancer cell lines such as SW 480 and Caco-2 cells [6,16] are scarce, and data about the effects on human colon normal cells are missing.

Previous investigations have demonstrated that the dietary administration of ET-containing foods such as strawberries and raspberries to rats inhibited events associated with both the initiation and promotion/progression of chemically induced oral, oesophageal and colon cancer [17–19]. However, the molecular mechanism and the active components responsible for this chemoprevention are not fully clear. In this context, very recently, the inhibition of the growth of premalignant and malignant human oral cell lines by specific constituents of black raspberries (ferulic acid and *b*-sitosterol) has been reported [20].

In this study, we assayed the pomegranate ET punicalagin (PUNI; as a dietary ET) and free EA on both human colon cancer Caco-2 and colon normal CCD-112CoN cells. Our aim was to describe the molecular regulation of the apoptosis induced by PUNI and EA as well as to elucidate whether the true apoptosis-inducer compound was PUNI, EA or both molecules.

2. Materials and methods

2.1. Cell culture

The human colon cell line Caco-2 and the normal colon cells CCD-112CoN were obtained from American Type Culture Collection (Rockville, USA). Caco-2 cells were cultured in minimal essential medium (MEM) containing 2 mmol/L glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, nonessential amino acids and 10% fetal bovine serum. CCD-112CoN cells were cultured in the same medium plus 100 mmol/L sodium piruvate and $NaHCO_3$ (2.2 g/L) at 37°C and humidified atmosphere (5% CO_2). Doubling time (required time to double cell population) was calculated for Caco-2 and CCD-112CoN cells according to Xiao et al. [21], using the formula $T_D = t \times \log_2 / (\log N_t - \log N_o)$.

2.2. Cell proliferation

The effect of the above treatments on cell proliferation was assessed using the crystal violet assay [22]. Caco-2 cells were suspended at a final concentration of 5×10^3 cells/well in 96-well plates and after 48 h were treated with increasing EA and PUNI concentrations, i.e., 1–30 μmol/L EA and 1–100 μmol/L PUNI for 72 h. Ellagic acid was purchased from Sigma (St. Louis, MO, USA). Pure PUNI standard was obtained from pomegranate peel according to a protocol previously reported [23]. To check for a possible synergistic effect between EA and PUNI, a mixture of both compounds at two different concentrations was tested, i.e., 10 μmol/L EA+10 μmol/L PUNI and 1 μmol/L EA+1 μmol/L PUNI.

Normal colon CCD-112CoN cells were seeded in 24-well plates at 5×10^3 cells/well and treated with 30 μmol/L of EA or 100 μmol/L PUNI for 12 days. Cell proliferation was also estimated using the crystal violet assay. It is noticeable that in the case of CCD-112CoN cell culture, the media were replaced every 3 days (refreshing EA and PUNI concentrations). Cell proliferation assays were repeated three times.

2.3. Stability of PUNI and EA in Caco-2 cell cultures. Identification of EA-derived metabolites in cell media

Three sets of experiments were performed. Samples were analysed at 1, 2, 8, 24 and 48 h. The experiments were repeated four times.

(i) One hundred micromoles per liter of PUNI or 30 μmol/L EA was added into MEM culture without cells (chemical stability).
(ii) One hundred micromoles per liter of PUNI or 30 μmol/L EA was added into MEM culture without cells. However, this medium was previously used to grow Caco-2 cells for 48 h (cells were removed by centrifuging the culture). This assay was carried out to assess whether the Caco-2 enzymes involved in PUNI or EA metabolism were constitutively excreted to the medium without the initial presence of PUNI or EA in the culture.
(iii) One hundred micromoles per liter of PUNI or 30 μmol/L EA was added into MEM culture together with Caco-2 cells. Then, at the different times specified above, the culture was centrifuged, and the media were analysed.

Culture media corresponding to the above set of assay conditions were filtered through a 0.45-μm membrane filter Millex-HV_{13} (Millipore, Bedford, MA, USA). Aliquots of 100 μl were injected onto a reverse-phase C_{18} LiChroCART column (25×0.4 cm, particle size 5 μm) using a Merck-Hitachi HPLC system with a model L-7100 pump and a Merck-Hitachi 7455 diode array detector. The mobile phase was water/acetic acid (99:1, v/v, solvent A) and acetonitrile (solvent B) at a flow rate of 1 ml/min. The gradient started with 1% B in A during 5 min to reach 20% B in A at 20 min, 40% B in A at 30 min, 95% B in A at 31 min. Chromatograms were recorded at 360 nm.

The media were also treated with 40 U/ml β-glucuronidase from bovine liver (G-0251, EC 3.2.1.31; 1000 U/g solid, Sigma) and 0.3 U/ml sulfatase from *Helix pomatia* (S-9626, EC 3.1.6.1; 10,000 U/g solid, Sigma) to assess the possible conjugation of sulphate and/or glucuronide moieties upon metabolism of EA and/or PUNI by Caco-2 cells.

2.4. Cellular uptake

Caco-2 cells were grown up to 80% confluence in 9-cm diameter Petri dishes and were incubated during 8, 24 and 48 h with either 100 μmol/L of PUNI or 30 μmol/L of EA in EMEM media without phenol red and with the supplements

specified above in the cell culture section. After treatment, the media were recovered and acidified with formic acid (1.5% in media) and filtered through a Sep-Pak cartridge (a reverse-phase C-18 cartridge; Millipore) which retains phenolic compounds. The cartridges were previously activated with 10 ml of MeOH and 10 ml of water. Every 10 ml of media was eluted with 2 ml of MeOH, and all methanolic fractions were collected and concentrated at 40°C under reduced pressure, filtered through a 0.45-μm membrane filter Millex-HV_{13} and then analysed by LC-MS/MS. Cells were washed twice with PBS and then lysed and scrapped with 2 ml of MeOH, sonicated on ice during 5 min, centrifuged, and the supernatant was concentrated under reduced pressure, and an aliquot (100 μl) was analysed by LC-MS/MS.

Cell extracts were also treated with sulphatase and glucuronidase as described above. Experiments to assess cellular uptake were performed in triplicate.

2.5. LC-MS/MS analyses

The HPLC system equipped with both a photodiode array detector and a mass detector in series consisted of a HPLC binary pump, autosampler and degasser controlled by software from Agilent Technologies (Waldbronn, Germany). The mass detector was an ion-trap mass spectrometer (Agilent) equipped with an electrospray ionisation system (capillary voltage, 4 kV; dry temperature, 350°C). Mass scan (MS) and MS/MS daughter spectra were measured from *m*/*z* 150 up to 1100 *m*/*z*. Collision-induced fragmentation experiments were performed in an ion trap using helium as collision gas, and the collision energy was set at 50%. Mass spectrometry data were acquired in the negative ionisation mode.

Chromatographic separations of samples were carried out on a reverse-phase C_{18} LiChroCART column (25×0.4 cm, particle size 5 μm, Merck, Darmstadt) using water/acetic acid (99:1, v/v) (A) and acetonitrile (B) as the mobile phases at a flow rate of 1 ml/min. The gradient started with 5% B in A to reach 40% B in A at 30 min and 95% B in A at 31 min. UV chromatograms of extracts were recorded at 280, 320 and 360 nm.

2.6. Flow cytometry analysis of cell cycle

After the treatments, cells (1×10^6) were fixed in ice-cold ethanol/PBS (70:30) for 30 min at 4°C, further resuspended in PBS with 100 μg/ml RNase and 40 μg/ml propidium iodide, and incubated at 37°C for 30 min. DNA content (20,000 cells) was analysed using a FACScan instrument equipped with FACStation running Cell Quest software (Becton Dickinson). The analyses of cell-cycle distribution were performed at least in triplicate for each treatment. The coefficient of variation, according to the FACStation Cell Quest software, was always less than 10%.

2.7. Determination of apoptosis by annexin-V assay

Phosphatidylserine externalisation and membrane integrity were quantified using the annexin V-FITC detection kit from Sigma. The protocol was that specified by the manufacturer with some modifications. Floating and adherent cells (5×10^4 cells) were washed twice with PBS and resuspended in 100 μl of binding buffer with 2.5 μl of annexin V-FITC and 5 μl of propidium iodine. After allowing to stain at room temperature, 400 μl of binding buffer was added, and samples were measured using a FACScan instrument equipped with FACStation running Cell Quest software (Becton Dickinson). The annexin-V assay was repeated three times.

2.8. Western blot

After 24, 48 and 72 h of treatment, the cells were washed twice with PBS and lysed in cold lysis buffer as described by Pozo-Guisado et al. [24]. Lysates were centrifuged at 15,000×*g* for 20 min at 4°C, and protein concentration was measured by Bradford's method [25]. To determine cyclins A, E, and cytochrome *c*, 20 μg protein/lane was used and 40 μg/lane for cyclin B1, bcl-XL, procaspase 3, 8 and 9 (this latter only in CCD-112CoN normal cells). β-Actin antibody (Sigma) was routinely assayed for monitoring total protein load. Proteins were separated by 10–12% SDS-PAGE and transferred to polyvinylidine diflouride membranes (Amersham Biosciences, UK) by electroblotting. Membranes were incubated for 2 h with the primary antibodies (Sigma) and 1 h with the secondary antibody conjugated to horseradish peroxidase (Sigma). Unless otherwise stated, proteins were detected using an ECL plus detection system (Amersham) according to the manufacturer's instructions. For quantification, the density of the bands was detected with scanning densitometry, using the Syngene Genetools Analysis Software (Syngene, USA). The Western blot assays (cyclins A, B1 and E, cytochrome *c*, bcl-XL, procaspases 3, 8 and 9) were repeated at least in triplicate.

