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Anthocyanins induce cell cycle perturbations and apoptosis in different human cell lines

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To investigate the mechanistic basis for the biological properties of anthocyanins, two aglycone anthocyanins [delphinidin (DY) and cyanidin (CY)] were used to examine their effects on cell cycle progression and on induction of apoptosis in human cancer cells (uterine carcinoma and colon adenocarcinoma cells) and in normal human fibroblasts. These compounds differ in the number and position of hydroxyl groups on the β ring in the molecular structure. Cellular uptake of anthocyanins was confirmed by HPLC analysis and no metabolites were detected. The clonogenic assay showed that CY induces a dose-dependent growth inhibitory effect only in fibroblasts. This effect was confirmed by flow cytometric analysis, showing a significant reduction of cells in S phase. In contrast, DP inhibited cell growth in normal and tumour cell lines. This event is accompanied in fibroblasts by an accumulation of cells in the S phase suggesting a block in the transition from S to G_2 phase. On the other hand, in tumour cell lines we observed a reduction of cells in G_1 phase, paralleled by the appearance of a fraction of cells with a hypodiploid DNA content, thus demonstrating an apoptotic effect by DP. The occurrence of apoptosis induced by DP was confirmed by morphological and biochemical features, including nuclear condensation and fragmentation, annexin V staining, DNA laddering and poly(ADP-ribose) polymerase-1-proteolysis. Furthermore, the mitochondrial membrane potential of apoptotic cells after treatment with DP was significantly lost. The different effects exerted by DP as compared with CY suggest that the presence of the three hydroxyl groups on the β ring in the molecular structure of DP may be important for its greater biological activity.

Introduction

Anthocyanins are plant pigments, widely found in many berries, in dark grapes, cabbages and other pigmented foods, plants and vegetables (1,2). They belong to the widespread class of phenolic compounds collectively named flavonoids. The differences between individual anthocyanins are related to the number of hydroxyl groups, the nature and number of sugars and the position of these attachments (3). Depending on the nutrition habits, the daily intake of anthocyanins in humans has been estimated to be up to 200 mg/day (4). Anthocyanins have been found to be absorbed unmodified from the diet (5,6), and to be incorporated in cultured cells, both in the plasma membrane and in the cytosol (7). Since the moderate consumption of such compounds through the intake of the products such as red wine (8) or bilberry extract (9) is associated with a lower risk of coronary heart disease, it has been proposed that anthocyanins may exert therapeutic activities on human diseases associated with oxidative stress, e.g. coronary heart disease and cancer (10). The antioxidant properties of anthocyanins have been demonstrated by both *in vitro* and *in vivo* experiments (3,11–16). It has also been suggested that anthocyanins play an important role in the prevention against mutagenesis and carcinogenesis mediating some physiological functions related to cancer suppression (17). Anthocyanins show inhibitory effects on the growth of some cancer cells (18–21) and also inhibit cell transformation (22).

Recently, we have demonstrated that anthocyanins protect against DNA damage induced by oxidative agents in rat smooth muscle and hepatoma cells (11). In the present study we analysed the effect of two aglycone anthocyanins, delphinidin (DP) and cyanidin (CY), on cell cycle progression and on induction of apoptosis in human cancer cells (uterine carcinoma and colon adenocarcinoma cells) and in normal human fibroblasts. The results were compared with the effects exerted by *trans*-resveratrol (RE), another plant polyphenol found in grapes and red wine and implied in chemoprevention (23–29).

Abbreviations: CY, cyanidin; DP, delphinidin; NHF, normal human embryonic fibroblasts; PI, propidium iodide; RE, resveratrol.

Materials and methods

Chemicals

DP and CY were obtained from Extrasynthese (Genay, France), while RE (99% purity) was obtained from Sigma-Aldrich-Fluka (St Louis, MO).

All the chemicals used, unless otherwise specified, were purchased from Sigma (St Louis, MO). Anthocyanins and RE were dissolved in dimethylsulphoxide (DMSO).

Cell culture and treatments

Normal human embryonic fibroblasts (NHF), from Istituto Zooprofilattico, Brescia, Italy, uterine carcinoma (HeLa S3 cells) and human colon adenocarcinoma (CaCo-2 cells), from European Tissue Culture Collection were cultured in Eagle's minimal essential medium, in Dulbecco's modified Eagle's medium (D-MEM), and D-MEM with GlutaMAX™-I, respectively. All the media used were supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen Co., Paisley, Scotland, UK).

Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2.

The treatments were performed for 24 h with different concentrations of DP, CY or RE, with the concentration of DMSO never exceeding 0.4%. Negative controls consisted of untreated cells and cells that received the solvent alone. In the figures the solvent alone is showed as control.

