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Apoptosis-inducing factor and caspase-dependent apoptotic pathways triggered by different grape seed extracts on human colon cancer cell line Caco-2

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Consumption of grape seed extract (GSE) is widely marketed as a dietary supplement and is considered safe for human health. Nevertheless, the analytical composition of GSE from different grape cultivars, growing in special agronomic constraints, differs greatly in flavan-3-ols content. The major concern with GSE studies is a lack of availability of uniformly standardised preparations, which raises an important question whether different GSE samples have comparable activity and trigger the same mechanisms of action on a given biological system. Therefore, it is tempting to speculate that GSE, obtained from different cultivars, could exert differentiated anticancer effects. The focus of the present study is to determine the selective biological efficacy of GSE obtained from three different sources on the human colon cancer cell line Caco-2. Irrespective of its source, high doses of GSE induced a significant inhibition on Caco-2 cell growth. Moreover, apoptosis was enhanced through both caspase-dependent and caspase-independent mechanisms, leading to an early apoptosis-inducing factor release and, further, to a dramatic increase in caspase 7 and 3 activity. However, a significant difference in apoptotic rates induced by the three grape sources clearly emerged when treating cancer cells with low and intermediate GSE concentrations (25 and 50 μg/ml).

Grape seed extracts: Apoptosis-inducing factor: Apoptosis: Flavan-3-ols

Compelling evidence from epidemiological studies has shown that consumption of a fruit and vegetable-based diet significantly reduces the risk of cancer, especially tumours of the digestive tract[1]. Consequently, the focus of cancer research in recent years has been shifting towards the isolation and characterisation of potential chemopreventive agents present in fruits and vegetables[2]. In this regard, many phytochemicals, such as bioflavonoids, proanthocyanidins and phytoestrogens, have shown promising chemopreventive and/or anticancer efficacy in various cell cultures and animal models[3]. Especially, the composite class of condensed tannins or proanthocyanidins[4] has been demonstrated to exert broad-based and outstanding anticancer effects. Furthermore, according to ancient medical traditions, epidemiological studies have confirmed that consumption of foods with a high content of flavan-3-ols (catechins, condensed tannins, gallate derivatives of cyanidins), flavonoids and anthocyanins significantly reduces the risk for degenerative diseases and several kinds of tumours[5,6]. Extracts from grape seeds (GSE) are a prominent, rich source of proanthocyanidins, and several medical reports suggest that consumption of grapes and wine could display beneficial chemopreventive effects on degenerative diseases[7]. Namely, in several ongoing studies, GSE have been shown to reduce the incidence of carcinogen-induced mammary tumours in rats and skin cancers in mice and to inhibit the growth of human cancer cells both *in vitro* and *in vivo*, after transplantation into animals[8–10]. A chemopreventive and anticancer efficacy of GSE was also documented in colon cancer[11,12]. GSE treatment inactivates in Caco-2 cells the phosphoinositide 3-kinase/protein kinase B (PI3-kinase/PKB) pathway, leading to a concomitant decrease in Bcl-2 antagonist of cell death (BAD), cAMP response element-binding protein (CREB) and forkhead in rhabdomyosarcoma (FKHR) phosphorylation.

Abbreviations: AIF, apoptosis-inducing factor; GSE, grape seed extract; PARP, poly-ADP-ribose polymerase.

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In turn, GSE treatment induces caspase-dependent activation of apoptosis, through increased caspase 3 activity and enhanced cleavage of poly-ADP-ribose polymerase (PARP)[11]. This is of paramount importance, bearing in mind that colorectal cancer is the third most common cause of cancer-related mortality in Western countries[13] and that, despite improvements in the management of colon cancer patients, there is little change in survival rates over the past 50 years[14].

Consumption of foods with high GSE content is widely marketed as a dietary supplement and it is considered safe for human health[15]. Nevertheless, the analytical composition of GSE from different sources and namely from different grape cultivars, growing in special agronomic constraints, differs greatly in tannin content, as we have previously shown[16]. The major concern with GSE studies is a lack of availability of uniformly standardised preparations. As outlined by Agarwal and co-workers[12], this fact raises an important question whether different GSE samples have comparable activity and trigger the same molecular mechanisms on a given biological system. Therefore, it is tempting to speculate that GSE obtained from different cultivars could exert differentiated anticancer effects, through the involvement of distinct signalling pathways. The focus of the present study is to determine the selective biological efficacy of seed and skin flavan-3-ol constituents, obtained from different grape sources on the human colon cancer cell line Caco-2.

Materials and methods

Cell culture

The human colorectal cancer cell line Caco-2 was obtained from the European Collection of Cell Cultures (ECACC). Primary human dermal fibroblasts were isolated from healthy dermis by a collagenase type II digestion.

Cells were seeded into 25 cm^2 flasks (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA) in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum and antibiotics (penicillin 100 IU/ml, streptomycin 100 μg/ml, gentamycin 200 μg/ml). The cultures were kept at 37°C in an atmosphere of 5 % CO_2 in air and the medium was changed every third day. At confluence, the cells were subcultured after removal with 0·05 % trypsin–0·01 % EDTA.