2.9. Determination of procaspase 9 activation in Caco-2 cells

Procaspase 9 could not be detected using Western blot in Caco-2 cells. Therefore, in this case, analysis of caspase 9 activity was performed using the Apoptosis Detection Kit Caspase 9 Assay from Serotec (Oxford, UK) according to the manufacturer's instruction. The methodology is based on fluorochrome inhibitors of caspases (FLICA). The green fluorescent signal is a direct measure of the amount of active caspase 9 present in the cell at the time the reagent was added. Cells that contain the bound labelled reagent can be analysed by 96-well plate-based fluorometry, fluorescence microscopy or flow cytometry.

Caco-2 cells were treated with 30 μmol/L EA, 100 μmol/L PUNI or 1 μmol/L of staurosporine (as positive control) for 24, 48 and 72 h. Cells were pelleted by centrifugation, adjusted to $0.5–1\times10^6$ cells/100 μl and incubated for 1 h at 37°C and 5% CO_2, protecting samples from light and adding 10 μl of the FLICA reagent and shaking gently every 15 min. Afterwards, cells were washed twice and measured by flow cytometry using a FACScan instrument equipped with FACStation running Cell Quest software (Becton Dickinson).

The determination of procaspase 9 activation in Caco-2 cells was repeated three times.

2.10. Preparation of mitochondrial and cytosolic extracts

Cells were washed with ice-cold PBS and resuspended in ice-cold HMKEE buffer as described by Oh and Lee [26]. Mitochondrial and cytosolic extracts were obtained at least from three independent experiments.

2.11. Measure of surface Fas expression and Fas/Fas-L interaction

Surface expression on CD95/Fas was evaluated in Caco-2 cells (control and treated cells) by flow cytometry. Cells were incubated on ice with antihuman Fas (CD95/Apo1) clone DX2 (Sigma) or with an isotypic control (Dako Diagnostika, Hamburg, Germany), and after 1 h, the cells were washed and incubated for 1 h with FITC-labeled antimouse (Sigma). Cells were analysed (20,000 events were acquired) with a FACScan flow cytometer (Becton Dickinson).

To determine Fas/Fas-L interactions, the cells were pre-incubated with 1 μg/ml of anti-Fas clone ZB4 (Upstate, Lake Placid, USA) or with a nonspecific IgG_1 (Dako Diagnostika) for 1 h. Afterwards, the cells were treated with PUNI (100 μM), EA (30 μM) or Fas ligand 1 μg/ml (Alexis, Gruenberg, Germany) for 48 h, and then apoptosis was determined by the annexin-V assay and procaspase 3 activation. The experiments for measuring surface Fas expression and Fas/Fas-L interaction were repeated in triplicate.

2.12. Morphological evaluation of apoptosis

Cells treated with 1–30 μM EA and 1–100 μM PUNI for 24, 48 and 72 h were fixed with MeOH/acetic acid (3:1) and stained with 50 mg/ml Hoechst 33258 dye at 37°C for 20 min. Afterwards, the cells were examined under a Nikon Diaphot-TMD inverted microscope (Nikon, Tokyo, Japan). Morphological evaluation of apoptosis was carried out twice for Caco-2 cells and three times for normal colon CCD-112CoN cells.

2.13. Statistics

Two-tailed unpaired Student's *t*-test was used for statistical analysis of the data. A P value $<.01$ was considered significant. In the case of annexin-V analyses, statistical significance was determined by analysis of variance using the Tukey–Kramer multiple comparisons test. Graphs of the experimental data were carried out by using the Sigma Plot 6.0 program for Windows.

3. Results and discussion

Polyphenol-rich dietary foodstuffs have attracted attention due to their potential cancer chemopreventive and chemotherapeutic properties. The anticarcinogenic effect of the commercially available polyphenol EA has been extensively studied in a number of cancer cells [5–8]. However, free EA is not usually found as such in dietary foodstuffs but forms part of the polymeric hydrolysable ETs, which are present in the Western diet through the intake of strawberries, walnuts, raspberries, pomegranates, oak-aged red wine, etc. [9]. In spite of being a more realistic diet-related model than EA, the studies regarding the effect of dietary ETs on cancer cells are much more limited than those related to EA, which could be partially due to the lack of commercially available pure ETs.

We have recently published that dietary ETs were not detected in plasma and urine of human volunteers [14,15] and that they were only detected in the faeces of some volunteers [15]. We have also shown that ETs are hydrolysed in the gastrointestinal tract to release EA, which is further metabolised by colonic microflora to bioavailable hydroxy-dibenzo-pyran-6-one derivatives ('urolithin' derivatives) [15,27]. In addition, the metabolism of EA to urolithins is greatly influenced by the individual colonic microflora, yielding a high interindividual variability in the production of these metabolites from EA, which parallels that reported for equol from isoflavones [15,27]. Since EA bioavailability is extremely low in humans and very far from the 'micromolar' active concentrations, conclusions arisen from the assay of EA on cancer cells from potential target organs such as breast, brain, etc., can be severely questioned from a dietary point of view.

Taking into account all of the above, there are two key points in the present study that considerably improve the knowledge about the effect of EA and ellagitannins on cancer cells. One deals with the description of the detailed apoptotic pathway that occurred in Caco-2 cells upon treatment with EA and PUNI (the pomegranate PUNI as dietary ellagitannin), whereas no apoptosis was observed in normal colon cells. Caco-2 cells were chosen as a widely accepted model for colon cancer. In the present study, Caco-2 cells were not cultured up to confluence since differentiation can lead to the loss of important tumorigenic-related features [28]. The second key point deals with the metabolism of PUNI and EA revealing that PUNI was hydrolysed to yield EA, which seemed to be the real apoptosis inducer, and thus PUNI (and probably most of ellagitannins) should be considered as an 'apoptotic precursor' through the release of its hydrolysis product, EA, to the medium. Although a recent study described the antiproliferative and apoptotic effects of PUNI in a number of cancer lines [29], the molecular regulation of the apoptotic effect, together with the metabolism of PUNI by cancer cells, was not reported, however.

3.1. Cell proliferation and cell cycle in Caco-2 cells

The effect on proliferation and cell cycle of both ETs and EA on different cancer cell lines has been previously reported [6,16,30]. In our study, proliferation of Caco-2 cells exposed to different concentrations of both EA and PUNI was inhibited in a dose- and time-dependent manner (Fig. 2A). The decrease was evident at 24 h, especially in the presence of PUNI 100 μM. It is remarkable that no synergistic but rather

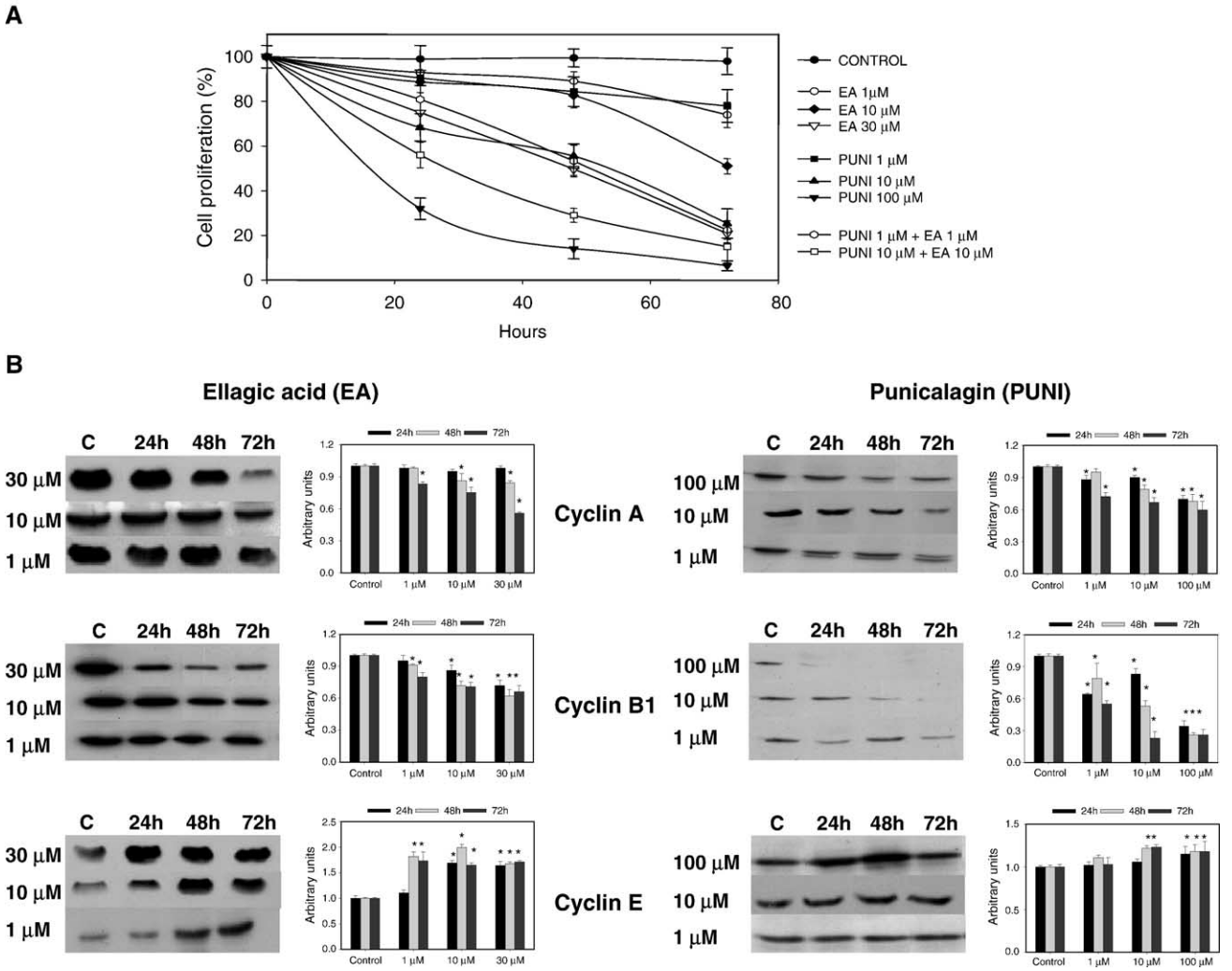

Fig. 2. Effect of EA and PUNI on (A) cell proliferation and (B) cyclins A, B1 and E expression in Caco-2 cells. Conditions as described in Materials and Methods. (A) Mean value ($n=3$) is shown. Coefficient of variation (CV) [(standard deviation/mean)×100] was always less than 10%. (B) Cyclins were detected using an ECL plus detection system when EA was assayed and using conventional ECL detection when PUNI was assayed. Gel ($n=3$) densitometry analysis is shown on the right of the corresponding Western blot. (*) Differences with respect to the control with $P<.01$.