Cellular uptake

HPLC analyses were carried out to ascertain the uptake of the different substances by the cell lines. Cells were treated for 24 h with DP or CY (200 μM) or with RE (100 μM). Anthocyanins cellular uptake was determined by HPLC, according to Youdim *et al*. (7), as this procedure allows us to discriminate cytosolic from cell membrane content. The same approach was adopted with RE, which was analysed according to Juan *et al*. (30).

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Cell viability

Cells were plated in 24-well tissue culture plates at the concentration of 6×10^4 or 1×10^5 cells/well for HeLa cells or for NHF and CaCo-2 cells, respectively, treated for 24 h with DP, CY or RE at the concentrations of 50, 100, 150 and 200 μM and cell viability was determined by the 3-[4,5-dimethylthiazolil-2-yl]-2,5-diphenyl-tetrazoliumbromide (MTT) colorimetric assay (31), as reported previously (11).

Clonogenic efficiency assay

Clonogenic survival was analysed by plating 100 cells/dish for NHF and CaCo-2 cells and 50 cells/dish for HeLa cells. Cells were treated for 24 h with DP, CY or RE at the same concentrations used for cell viability assay, then washed twice in PBS and fresh medium was added. After 7 days, the colonies were stained with crystal violet and counted. The clonogenic efficiency was calculated as the mean percentage with respect to control cells.

Cell cycle analysis

In order to study the effect of anthocyanins on cell proliferation, cells were plated in 25 cm^2 culture flasks at the concentration of 5×10^5. After 24 h of treatment with DP, CY (100, 150 and 200 μM) or RE (100 μM), both adherent and floating cells of each sample were harvested and fixed in cold 70% ethanol. Fixed cells were washed in PBS and re-suspended in PBS containing 5 μg/ml propidium iodide (PI) and 1 mg/ml RNase A. Cells were analysed with a Coulter Epics XL (Coulter Co., Miami FL) flow cytometer. 10 000 cells were measured for each sample.

Evaluation of apoptosis

Analysis of cell morphology. Apoptotic cells were identified through microscopic observation of samples stained with Hoechst 33258. Cells were seeded on coverslips at a concentration of 3×10^4 (HeLa) or 5×10^4 (CaCo-2 and NHF). After 24 h of treatment with DP (100, 150 and 200 μM) or RE (100 μM), cells were fixed for 5 min with 1% paraformaldehyde at room temperature and then with ice-cold 70% ethanol for 15 min at −20°C. After washing with PBS, DNA was stained with 0.5 μg/ml Hoechst 33258 for 10 min at room temperature.

Detached cells were re-suspended in methanol/acetic acid (3:1), pipetted onto a glass coverslip and stained with Hoechst 33258. The number of apoptotic cells was evaluated through fluorescence observation using a Nikon Eclipse E400 microscope at the 400× magnification. For each sample, 300 cells were counted.

Flow cytometric detection of apoptotic cells by annexin V labelling and propidium iodide staining. Cells were plated in 25 cm^2 culture flasks at a concentration of 5×10^5. To detect early and late apoptosis, after 24 h of treatment, both adherent and floating cells were harvested together and incubated for 10 min in serum-free culture medium containing FITC-conjugated annexin V (3 μl/10^6 cells) (Bender Med Systems) and 2 μg/ml PI. In each experiment, dual parameter cytograms of the red-versus-green fluorescence signals were obtained and at least 10 000 events were measured in the gated regions chosen for calculations.

Analysis of DNA fragmentation. 2×10^6 HeLa cells were plated in 75 cm^2 culture. To visualize internucleosomal DNA fragmentation in treated cells, adherent and floating cells were harvested separately and rinsed twice in cold PBS containing 5 mM EDTA. Briefly, cells were re-suspended twice in lysis buffer containing 1% Nonidet-P40, 20 mM EDTA and 50 mM Tris–HCl, pH 8.0. The recovered supernatants were combined and incubated with 1% SDS and 0.5 mg/ml RNase A at 56°C for 3 h and thereafter treated with 1 mg/ml proteinase K at 37°C for 3 h. The DNA was precipitated by the addition of 0.1 vol of 10 M ammonium acetate and 2 vol of ethanol and analysed by agarose gel electrophoresis. DNA was visualized by staining with ethidium bromide. Gene Ruler 1 kb DNA Ladder (MBI Fermentas, Leiden, The Netherlands) was used as size marker.

Detection of the mitochondrial membrane potential ($\Delta\Psi m$). The mitochondrial membrane potential was analysed using the J-aggregate forming lipophilic compound 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) (Molecular Probes, Eugene, OR), a cationic fluorescent dye capable of selectively entering mitochondria. At low mitochondrial membrane potential, the dye emits a green fluorescence, while at higher membrane potential, it forms red fluorescent 'J' aggregates. The analysis was performed by cytofluorimetric analysis according to Cossarizza *et al.* (32). HeLa cells were plated in 75 cm^2 culture flasks at a concentration of 2×10^6. After 24 h of treatment with 200 μM DP or 100 μM RE, both adherent and floating cells (1×10^6) were incubated with 5 μg/ml JC-1 (1 mM stock solution in DMSO) for 20 min at room temperature in the dark. After centrifugation, cells were washed twice with PBS, re-suspended in 0.5 ml PBS and analysed by flow cytometry.

PARP-1 proteolysis. HeLa cells were plated in 75 cm^2 culture flasks at a concentration of 2×10^6. After the treatments, adherent and floating cells were recovered separately, washed twice with PBS and re-suspended at the concentration of 2.5×10^6/200 μl in denaturing buffer, according to Donzelli *et al.* (33), disrupted by sonication on ice, twice for 30 s (60 W) and heated for 5 min at 90°C. Samples were electrophoresed in a 7.5% SDS–PAGE mini-gel and transferred onto a nitrocellulose filter. The membrane was then saturated overnight with 5% milk in PBS containing 0.2% Tween-20 and incubated for 1 h with anti-PARP-1 monoclonal antibody C-2-10 (diluted 1:500 in PBS containing 0.2% Tween-20) (Alexis, Vinci-Biochem, Italy). After several washes with PBS containing 0.2% Tween-20, the membrane was incubated for 1 h in the presence of rabbit anti-mouse IgG antibody conjugated to horseradish peroxidase (diluted 1:5000 in PBS containing 0.2% Tween-20) (Amersham, Cologno Monzese, Italy). Visualization of immunoreactive bands was performed by an ECL system (Amersham).

Statistical analysis

Results are expressed as mean ± standard deviation. Statistical significance was calculated in all experimental sets using the Student's *t*-test.

Results

Cellular uptake

The cellular uptake of the two anthocyanins DP, CY and RE in the three cell lines was compared. As shown in Figure 1, for each compound the highest content was found in the cytoplasm. DP and CY showed comparable values of uptake, independently of the cell lines used. Cytoplasmic concentrations, expressed in pmol/10^6 cells, ranged from 58.01 for CY in NHF to 84.58 for DP in CaCo-2 cells. Maximum uptake was found for RE in HeLa cells. No metabolites were spotted for anthocyanins, whereas piceatannol, a RE derivative, was detected only in HeLa cells.

Effect of anthocyanins on cell viability and on clonogenic efficiency

The cytotoxic effect of the compounds was evaluated measuring cell viability by the MTT test (Figure 2A). CY did not affect cell viability at all the concentrations analysed in the three cell lines. DP was not cytotoxic for NHF and CaCo-2 cells, which exhibited a cell viability higher than 80%. In HeLa cells, 150 and 200 μM DP reduced cell viability to 59 and 50%, respectively. In NHF and CaCo-2 cells, RE induced a weak cytotoxicity at the highest concentration with a cellular survival of 66 and 63%, respectively, whereas the same effect was detected in HeLa cells even at a lower concentration (59%, 100 μM).

Figure 2B shows that in the three cell lines DP and RE induced a dose-dependent reduction in clonogenic efficiency, whereas the highest inhibition (76%) by CY was detected in NHF at the concentration of 200 μM.

Effect of anthocyanins on cell cycle

To further investigate the effect of anthocyanins on cell proliferation, the distribution in each phase of the cell cycle was determined by flow cytometric analysis of DNA content. As reported in Figure 3, a significant reduction ($P < 0.05$) of cells in S phase and an accumulation in the G_1 phase were observed in NHF treated with 150 and 200 μM CY. The reduction by ~50% of the number of cells in S phase induced by CY in NHF was also confirmed by cytometric evaluation of BrdU incorporation. However, cells in S phase exhibited levels of BrdU labelling comparable with those measured in control cells (data not shown). In contrast, no significant effect was detected in HeLa and CaCo-2 cells treated with CY.

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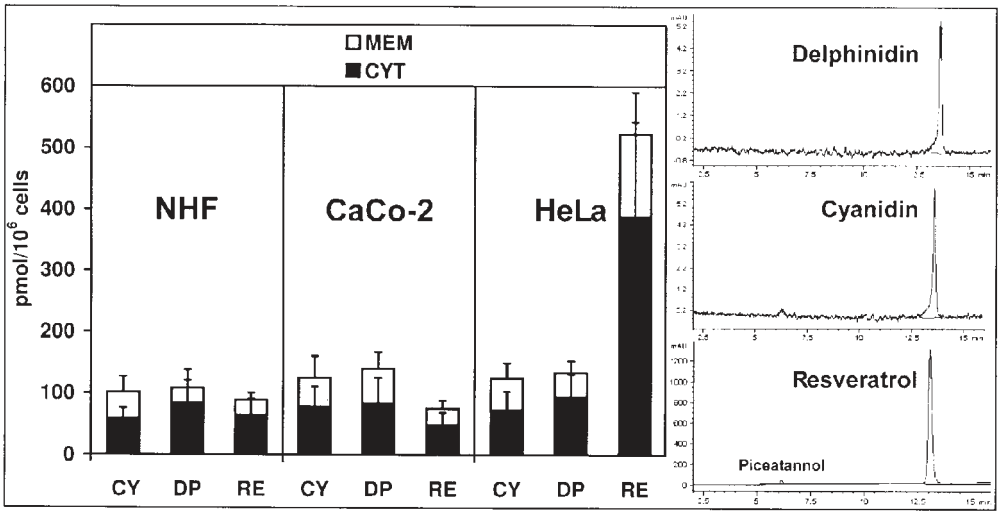