an additive effect was observed when a mixture of EA and PUNI (10 μmol/L EA plus 10 μmol/L PUNI and another assay with 1 μmol/L EA plus 1 μmol/L PUNI) was assayed (Fig. 2A). Cell-cycle progression is regulated by the activity of cyclins, a family of proteins that activate the so-called cyclin-dependent kinases (Cdks). Cyclin A is required for S-phase and passage through G_2, cyclin E activates Cdk2 protein near the start of S-phase and cyclin B1 is a critical regulator of mitosis. In general, uncontrolled expression of cyclins and/or Cdks leads to either tumorigenesis or cell-cycle arrest [31]. Overall, both EA and PUNI down-regulated cyclins A and B1, and upregulated cyclin E in a dose- and time-dependent manner (Fig. 2B) which provoked Caco-2 cell-cycle arrest in S phase (results not shown).

3.2. Apoptosis in Caco-2 cells

The role for apoptosis has been proposed in a variety of human diseases [32]. Suppression of apoptosis contributes to carcinogenesis by several mechanisms, such as facilitating the accumulation of gene mutations, permitting growth factor-independent cell survival, promoting resistance to immune-based cytotoxicity and allowing bypassing of cell-cycle checkpoints, which would normally induce apoptosis. Although the existence of multiple programs of cell death is strongly supported by the vast amount of information disclosed in recent years, two major (and most representative) apoptotic pathways have been described [33]. One is dependent on the release of proapoptotic proteins from mitochondria to cytosol [34], while the other major pathway involves apoptosis induction via membrane receptors including CD95/CD95L (Fas/Fas-L) and other members of the TNF superfamily of cell death receptors [35]. Caspases are the main executioners of apoptosis [36]. There are the so-called initiator caspases (caspases 8 and 9) that respond to a number of stimuli to trigger the activation of the so-called effector caspases

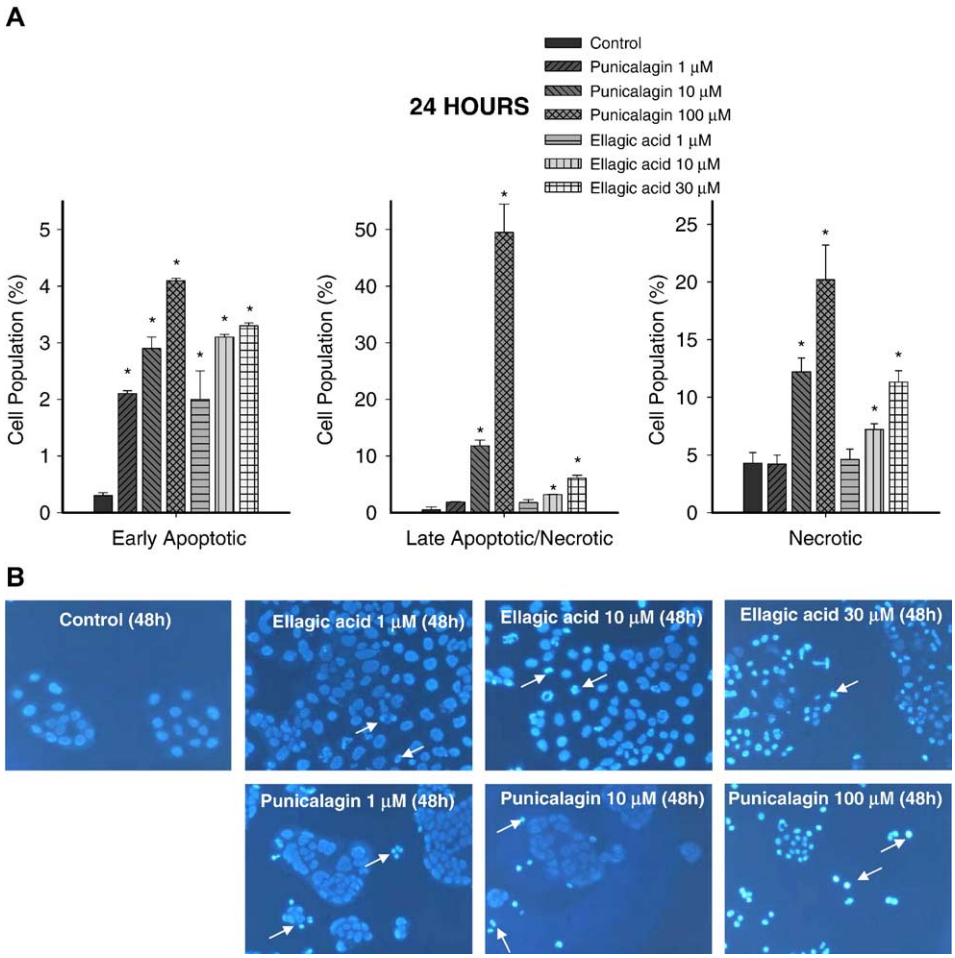

Fig. 3. (A) Effect of different EA and PUNI concentrations on the percentage of early apoptotic, late apoptotic/necrotic and necrotic Caco-2 cells after 24 h. Data are the mean of three separate experiments. (*) Differences with respect to the control with $P<.01$. (B) Morphological evaluation of apoptosis in Caco-2 cells with the Hoechst 33258 dye upon treatment with EA and PUNI. The arrows designate typical apoptotic nuclei with condensed chromatin.

(caspases 3 and 7) which launch the events necessary to mediate the apoptotic phenotype.

In our study, Caco-2 cells underwent apoptosis upon treatment with PUNI and EA. The population of single annexin V-positive cells (early apoptotic), annexin V plus propidium iodide-positive cells (late apoptotic/necrotic) and single propidium iodide-positive cells (necrotic) increased with increasing drug concentration (Fig. 3A). Quantitative analyses indicated a significant increase ($P<.01$) of apoptosis after 24 h (Fig. 3A) and reaching the highest early apoptotic cell population at 24 and 48 h in the presence of EA and PUNI, respectively (results not shown). Despite reaching approximately the same early apoptotic population upon treatment with both EA and PUNI, however, a much higher number of positive iodide cells (late apoptotic/necrotic) were observed with PUNI at 24 h and especially at 72 h (results not shown).

In contrast to normal cells, the nuclei of apoptotic cells have highly condensed chromatin that can be uniformly stained by the DNA-binding fluorochrome dye Hoechst 33258. Apoptotic nuclei can take the form of crescents around the periphery of the nucleus, or the entire nucleus can appear to be one or a group of featureless, bright spherical beads. Both EA and PUNI provoked the above chromatin condensation on Caco-2 cells confirming the apoptosis phenomenon (Fig. 3B).

3.3. The (intrinsic) mitochondrial apoptotic pathway. Treatment with EA or PUNI leads to cytochrome c release and procaspase 9 and 3 activation in Caco-2 cells

The release of cytochrome *c* from the mitochondria into the cytosol is characteristic in the (intrinsic) mitochondrial apoptotic pathway. To this purpose, cytochrome *c* levels were determined in both cytosolic and mitochondrial Caco-2 extracts after treatment with different EA and PUNI concentrations. There was a dose- and time-dependent release of cytochrome *c* from the mitochondria into the cytosol after incubation of Caco-2 cells with both EA and PUNI (Fig. 4A). It is noticeable that maximum cytochrome *c* release was observed at 24 h with 10 and 30 μmol/L EA, whereas this maximum was delayed and appeared at 48 h with EA 1 μmol/L and PUNI from 1 to 100 μmol/L (Fig. 4A). In addition, the expression of bcl-XL, belonging to the 'pro-survival' (antiapoptotic) proteins of the bcl-2

family, was also explored. This is especially interesting since the down-regulation of the bcl-XL protein expression has been proposed as a new and promising treatment strategy for human (colon) cancer [37,38]. This anti-apoptotic protein preserves mitochondria integrity preventing cytochrome *c* release into the cytosol [39]. In our study, the treatments with EA (and also with PUNI as EA precursor) attenuated the protein levels of bcl-XL in treated Caco-2 cells (Fig. 4B). As cytochrome *c* release usually induces the activation of procaspase 9, we further determined procaspase 9 activation. As shown in Fig. 5A, treatment of Caco-2 cells with 30 μmol/L EA and 100 μM PUNI increased the proteolytic activity of caspase 9, which was first detected with this assay method after 48 h (with PUNI) and became evident after 72 h also with EA and staurosporine 1 μM (the latter as positive control).