Fig. 1. Cellular uptake of CY, DP and RE on NHF, CaCo-2 and HeLa cells. Cells were incubated with 200 μM anthocyanins or 100 μM RE for 24 h and the cellular uptake was evaluated by HPLC. Three independent experiments were performed. Bars indicate standard deviation of the mean. In the right panel three representative chromatograms are reported (MEM, membrane; CYT, cytoplasm).

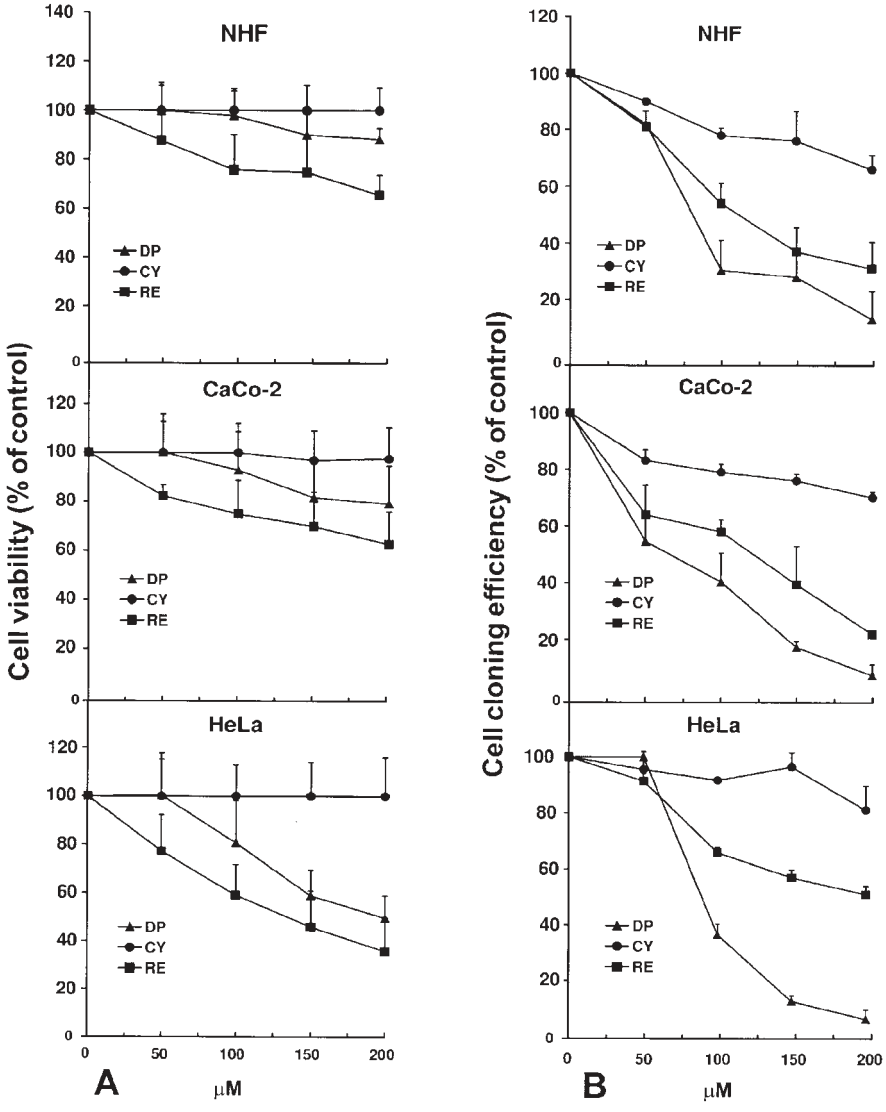

Fig. 2. Effect of anthocyanins and RE on NHF, CaCo-2 and HeLa cells on cytotoxicity and clonogenic efficiency. (**A**) Cells were incubated with DP, CY or RE for 24 h and cytotoxicity was determined by the MTT assay. (**B**) Cells were incubated 24 h with DP, CY or RE and grown for 7 days before counting colonies. Data are mean ± SD of three independent experiments; values are expressed in percentage and referred to control cells.