Procaspase 3 activation as possible effector in the apoptosis of Caco-2 cells induced by EA and PUNI was also explored. No remarkable changes were observed in the expression of procaspase 3 in Caco-2 cells with EA or PUNI after 24 h (Fig. 5B). A significant decrease in procaspase 3 expression (indicating the cleavage of procaspase 3 and the corresponding activation to caspase 3) was observed after 48 h of treatment with EA 10 and 30 μmol/L and PUNI

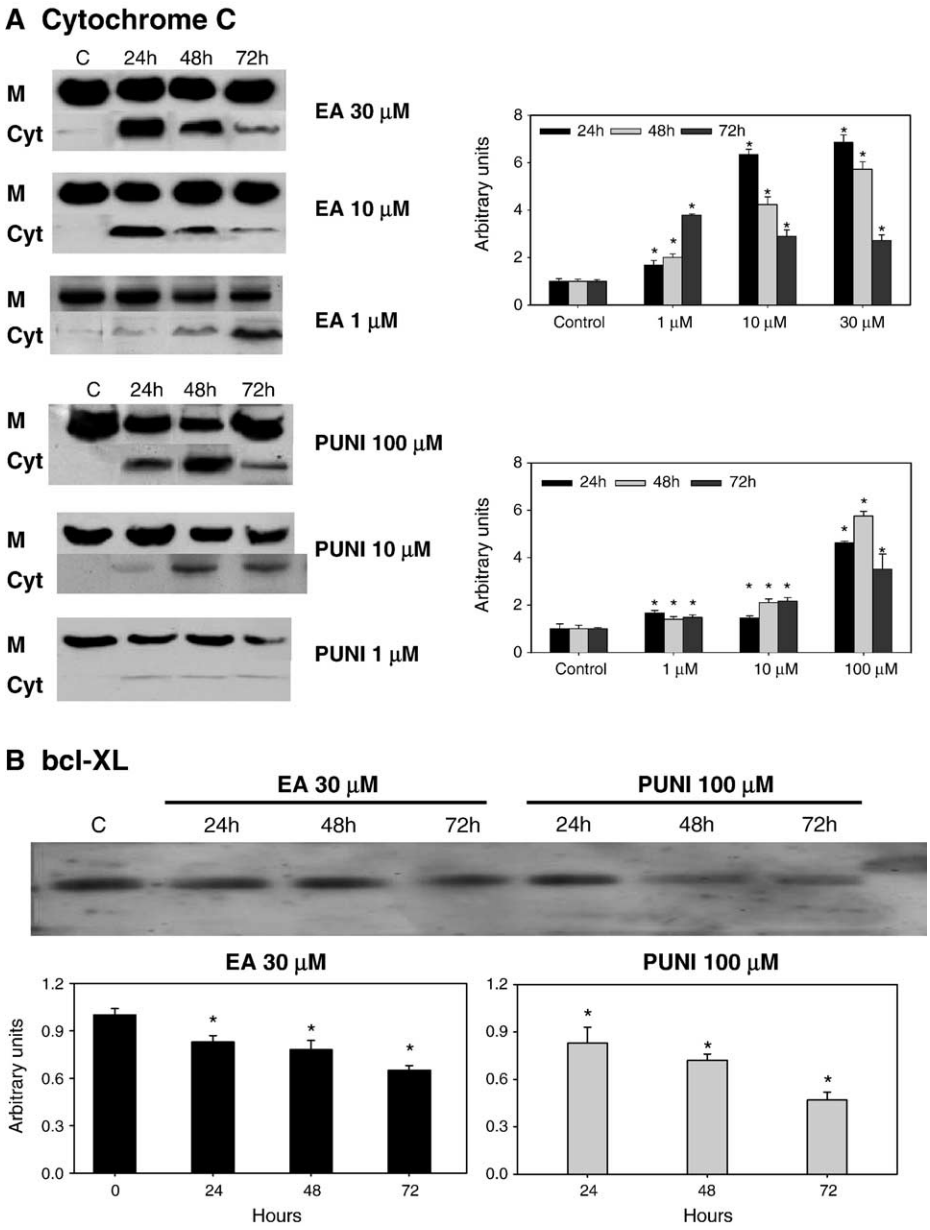

Fig. 4. (A) Western blot analysis of cytochrome *c* release from mitochondria (M) into the cytosol (Cyt) in Caco-2 cells upon treatment with EA and PUNI. Gel ($n=3$) densitometry analyses (bar plots) were carried out only in the cytosolic fraction. (*) Differences with respect to the control with $P<.01$. (B) Western blot analysis of bcl-XL protein upon treatment with 30 μmol/L EA and 100 μmol/L PUNI at different assay times. Gel ($n=3$) densitometry analyses are shown below. (*) Significant differences with respect to the control ($P<.01$).

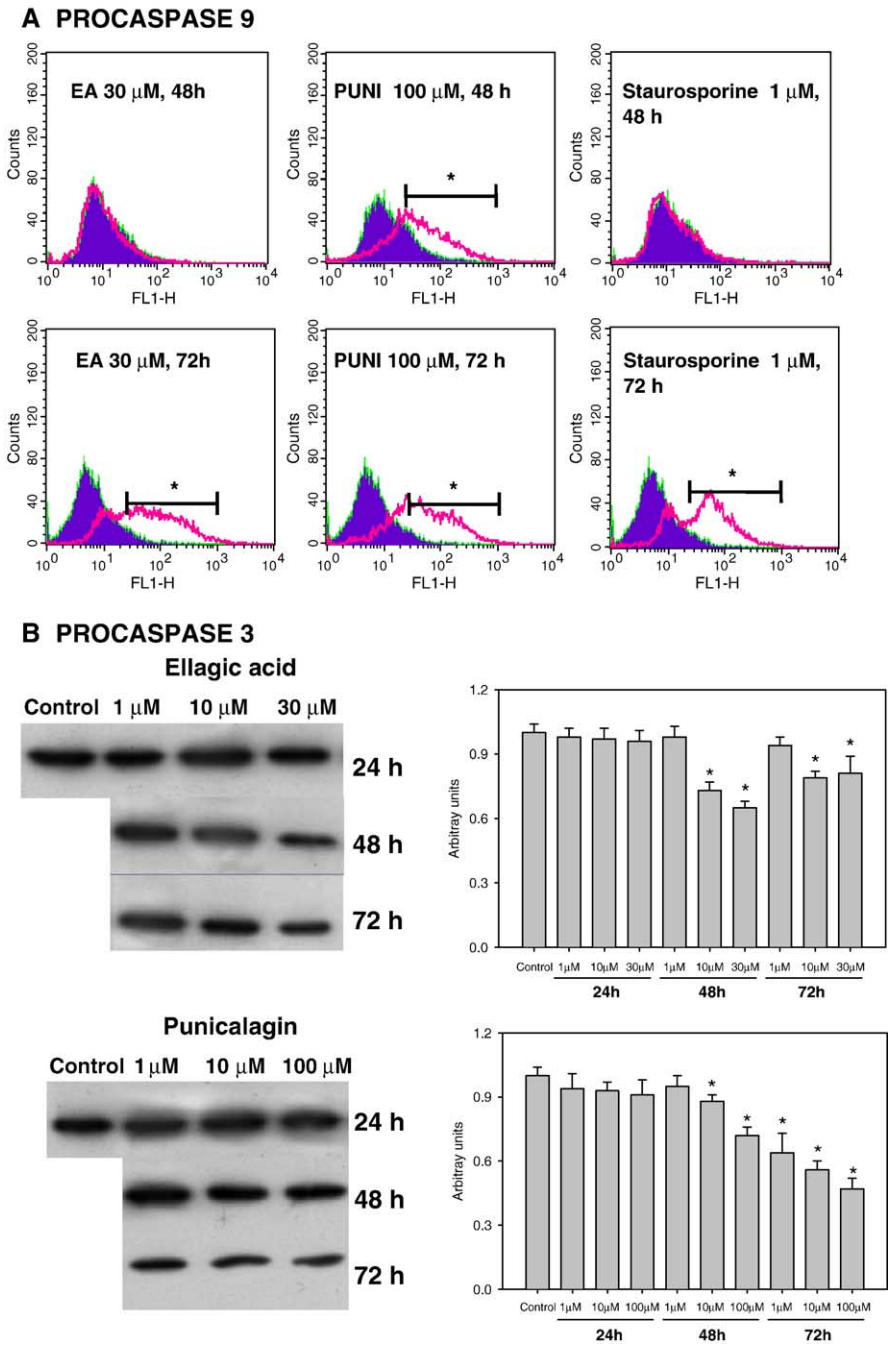

Fig. 5. (A) Flow cytometric analysis of procaspase 9 activation in Caco-2 cells with the carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspase 9 (FAM-LEHD-FMK). Staurosporine was assayed as positive control. (*) Significant differences with respect to the control ($P<.01$). (B) Western blot analysis of procaspase 3 activation in Caco-2 cells. Gel ($n=3$) densitometry analyses are shown on the right. (*) Significant differences with respect to the control ($P<.01$).

10 and 100 μmol/L (Fig. 5B). After 72 h, procaspase 3 activation was significant with all concentrations except with EA 1 μmol/L (Fig. 5B).