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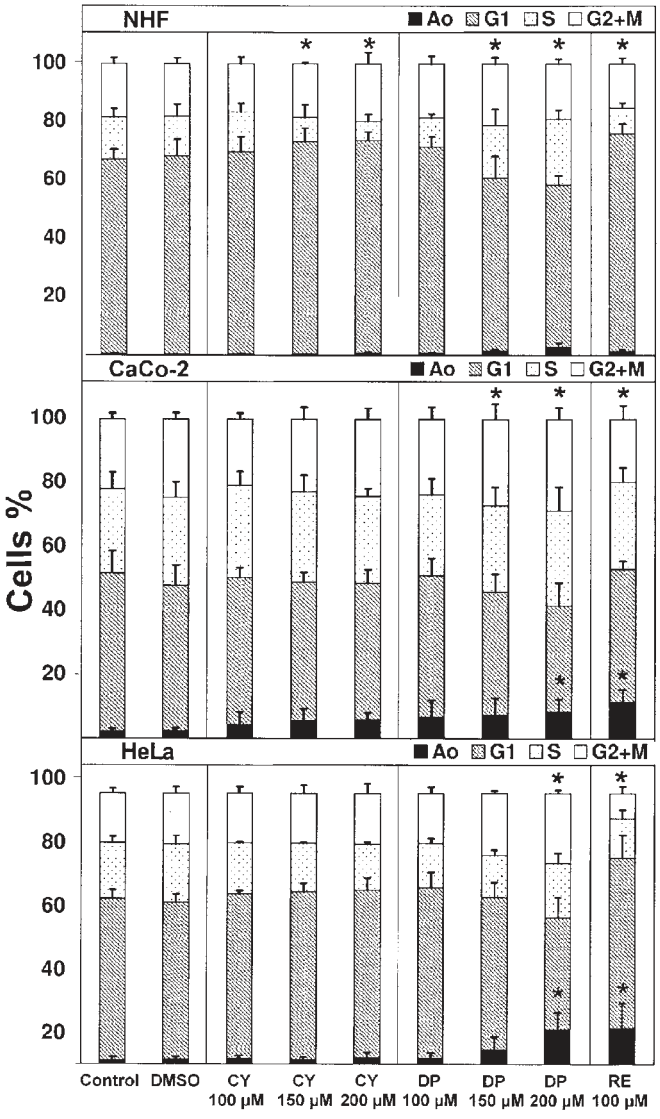

Fig. 3. Cell cycle analysis of NHF, CaCo-2 and HeLa cells treated with anthocyanins (CY, DP) and RE for 24 h. Cell cycle distribution was determined in samples stained with PI and measured by flow cytometry, as described under 'Materials and methods'. Mean values of the percentage of cells in each phase of cell cycle were obtained from three independent experiments. A_0 region corresponds to cells in the sub-G_1 peak. *Significantly different compared with control ($P < 0.05$ by Student's *t*-test).

DP at 150 and 200 μM concentrations, caused an accumulation of cells in S phase and a consequent reduction of the cellular fraction in G_1 phase in NHF. BrdU incorporation confirmed these results and showed that DNA synthesis was not affected (data not shown). A significant reduction of cells in G_1 phase and an accumulation in G_2/M phase was observed in HeLa and in CaCo-2 cells treated with DP at the concentration of 200 μM, and at 150/200 μM, respectively. This decrease was accompanied by the appearance of cells with subdiploid DNA content, thus suggesting the presence of apoptotic cells, which accounted for 12.6 ± 6.5% in HeLa cells and 8.6 ± 3.8% in CaCo-2 cells treated with 200 μM DP.

RE caused an accumulation of cells in G_1 phase with a consequent reduction in S phase in NHF and HeLa cells, whereas it induced a significant reduction of cells in G_1 phase in CaCo-2 cells. A sub-G_1 peak in both HeLa (13.3 ± 9%) and CaCo-2 cells (11.5 ± 3.9%) was detected, similar to that exerted by DP (200 μM) in HeLa cells. No significant sub-G_1 peak was detected in NHF treated with the three polyphenols.

Analysis of cell morphology, DNA fragmentation and flow cytometric detection of apoptotic cells by dual staining with annexin V and PI

To verify the presence of the typical cytological features of apoptosis in cells treated with DP, we analysed the nuclear morphology by staining DNA with the specific dye Hoechst 33258.

The calculation of the apoptotic index (A.I.) showed that DP determined a dose-dependent apoptosis in HeLa cells. In fact, 25.4 ± 4.7, 35.5 ± 5.2 and 40.6 ± 3.3% of cells were apoptotic after the treatment with 100, 150 and 200 μM, respectively. No effect was noted for DP in NHF, and a weak but not significant effect was detected only at the highest concentration in CaCo-2 cells (7.1 ± 4.2%). No evidence of apoptosis was found in NHF treated with 100 μM RE, whereas in HeLa and CaCo-2 cells the A.I. accounted for 32.6 ± 3 and 15.4 ± 7%, respectively. Figure 4(A) shows the morphological changes typical of apoptosis, such as nuclear shrinkage, chromatin condensation and fragmentation, visible in HeLa cells after 24 h of DP or RE treatment.

Figure 4(B) shows representative cytograms relative to annexin V staining of HeLa cells. Apoptotic cells were visible after the treatment with 200 μM DP and 100 μM RE. Moreover, DP and RE exerted a weak even not significant apoptotic effect in CaCo-2 cells, but not in NHF (data not shown).

Finally to further confirm the apoptotic effect induced by DP in HeLa cells, we investigated the occurrence of DNA laddering. As shown in Figure 4(D), oligonucleosomal DNA fragments were visible in floating HeLa cells incubated for 24 h with 200 μM DP and 100 μM RE.

Detection of the mitochondrial membrane potential ($\Delta\Psi m$) and PARP-1 proteolysis

To better define the features of DP- and RE-induced apoptosis in HeLa cells, we finally investigated two specific steps of the apoptotic pathway occurring in early (mitochondrial membrane depolarization) and intermediate (PARP-1 proteolysis) apoptotic cells.

Figure 5(A) shows typical examples of the fluorescence distribution of cells stained with the JC-1 after 24 h treatment with 200 μM DP and 100 μM RE. Both treatments induced a decrease in the number of cells with normal mitochondrial potential (at the right of the diagonal line) from 98.8% of control cells to 75.6% of DP-treated cells and 76.2% of RE-treated cells.

PARP-1 proteolysis was detected by western blotting in floating HeLa cells treated for 24 h with 200 μM DP and 100 μM RE (Figure 5B).

Discussion

In recent years, naturally occurring antioxidant micronutrients present in the diet and beverages have gained considerable attention as cancer chemopreventive agents (34–36). As the chemopreventive activity of a substance is often due to its antiproliferative and pro-apoptotic properties, we have tested the effect of two flavonoid compounds, the anthocyanins DP and CY, on cell growth and apoptosis in different human cell

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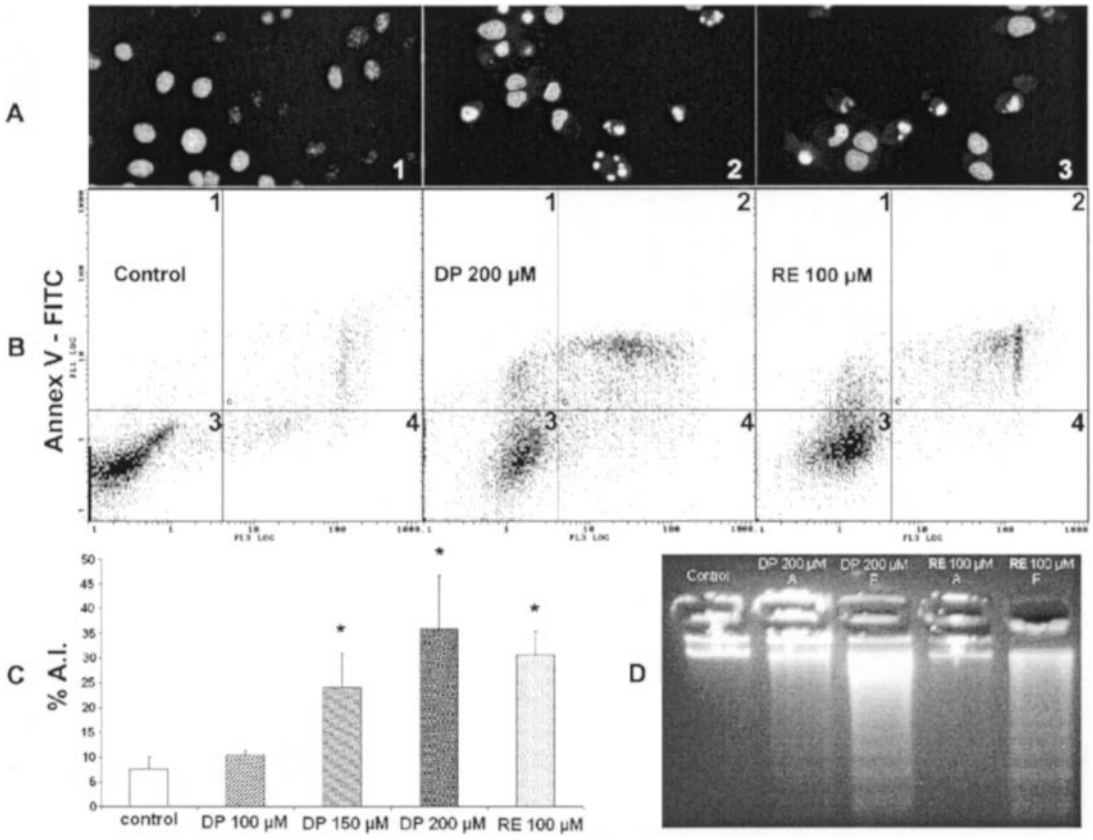