3.4. The (extrinsic) death receptor pathway. Apoptosis induced by EA or PUNI is not FAS-mediated, and no caspase 8 activation occurred

Death receptors, belonging to the TNF receptor gene superfamily, have cytoplasmatic sequences (so-called death domain) that permit death receptors to engage the cell's apoptotic machinery [35]. CD95 (Fas) expression was not detected in Caco-2 cells (results not shown) in accordance with previous reports [40]. However, a previous study described the induction of apoptosis in Caco-2 cells by wheat gliadins following Fas-dependent pathway [41]. Therefore, specific anti-Fas antibody (to block the potential interaction of PUNI or EA with the receptor) and Fas ligand (to promote apoptosis via Fas) were assayed in order to fully

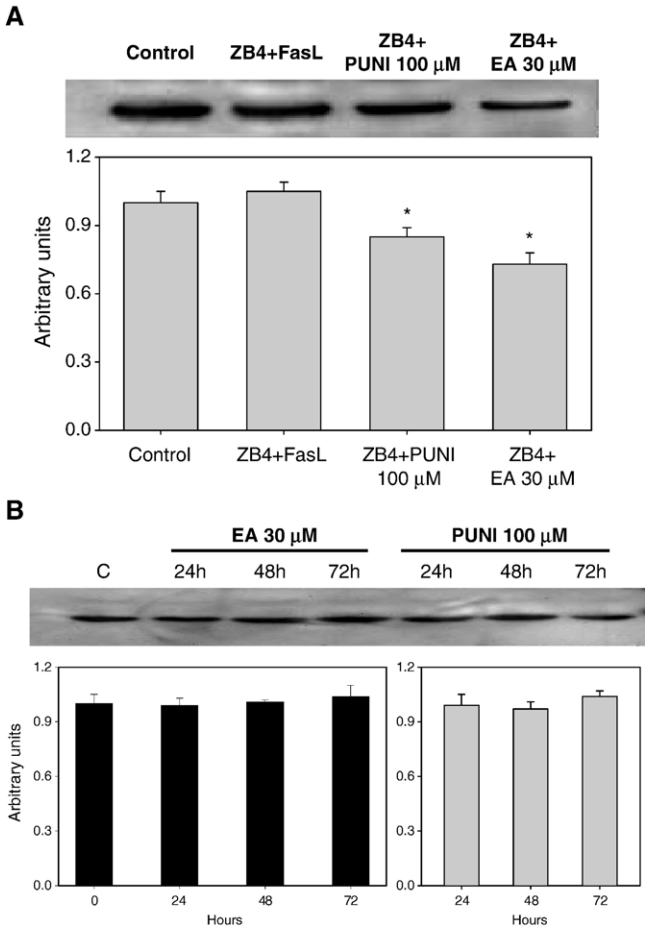

Fig. 6. Evaluation of EA and PUNI-induced apoptosis in Caco-2 cells via extrinsic pathway. (A) Western blot analysis of procaspase 3 activation via Fas receptor (representative experiment after 48 h of treatment). Gel ($n=3$) densitometry analyses are shown below. (*) Differences with respect to the control with $P<.01$. (B) Western blot analysis of procaspase 8 activation as common intermediate in the dead receptor (extrinsic) apoptosis pathway. Gel ($n=3$) densitometry analyses are shown below. (*) Significant differences with respect to the control ($P<.01$).

discard the possible involvement of Fas receptor in the apoptosis induced by EA or PUNI in Caco-2 cells. Our results demonstrate that no apoptosis was provoked by the Fas ligand (evaluated by the annexin-V assay and procaspase 3 activation), which indicated that Fas receptor (if present in Caco-2 cells) was not involved in the apoptosis induced by EA and PUNI in Caco-2 cells (Fig. 6A). In addition, the incubation of Caco-2 cells with the anti-Fas ZB4 antibody (to avoid possible interaction with the Fas receptor) in the presence of EA and PUNI did not avoid apoptosis (Fig. 6A), which further confirmed that apoptosis induced by EA and PUNI was not Fas-mediated.

The 'initiator caspase' 8 is a common intermediate in death receptors for triggering apoptosis via 'extrinsic pathway' [42]. In our model, neither PUNI nor its hydrolysis product, EA, activated the initiator caspase 8, suggesting that apoptosis via membrane dead receptors (Fas and TRAIL receptor signals) was very unlikely to occur. As

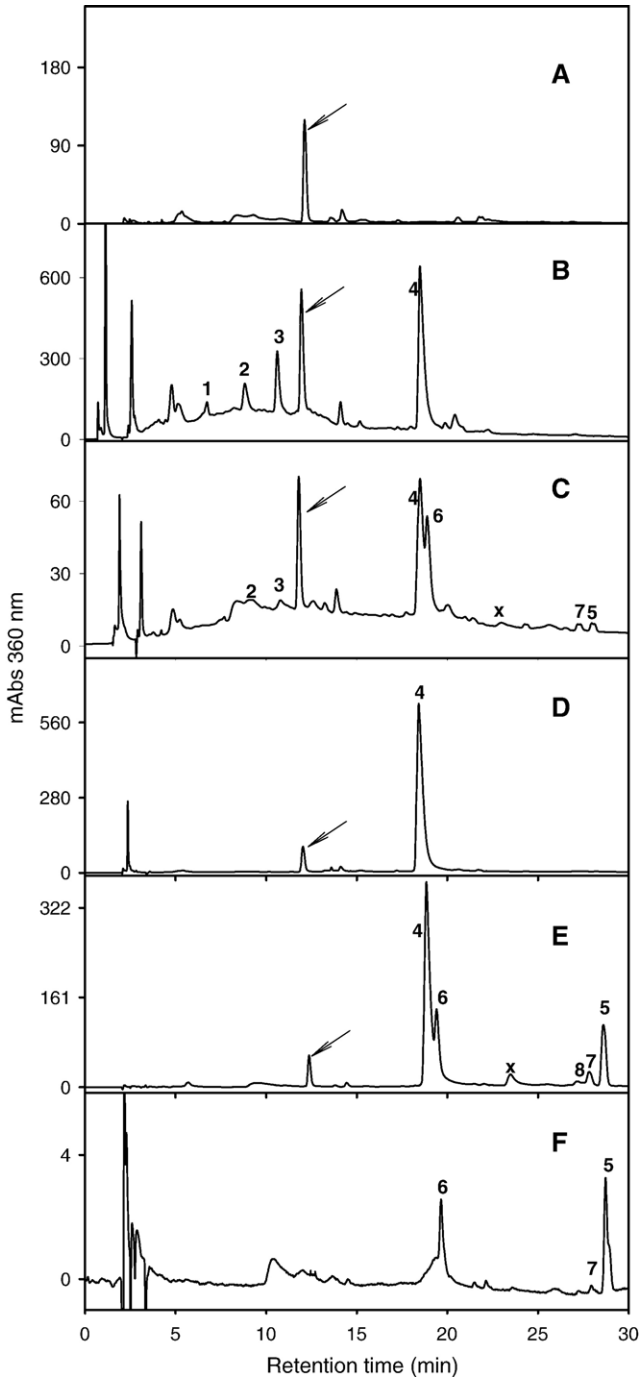

Fig. 7. (A–E) HPLC profiles at 360 nm of culture media and (F) cell extract. (A) Control culture medium after 48 h. The arrow designates a compound of the medium. (B) Cell medium in the presence of 100 μmol/L PUNI (isomers **1**, **2** and **3**) but without Caco-2 cells after 24 h. Compound **4** is EA. (C) Cell medium in the presence of 100 μmol/L PUNI with Caco-2 cells after 48 h showing the EA-derived metabolites: **5**, dimethyl ellagic acid; **6**, dimethyl ellagic acid-glucuronide; **7**, isomer of dimethyl ellagic acid-sulphate; ×, unidentified EA-derived metabolites (according to its EA-like UV spectrum). (D) Cell medium in the presence of 30 μmol/L EA but without Caco-2 cells after 48 h. (E) Cell medium in the presence of 30 μmol/L EA with Caco-2 cells after 48 h showing the same EA-derived metabolites described above when cells were incubated with PUNI and one more isomer of dimethyl ellagic acid-sulphate (**8**). (F) HPLC profile of lysed cells after treatment with 100 μmol/L EA for 48 h. No free EA (compound **4**) was detected.

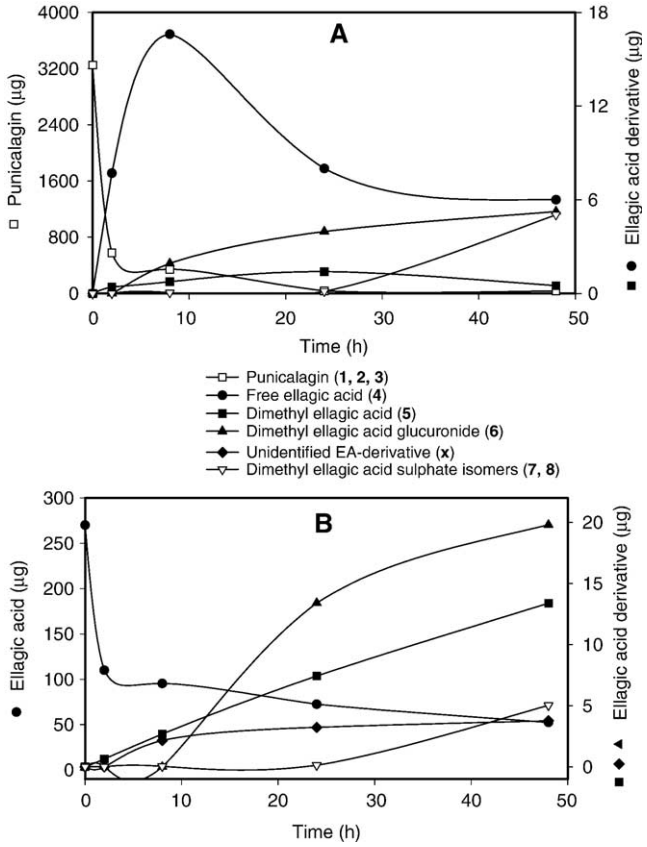

Fig. 8. Time-course evolution of the stability and metabolism of both PUNI (A) and EA (B) in the Caco-2 cell medium. Numbers in the legend refer to the peaks in Fig. 7. The mean value ($n=4$) is shown. Coefficient of variation was always less than 10%.

shown in Fig. 6B, no effect was observed in the expression of caspase 8, which again indicated that the death receptors are not likely to play a role in the EA- and PUNI-induced death of Caco-2 cells.