Fig. 4. DP- and RE-induced apoptosis in Hela cells. (**A**) Nuclear morphology of cells stained with Hoechst 33258. 1, Control cells; 2, cells treated with 200 μM DP for 24 h; 3, cells treated with 100 μM RE for 24 h (magnification: 400×). (**B**) Representative cytograms of annexin V versus PI fluorescence intensities as determined by flow cytometric analysis in HeLa cells incubated for 24 h in the absence or presence of DP or RE; within a cytogram, quadrant 1 and 2 represent early and late apoptotic cells, respectively; quadrant 3, viable cells; quadrant 4, necrotic cells. (**C**) A.I. of cells after a 24 h incubation with DP (100, 150 and 200 μM) and RE (100 μM). The values are the mean ± SD of three independent experiments. *Significantly different compared with control ($P < 0.05$ by Student's *t*-test). (**D**) DNA ladder in HeLa cells treated with 200 μM DP and 100 μM RE. A, attached cells; F, floating cells.

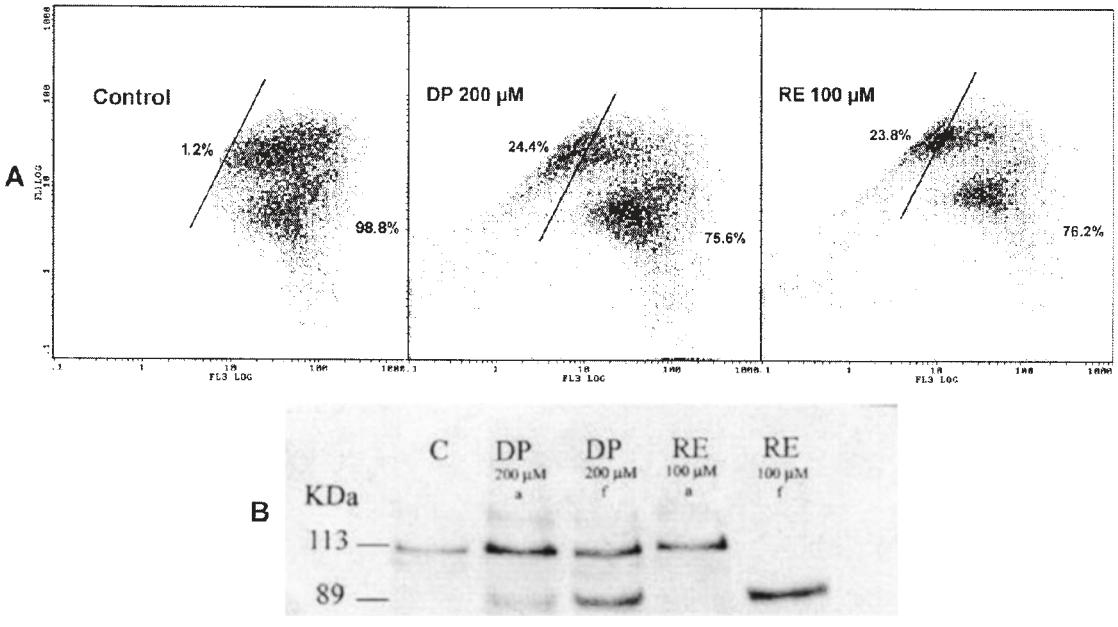

Fig. 5. Mitochondrial membrane potential and PARP-1 proteolysis in HeLa cells after 24 h treatment with 200 μM DP or 100 μM RE. (**A**) Flow cytometric determination of mitochondrial depolarization. Treated cells were incubated with 5 μg/ml JC-1 for 20 min, and the intensities of FL-1 and FL-3 fluorescence were measured. JC-1 fluorescence in the FL-1 channel increases as the mitochondrial membrane potential drops, while its fluorescence in FL-3 channel decreases. Percentage numbers at the right and the left of the diagonal line indicate proportion of cells with normal and depolarized mitochondria, respectively. (**B**) Western blotting analysis of PARP-1. Both the 113 kDa, full length enzyme and the 89 kDa proteolytic fragment were detected using the monoclonal antibody C-2-10; a, attached cells; f, floating cells.

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lines, comparing their effects to those exerted by RE, the antiproliferative and pro-apoptotic effects of which are well documented (23–29).

Data presented here indicate that in the concentration range of 100–200 μM CY is able to interfere with cell cycle only in normal cells, meanwhile DP inhibits cell proliferation in normal human fibroblasts and has a pro-apoptotic activity in cancer cells.

The range of concentrations used is consistent with many other studies (18–21) that investigated the antitumour effect of anthocyanins in culture cells. The maximum concentration of 200 μM DP adopted in this study, corresponding to 6.8 mg/100 ml, is within the concentration range of anthocyanins contained in blackberry juice used in a recent study regarding absorption and excretion of anthocyanins in humans (6). Nevertheless, from the pharmacokinetic point of view, anthocyanin bio-absorption in humans is generally poor, reaching the maximum blood concentration of ~13 μg/100 ml after supplementation compared with a baseline level of ~8 μg/100 ml (6).

It has been recently hypothesized that this apparent low bioavailability of anthocyanins *in vivo* may be due to their biotransformation into metabolites that are not detectable with current techniques or are rapidly degraded (37).

In our study at the more effective concentration, i.e. 200 μM, the uptake of the aglycones DP and CY was comparable in the three different cell lines, both substances were found both in membrane and cytoplasm, even if the concentration was generally higher in the cytoplasm. This is in accord with data reported by Youdim *et al*. and by Lazzè *et al*. (7,11) who demonstrated that anthocyanin cellular uptake is influenced by the sugar moiety. Moreover, in agreement with *in vitro* and *in vivo* studies, no metabolites were found after DP or CY treatment (5,7), whereas the presence of piceatannol, an RE metabolite, was detected in HeLa cells. It has been demonstrated that RE is converted to piceatannol in some tumour cell lines by the cytochrome P450 enzyme CYP1B1. This substance has been found to have an anticarcinogenic property (38,39).

The clonogenic assay showed that CY induces a dose-dependent inhibitory effect only in NHF. This effect was confirmed by flow cytometric analysis, where a significant reduction of cells in S phase, without affecting DNA synthesis, was detected. This finding was comparable with that exerted by RE, the effect of which on S phase in NHF was reported previously (25). The lack of the inhibitory effect by CY in tumour cell lines could be explained by the induction by CY of a checkpoint pathway blocking the $G_1 \rightarrow S$ transition of the cell cycle in normal cells. Instead in cancer cells some proteins involved in checkpoint pathway could be altered thus unable to be affected by CY.

In agreement with other studies (40,41), we demonstrated that DP inhibited cell growth and cell cycle progression in normal and tumour cell lines in a dose-dependent manner. In NHF, this substance induced an accumulation of cells in the S phase at the highest concentration suggesting a block in the transition from S to G_2 phase. On the other hand, we observed a reduction of tumour cells in G_1 phase, accompanied by the appearance of a sub-G_1 peak, thus demonstrating an apoptotic effect comparable with that exerted by RE in different tumour cell lines (27–29).

The induction of the apoptotic cell death by DP was supported by typical morphological and molecular hallmarks including chromatin fragmentation and phosphatidylserine exposure. However, these events were observed mainly in HeLa cells, which exhibit also DNA apoptotic laddering.

Several reports have suggested that chemotherapeutic agents trigger apoptosis by two major cell-intrinsic pathways, one that begins with ligation of cell surface death receptors and another that involves mitochondrial release of cytochrome C (42). Our observations allow the definition of a DP-mediated mitochondrial apoptotic pathway, which shows mitochondrial membrane depolarization and the typical PARP-1 degradation. This mechanism is comparable with that exerted by RE as demonstrated also in other cell lines (43,44). Moreover, the apoptosis induced by DP is independent of p53, as in HeLa cells p53 is proteolytically degraded by a viral protein (45).

The stronger effect exerted by DP as compared with CY suggests that the presence of three hydroxyl groups on the β ring in its structure may be important for its greater biological activity. This has been confirmed by quantum mechanics studies, where the abstraction of the hydrogen atom in 3′ position of the β ring of DP results in being highly exothermic (data not shown). A structure–activity relationship of anthocyanins was supported by the observation that the presence of at least two hydroxyl groups on the β ring of anthocyanin aglycon is essential for their biological activity (22,46). The relevance of number and position of hydroxylic groups was correlated previously to the biological effect of RE (25,47). In a similar way, the cancer preventive properties of RE have been put in correlation with its conversion by 3′ hydroxylation in the β ring into a compound (piceatannol) with known anticancer activity (38).

In conclusion, the results of this study demonstrate that: (i) CY has an antiproliferative effect only in normal fibroblasts; (ii) DP affects the proliferation capacity of normal cells and has a pro-apoptotic effect in tumour cell lines. The apoptosis by DP and RE is activated through a mitochondrial pathway; (iii) the different biological effects exerted by DP as compared with CY seem to be due to the three hydroxyl groups on the β-ring in its structure.

On the whole, from our data it is tempting to speculate that anthocyanins are promising substances for reducing cancer risk because of their antiproliferative potential and their apoptotic effects specifically in cancer cells. Clearly, additional pre-clinical studies using appropriate *in vivo* animal models as well as carefully designed pharmacokinetics studies are needed before clinical testing of anthocyanins as cancer preventive or therapeutic agents.

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