3.5. PUNI hydrolysis releases EA which is metabolised by Caco-2 cells

In the present study, both EA and PUNI induce apoptosis in Caco-2 cells seemingly by following the same pathway, i.e., intrinsic (mitochondrial) pathway (cytochrome *c* release, and activation of caspases 9 and 3). However, a more in-depth study of the process revealed that PUNI was hydrolysed in the cell medium yielding EA, which suggests that EA is the actual inducer of apoptosis since no apoptosis was detected upon PUNI treatment until accumulation of EA occurred. To the best of our knowledge, this is the first time that this conversion from ET to EA is described in cell cultures. Although further confirmation is needed for each specific ellagitannin, the apoptotic-mediated anticarcinogenic activity reported for a number of ETs could be really due to the EA released to the medium, and thus ETs could be considered as 'apoptotic precursors' [43].

Most polyphenols are very reactive polyhydroxylated compounds, and cells try to metabolise these molecules by blocking the hydroxyl reactive moieties [44]. In addition, polyphenols are rather unstable in cell cultures partially due to the neutral (physiological) pH [44,45]. Despite these well-known facts, however, the studies that investigate the effect of dietary natural polyphenols on cancer cells very rarely perform assays to check polyphenols stability, cellular uptake and metabolism [44–46]. In addition, from a dietary point of view, the assay of 'realistic' in vitro concentrations depending on the in vitro model is not usually approached [44,45]. To this purpose, we examined the LC-MS-MS profile of both cell media and Caco-2 extracts in the absence (control) and in the presence of EA 30 μmol/L or PUNI 100 μmol/L. It is of note that no oxidation products (quinone derivatives) were detected either in the medium or inside the cells (results not shown).

Control cell media contained one compound with a peak at 360 nm (designated with an arrow in Fig. 7A). The same cell media in the presence of PUNI (Fig. 7B) but in the absence of Caco-2 cells showed the three characteristic main PUNI isomers (compounds **1**, **2**, **3** with ion mass at m/z^- 1083). Compound **4** was identified as free EA (ion mass at m/z^- 301). Therefore, a significant amount of EA was observed in the cell media due to spontaneous hydrolysis of PUNI (Fig. 7B).

In the presence of Caco-2 cells, PUNI isomers were hardly detected in the media after 48 h (only peaks **2** and **3**; Fig. 7C). In addition to free EA (**4**; Fig. 7C), other EA derivatives detected (Fig. 7C) according to their UV spectra and ion mass were identified as dimethyl-EA (**5**, m/z^- 329), dimethyl-EA-glucuronide (**6**, m/z^- 505) and dimethyl-EA-sulphate (**7**, m/z^- 409) (Fig. 7C). The conjugation of EA-derived metabolites with glucuronic acid and sulphate was verified by incubating the samples with both glucuronidase and sulphatase (results not shown).

HPLC profiles of cell media (without Caco-2 cells) in the presence of EA showed the same peak corresponding to compound **4** (Fig. 7D) coincident with that described above in the case of PUNI (Fig. 7B,C). No other EA-related peak was observed in the cell media without cells (results not shown). In the presence of Caco-2 cells, the same EA derivatives described above (when cells were incubated with PUNI) were detected (Fig. 7E), including one more dimethyl-EA-sulphate isomer (**8**, m/z^- 409). Therefore, PUNI underwent spontaneous hydrolysis in the cell media to release EA (in the presence and in the absence of Caco-2 cells). However, only in the presence of Caco-2 cells was EA further metabolised to produce the above EA-derived metabolites.

These EA-derived metabolites were not detected when PUNI or EA was added to the media in the absence of Caco-2 cells but using the media in which Caco-2 cells were previously grown for 48 h (results not shown). This indicated that Caco-2 cells did not excrete enzymes constitutively to the medium involved in the metabolism of PUNI or EA. Under this assay condition, the same results shown in Fig. 7B and D were obtained.

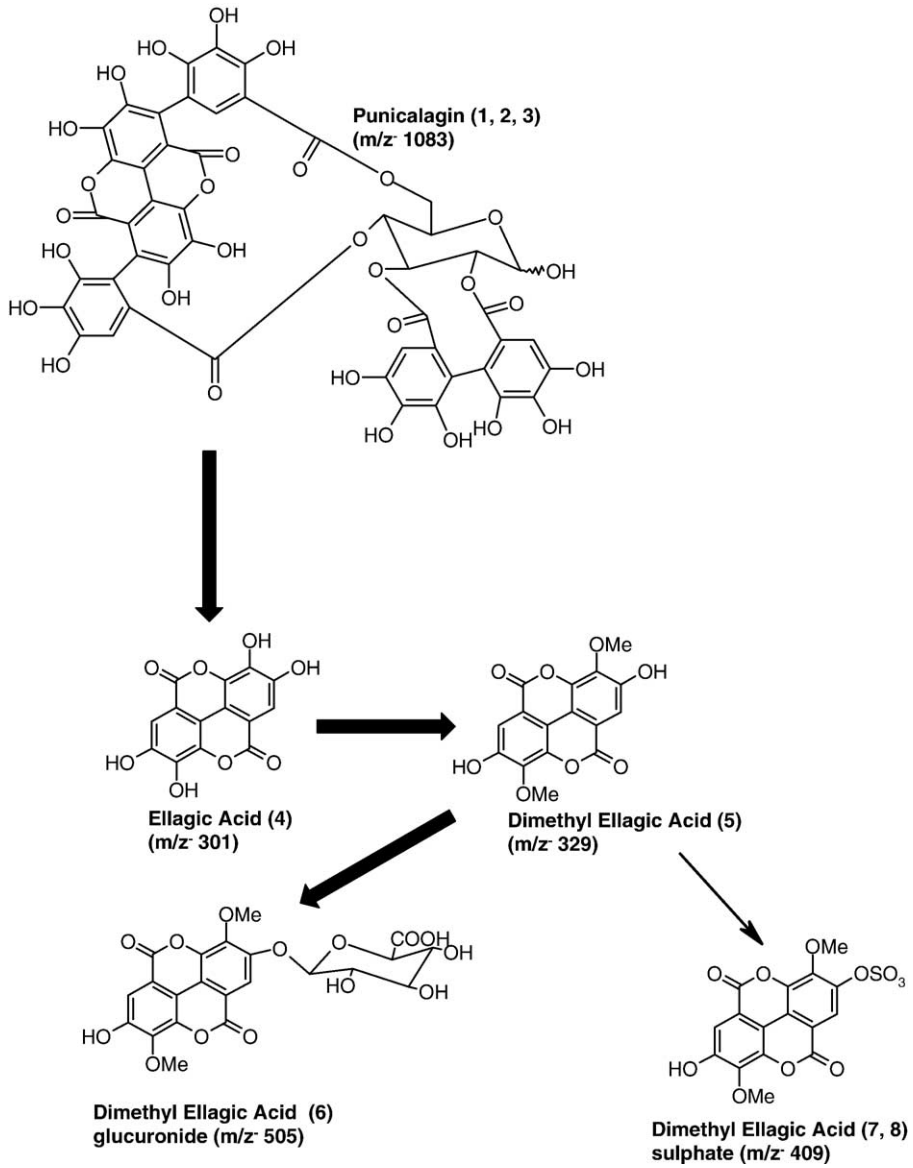

Fig. 9. Tentative metabolism pathway of PUNI and EA by Caco-2 cells. Thicker arrows designate the preferential pathway. Numbers that follow the name of compounds refer to the peaks in Fig. 7.

To assess the uptake of both EA and PUNI by Caco-2 cells, the corresponding cell lysates (from control and treated cells) were analysed (Fig. 7F). It is remarkable that no free EA was detected in any sample, and only EA-derived metabolites were detected, i.e., dimethyl-ellagic (**5**), dimethyl-EA-glucuronide (**6**) and one of the dimethyl-EA-sulphate isomers (**7**). These metabolites were easily detected upon treatment of Caco-2 cells with EA after 48 h. In the case of the cell extracts from PUNI treatments, no intact PUNI was detected inside the cells, and the above metabolites were hardly detected, and only compound **6** was easily identified (results not shown). These results indicated that EA-derived metabolites were produced inside the cells and were further excreted into the medium.

The methylation of EA by Caco-2 cells to produce dimethyl-EA and the further glucuronidation or sulphation of this dimethyl-derivative suggest the involvement of the Phase II enzymes catechol-*O*-methyltransferase (COMT), glucuronyl transferase and sulphate transferase. The role of these (detoxifying) enzymes in the metabolism of polyphenols has been described in different tissues, subcellular fractions and cell lines including Caco-2 cells [47,48]. Therefore, in the present study, Caco-2 cells tried to convert EA preferentially to dimethyl-EA-glucuronide derivatives to diminish the potential toxicity of the polyphenolic hydroxyl groups which have been reported to selectively exert great anticancer activity [49,50]. In this context, it should be noted that no free EA was observed inside the cells, indicating that free EA entered into the cells where EA was metabolised and further excreted to the medium (no EA metabolites were found in the medium without cells).

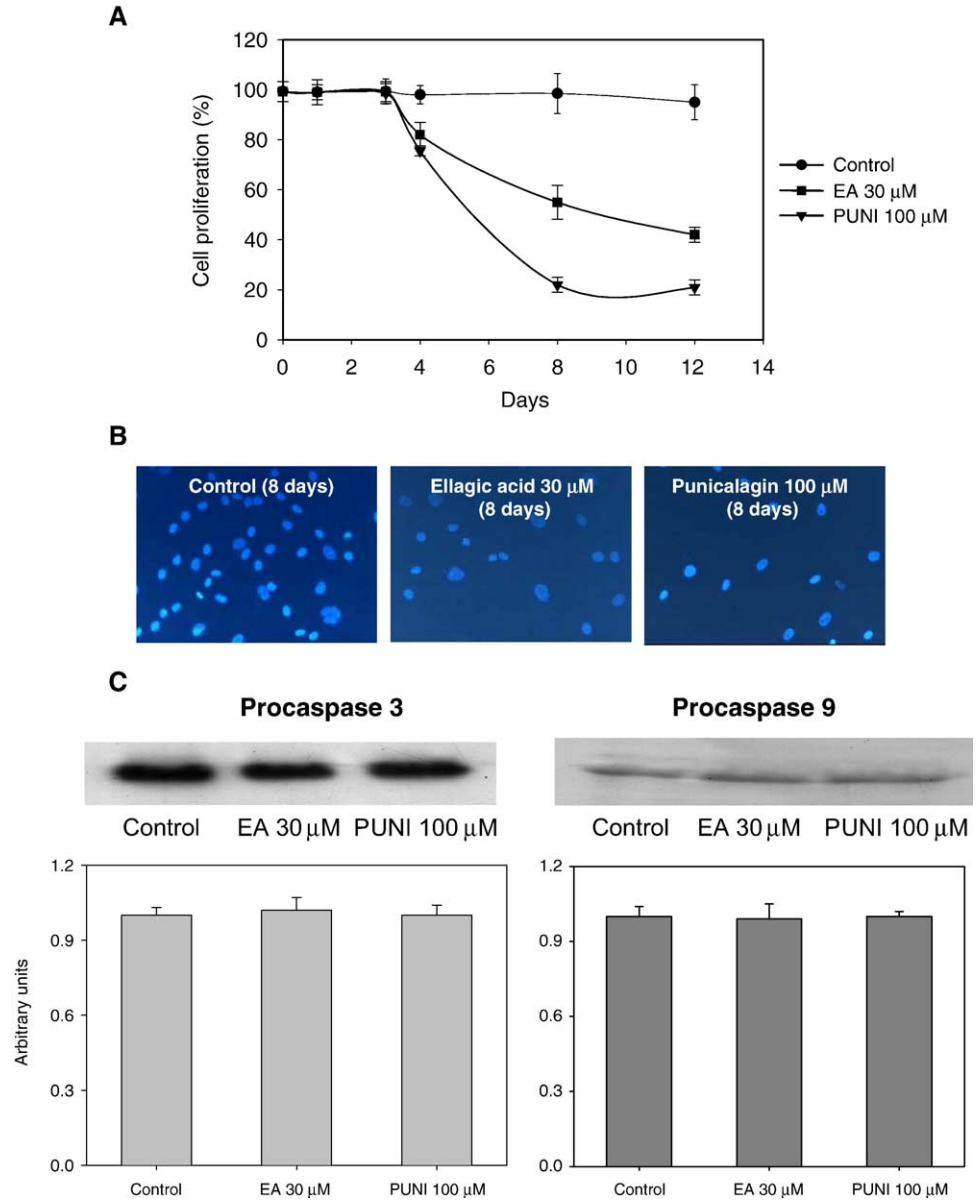

Fig. 10. Effect of EA and PUNI on colon normal CCD-112CoN cells. EA 30 μmol/L and PUNI 100 μmol/L were refreshed in the medium every 2 days. (A) Effect on cell proliferation. (B) Morphological evaluation of apoptosis in CCD-112CoN cells with the Hoechst 33258 dye after 8 days. (C) Western blot analysis of procaspase 3 and 9 in CCD-112CoN cells after 8 days. Gel ($n=3$) densitometry analyses are shown below. (*) Significant differences with respect to the control ($P<.01$).

The analysis of cell media (with Caco-2 cells) to study the time-course evolution of PUNI and EA as well as the EA-derived metabolites described above showed that PUNI quickly disappeared (open square, Fig. 8A) with concomitant increase of free EA (filled circle, Fig. 8A), which reached the maximum after 8 h and then decreased with accumulation of compounds **5**, **6** and **7** (Fig. 8A). This is important since no apoptosis was observed until EA accumulation occurred (results not shown). The analysis of cell media with EA-treated Caco-2 cells showed that free EA decreased quickly during the first 2 h and then more slowly (Fig. 8B) with concomitant accumulation of compound **5** and then subsequently appeared with the rest of EA-derived metabolites (Fig. 8B). The analysis of these results led us to propose a tentative pathway followed by Caco-2 cells to metabolise EA (Fig. 9). According to our results, PUNI (isomers **1**, **2** and **3**; Figs. 7–9) is hydrolysed in the medium, independent of Caco-2 cells, to yield EA (compound **4**; Figs. 7–9) which was first methylated by COMT to yield dimethyl EA (compound **5**; Figs. 7–9). From this dimethyl derivative, the preferential pathway was the glucuronidation to yield dimethyl EA glucuronide (compound **6**; Figs. 7–9), although some sulphatation also occurred to yield dimethyl EA sulphate (isomers **7** and **8**, Figs. 7–9).

3.6. Neither PUNI nor EA induces apoptosis in normal colon CCD-112CoN cells

Despite the huge number of reports regarding the effect of natural polyphenols on cancer cells, the assay on normal cells has been rather poorly studied [20,51]. This could be due to the much slower growth and difficult culture conditions with respect to their cancerous counterparts. It should be stressed that the effects on normal cells must be investigated in longer assays because of the much slower metabolism and growth of normal cells. In fact, some previous reports investigate the effect on both cancer and normal cells during the same time of exposure to different compounds, which could lead to questionable results. The normal colon CCD-112CoN cells showed a doubling time of 8 days in contrast to the 40 h of Caco-2 cells. In our study, both EA and PUNI (30 and 100 μmol/L, respectively) decreased the proliferation of normal colon CCD-112CoN cells from the fourth day of treatment (Fig. 10A). However, it should be stressed that assay conditions were more drastic than in the case of Caco-2 cells since the inhibition of cell proliferation in normal colon cells was observed only at the highest concentrations (100 μmol/L PUNI and 30 μmol/L EA) and after various changes of cell media (involving the 'refreshment' of PUNI and EA concentrations). However, and most important, no apoptosis was observed, suggesting the specific effect of EA (as apoptosis inducer) on Caco-2 cells through some key intermediate (not present in normal cells), which deserves further investigations. The absence of apoptosis was assessed by morphological evaluation (absence of chromatin condensation) at the eight day (when cell proliferation was significantly affected) (Fig. 10B). In addition, no activation of caspase 3 or 9 was observed (Fig. 10C), which further confirmed the absence of the apoptotic phenomenon in these cells upon treatment with either EA or PUNI.

In summary, our results suggest that ellagitannin PUNI could be considered as an 'apoptotic precursor' through the release of its hydrolysis product, EA, to the medium. Ellagic acid entered into the cells and was further metabolised to produce different dimethyl-EA derivatives. Ellagic acid provoked different effects on Caco-2 cells including down-regulation of cyclins A and B1 and upregulation of cyclin E with cell-cycle arrest in S phase and triggered apoptosis via intrinsic pathway through bcl-XL down-regulation with mitochondrial release of cytochrome *c* into the cytosol and activation of initiator caspase 9 and effector caspase 3. No apoptosis was observed in colon normal CCD-112CoN cells.

To the best of our knowledge, the above approach, which combines the description of the molecular regulation of the apoptotic pathway, the metabolism of the assayed compounds by cancer cells and the potential effect on normal cells, has not been reported so far. With all the caution called for in drawing conclusions from in vitro cell line experiments regarding the responses of cancer cells in vivo, the data presented in this study suggest that the anticarcinogenic effect of dietary ETs could be mainly due to their hydrolysis product EA, which could effectively induce apoptosis in colon cancer cells.

Acknowledgments

This investigation has been supported by Spanish CICYT, projects AGL2003-02195 and AGL-2004-03989, and by the Fundación Séneca, project PB/18/FS/02. Mar Larrosa has a fellowship from the Spanish Consejo Superior de Investigaciones Científicas (CSIC) and ESF (I3P Programme).

References

[1] Arai Y, Watanabe S, Kimira M, Simio K, Mochizuki R, Kinae N. Dietary intakes of flavonols, flavones and isoflavones by Japanese women and the inverse correlation between quercetin intake and plasma LDL cholesterol. J Nutr 2001;130:2378–83.

[2] Bazzano LA, He J, Ogden LG, Loria CM, Vupputuri S, Myers L, et al. Fruit and vegetable intake and risk of cardiovascular disease in US adults the first national health and nutrition examination survey epidemiologic follow-up study. Am J Clin Nutr 2002;76:93–9.

[3] Flood A, Velie EM, Chaterjee NS, Amy F, Thompson FE, Lacey JV, et al. Fruit and vegetable intakes and the risk of colorectal cancer in the breast cancer detection demonstration project follow-up cohort. Am J Clin Nutr 2002;75:936–43.

[4] Geleijnse JM, Launer LJ, van der Kuip DAM, Hofman A, Witteman JCM. Inverse association of tea and flavonoid intakes with incident myocardial infarction: the Rotterdam study. Am J Clin Nutr 2002;75:880–6.

[5] Li TM, Chen GW, Su CC, Ling JG, Yeh CC, Cheng KC, et al. Ellagic acid induced p53/p21 expression, G1 arrest and apoptosis in human bladder cancer T24 cells. Anticancer Res 2005;25:971–9.

[6] Losso JN, Bansode RR, Trappey A, Bawadi HA, Truax R. In vitro anti-proliferative activities of ellagic acid. J Nutr Biochem 2004;15:672–8.

[7] Mertens-Talcott SU, Bomser JA, Romero C, Talcott ST, Percival SS. Ellagic acid potentiates the effect of quercetin on p21waf1/cip1, p53, and MAP-kinases without affecting intracellular generation of reactive oxygen species in vitro. J Nutr 2005;135:609–14.

[8] Narayanan BA, Geoffroy O, Willingham MC, Re GG, Nixon DW. p53/p21(WAF1/CIP1) expression and its possible role in G1 arrest and apoptosis in ellagic acid treated cancer cells. Cancer Lett 1999;136:215–21.

[9] Clifford MN, Scalbert A. Ellagitannins — nature, occurrence and dietary burden. J Sci Food Agric 2000;80:118–25.

[10] Kuo PL, Hsu YL, Lin TC, Chang JK, Lin CC. Induction of cell cycle arrest and apoptosis in human non-small cell lung cancer A549 cells by casuarinin from the bark of *Terminalia arjuna* Linn. Anti-Cancer Drugs 2005;16:409–15.

[11] Pan MH, Lin JH, Lin-Shiau SY, Lin JK. Induction of apoptosis by penta-*O*-galloyl-β-D-glucose through activation of caspase-3 in human leukemia HL-60 cells. Eur J Pharmacol 1999;381:171–83.

[12] Tanimura S, Kadomoto R, Tanaka T, Zhang YJ, Kouno I, Kohno M. Suppression of tumor cell invasiveness by hydrolyzable tannins (plant polyphenols) via the inhibition of matrix metalloproteinase-2/-9 activity. Biochem Biophys Res Commun 2005;330:1306–13.

[13] Hollman PCH, Katan MB. Absorption, metabolism and bioavailability of flavonoids. In: Rice-Evans C, Packer L, editors. Flavonoids in health and disease. New York: Marcel Dekker; 1998. p. 483–522.

[14] Cerdá B, Espín JC, Parra A, Martínez P, Tomás-Barberán FA. The potent in vitro antioxidant ellagitannins from pomegranate juice are metabolized into bioavailable but poor antioxidant hydroxy-

6H-dibenzopyran-6-one derivatives by the colonic microflora of healthy humans. Eur J Nutr 2004;43:205–20.
[15] Cerdá B, Tomás-Barberán FA, Espín JC. Metabolism of chemopreventive and antioxidant ellagitannins from strawberries, raspberries, walnuts and oak-aged wines in humans: identification of biomarkers and individual variability. J Agric Food Chem 2005;53: 227–35.
[16] Narayanan BA, Re GG. IGF-II down regulation associated cell cycle arrest in colon cancer cells exposed to phenolic antioxidant ellagic acid. Anticancer Res 2001;21:359–64.
[17] Harris GK, Gupta AM, Nines RG, Kresty LA, Habib SG, Frankel WL, et al. Effects of lyophilized black raspberries on azoxymetane-induced colon cancer and 8-hydroxy-2′ -deoxyguanosine levels in the Fischer 344 rat. Nutr Cancer 2001;40:125–33.
[18] Kresty LA, Morse MA, Morgan C, Carlton PS, Lu J, Gupta A, et al. Chemoprevention of esophageal tumorigenesis by dietary administration of lyophilised black raspberries. Cancer Res 2001;61:6112–9.
[19] Stoner GD, Kresty LA, Carlton PS, Siglin JC, Morse MA. Isothiocyanates and freeze-dried strawberries as inhibitors of esophageal cancer. Toxicol Sci 1999;52:95–100.
[20] Han C, Ding H, Casto B, Stoner GD, D'Ambrosio SM. Inhibition of the growth of premalignant and malignant human oral cell lines by extracts and components of black raspberries. Nutr Cancer 2005;51:207–17.
[21] Xiao DS, Wen JF, Li JH, Hu ZL, Zheng H, Fu CY. Effect of deleted pancreatic cancer locus 4 gene transfection on biological behaviors of human colorectal carcinoma cells. World J Gastroenterol 2005;21: 348–52.
[22] Wolter F, Akoglu B, Clausnitzer A, Stein J. Downregulation of the cyclin D1/Cdk4 complex occurs during resveratrol-induced cell cycle arrest in colon cancer cell lines. J Nutr 2001;131:2197–203.
[23] Cerdá B, Llorach R, Cerón JJ, Espín JC, Tomás-Barberán FA. Evaluation of the bioavailability and metabolism in the rat of punicalagin, and antioxidant polyphenol from pomegranate juice. Eur J Nutr 2003;42:18–28.
[24] Pozo-Guisado E, Álvarez-Barrientos A, Mulero-Navarro S, Santiago-Josefat B, Fernández-Salguero PM. The antiproliferative activity of resveratrol results in apoptosis in MCF-7 but not in MDA-MB-231 human breast cancer cells: cell-specific alteration of the cell cycle. Biochem Pharmacol 2002;64:1375–86.
[25] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal Biochem 1976;210:727–35.
[26] Oh SH, Lee BH. Induction of apoptosis in human hepatoblastoma cells by tetrandrine via caspase-dependent Bid cleavage and cytochrome *c* release. Biochem Pharmacol 2003;66:725–31.
[27] Cerdá B, Periago P, Espín JC, Tomás-Barberán FA. Identification of urolithin A as a metabolite produced by human colon microflora from ellagic acid and related compounds. J Agric Food Chem 2005;53: 5571–6.
[28] Sambuy Y, De Angelis I, Ranaldi G, Scarino ML, Stammati A, Zucco F. The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. Cell Biol Toxicol 2005;21:1–26.
[29] Seeram NP, Adams LS, Henning SM, Niu Y, Zhang Y, Nair MG, et al. In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. J Nutr Biochem 2005;16:360–7.
[30] Ito H, Miyake M, Nishitani E, Mori K, Hatano T, Okuda T, et al. Anti-tumor promoting activity of polyphenols from *Cowania mexicana* and *Coleogyne ramosissima*. Cancer Lett 1999;143:5–13.
[31] Le NTV, Richardson DR. The role of iron in cell cycle progression and the proliferation of neoplastic cells. Biochim Biophys Acta 2002; 1603:31–46.
[32] Fadeel B, Orrenius S, Zhivotovsky B. Apoptosis in human disease: a new skin for the old ceremony? Biochem Biophys Res Commun 1999;266:699–717.
[33] Lorenzo HK, Susin SA. Mitochondrial effectors in caspase-independent cell death. FEBS Lett 2004;557:14–20.
[34] Green DR, Reed JC. Mitochondria and apoptosis. Science 1998;281:1309–12.
[35] Ashkenazi A, Dixit A. Death receptors: signalling and modulation. Science 1998;281:1305–8.
[36] Cohen GM. Caspases: the executioners of apoptosis. Biochem J 1997;326:1–16.
[37] Wu S, Zhu H, Gu J, Zhang L, Teraishi F, Davis JJ, et al. Induction of apoptosis and down-regulation of Bcl-XL in cancer cells by a novel small molecule, 2[[3-(2,3-dichlorophenoxy)propyl]amino]ethanol. Cancer Res 2004;64:1110–3.
[38] Zhu H, Guo W, Zhang L, Davis JJ, Teraishi F, Wu S, et al. Bcl-XL small interfering RNA suppresses the proliferation of 5-fluorouracil-resistant human colon cancer cells. Mol Cancer Ther 2005;4:451–6.
[39] Yang J, Liu XS, Bhalla K, Kim CN, Ibrado AM, Cai JY, et al. Prevention of apoptosis by Bcl-2: release of cytochrome *c* from mitochondria blocked. Science 1997;275:1129–32.
[40] Petak I, Danam RP, Tillman DM, Vernes R, Howell SR, Berczi L, et al. Hypermethylation of the gene promoter and enhancer region can regulate Fas expression and sensitivity in colon carcinoma. Cell Death Diff 2003;10:211–7.
[41] Giovannini C, Matarrese P, Scazzocchio B, Varì F, D'Archivio M, Straface E, et al. Wheat gliadin induces apoptosis of intestinal cells via an autocrine mechanism involving Fas–Fas ligand pathway. FEBS Lett 2003;540:117–24.
[42] Tamm I, Schriever F, Dorken B. Apoptosis: implications of basic research for clinical oncology. Lancet Oncol 2001;2:33–42.
[43] Liu M, Wang Z, Li H, Wu R, Liu Y, Wu Q. Mitochondrial dysfunction as an early event in the process of apoptosis induced by woodfordin I in human leukemia K562 cells. Toxicol Appl Pharmacol 2004;194: 141–55.
[44] Larrosa M, Tomás-Barberán FA, Espín JC. The grape and wine polyphenol piceatannol is a potent inducer of apoptosis in human SK-Mel-28 melanoma cells. Eur J Nutr 2004;43:275–84.
[45] Larrosa M, Tomás-Barberán FA, Espín JC. Resveratrol and its analogous 4-hydroxystilbene induce growth inhibition, apoptosis, S-phase arrest and upregulation of cyclins A, E and B1 in human SK-Mel-28 melanoma cells. J Agric Food Chem 2003;51:4576–84.
[46] Hong J, Hong L, Meng X, Ryu JH, Hara Y, Yang CS. Stability, cellular uptake, biotransformation, and efflux of tea polyphenol (−)-epigallocatechin-3-gallate in HT-29 human colon adenocarcinoma cells. Cancer Res 2002;62:7241–6.
[47] Manna C, Galletti P, Maisto G, Cucciolla V, D'Angello S, Zapia V. Transport mechanism and metabolism of olive oil hydroxytyrosol in Caco2-cells. FEBS Lett 2000;470:341–4.
[48] Kern SM, Bennett RN, Needs PW, Kron P, García-Conesa MT. Characterization of metabolites of hydroxycinnamates in the in vitro model of human small intestinal epithelium caco-2 cells. J Agric Food Chem 2003;51:7884–91.
[49] Stivala LA, Savio M, Carafoli F, Perusca P, Bianchi L, Maga G, et al. Specific structural determinants are responsible for the antioxidant activity and cell cycle effects of resveratrol. J Biol Chem 2001;276: 22586–94.
[50] Morris GZ, Williams RL, Elliott MS, Beebe SJ. Resveratrol induces apoptosis in LNCaP cells and requires hydroxyl groups to decrease viability in LNCaP and DU 145 cells. Prostate 2002;52:319–29.
[51] Gosslau A, Chen M, Ho CT, Chen KY. A methoxy derivative of resveratrol analogue selectively induced activation of the mitochondrial pathway in transformed fibroblasts. Br J Cancer 2005;92: 513–21.