Sample preparation and analysis

Italia white grape, and *Palieri* and *Red Globe* red grape cultivars from an experimental vineyard located in the Puglia region (Italy) were kindly provided by the Agricultural Research Council – Research Unit for grape and winegrowing in the Mediterranean environment (CRA-UTV; Turi, BA, Italy). Fresh grape berry samples were skinned, seeds were separated from the pulp and then the skins and seeds were gently wiped with filter paper to eliminate pulp residues. Homogeneous and dry material from skins and seeds was obtained, extracted with methanol, purified and analysed by electrospray ionisation MS according to a previously published method[17]. GSE were re-suspended in 70 % ethanol at a concentration of 30 mg/ml and stored in the dark at −20°C. To obtain a 100 μg/ml concentration (the highest concentration of GSE employed in our experiments), GSE stock solutions were diluted 1:300.

Cell proliferation assay

Caco-2 cells and human dermal fibroblasts were seeded in twelve-well culture plates (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA) at concentrations ranging between 1×10^4 cells/well and 3×10^4 cells/well in a standard medium. After a zero time (T_0) cell count, the cells were stimulated with 70 % ethanol (1:300, control), or with *Italia*, *Palieri* or *Red Globe* GSE at 25, 50 or 100 μg/ml and incubated at 37°C in an atmosphere of 5 % CO_2 in air. The cells were then detached from wells by trypsinisation and cell count was performed by a particle count and size analyser (Beckman Coulter, Inc., Fullerton, CA, USA) after 24, 48, 72 and 96 h. For each data point, two replicate wells were used, and every experiment was performed six times.

Apoptotic cell death assay

Caco-2 cells were cultured at confluence into 25 cm^2 flasks (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ, USA) in a standard medium and stimulated with 70 % ethanol (1:300, control) or with *Italia*, *Palieri* or *Red Globe* GSE at 25, 50 or 100 μg/ml and incubated at 37°C in an atmosphere of 5 % CO_2 in air. After 24 h, the cells were trypsinised, washed twice with PBS and stained with fluorescein isothiocyanate-labelled annexin V and 7-aminoactinomycine-D (7-AAD) according to the manufacturer's instructions (Instrumental Pro3 Laboratory, Cavenago, MI, Italy). Then, the samples were analysed by flow cytometry (EPICS Coulter XL; Beckman Coulter Inc., Fullerton, CA, USA) for the quantification of apoptotic cells. The fluorescence of 20 000 events was measured and an excitation wavelength of 488 nm was used in combination with standard filters to discriminate between the FL1 and FL3 channels, forward scatter and side scatter.

Immunoblot analysis

Following treatment with GSE at 50 μg/ml, Caco-2 cells were washed twice with ice-cold PBS and scraped in the following lysis buffer: 50 mM-2-amino-2-hydroxymethyl-propane-1,3-diol-HCl, pH 7·4; 150 mM-NaCl; 0·2 % NP-40; 1 % 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS); 2 mM-EDTA dissolved in tetra-distilled water. A mix of protease inhibitors (Complete-Mini Protease Inhibitor Cocktail Tablets; Roche, Mannheim, Germany) was added just before use. Cellular extracts were then sonicated and centrifuged at 14 000 rpm for 10 min. The protein content of supernatant fractions was determined by using the Bradford assay. For immunoblot analyses, cellular extracts were separated on SDS-PAGE gels with a concentration of acrylamide specific for the proteins studied. Proteins were blotted onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) and probed with the following antibodies: anti-apoptosis-inducing factor (AIF) (anti-AIF, sc-5586; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); anti-cleaved PARP (Sigma, St Louis, MO, USA); anti-cleaved caspase 9 (no. 9501S; Cell Signaling Technology, Inc., Boston, MA, USA); anti-cleaved

caspase 7 (no. 9491S; Cell Signaling Technology, Inc.); anti-cleaved caspase 3 (no. 9661S; Cell Signaling Technology, Inc.); anti-α-tubulin (Sigma, St Louis, MO, USA). Antigens were detected with an enhanced chemiluminescence (ECL) kit from Amersham (Amersham Biosciences, Little Chalfont, Bucks, UK) according to the manufacturer's instructions.

Densitometry

All Western blot images were acquired and analysed through an Imaging Fluor S densitometer (Bio-Rad Laboratories). The optical density of each condition was normalised against the signal of the internal control α-tubulin.

Statistical analysis

Data were expressed as mean values and standard deviations and statistical analysis was performed using ANOVA, followed by the Bonferroni *post hoc* test. Pearson correlation coefficients between dose and inhibition potential, on both the entire dataset and inside each cultivar, were computed to assess the statistical relevance of the exerted effect at different scales of definition. Factorial ANOVA was applied on the entire dataset to dissect dose and time effects of treatment. The mixed model of errors was adopted (model III), in order to take into consideration a possible mix of fixed and random effects of the analysed sources of variation. Both the general significance of the entire model from randomness (general effect) and single effect significance were estimated. Differences were considered significant at the level of $P<0{\cdot}05$. Statistical analysis was performed by using GraphPad Instat software (GraphPad Software, Inc., San Diego, CA, USA).

Results

Grape skin extracts

Both human dermal fibroblasts and colon cancer cells treated with grape skin extracts did not show any significant change in their proliferative rate (data not shown). In addition, no relevant apoptosis was observed in these conditions (data not shown). In conclusion, grape skin extracts seem to be devoid of significant anticancer effects, even if significant amounts of stilbenes and anthocyanins are present. Some reports have outlined that both stilbenes (resveratrol)[18,19] and anthocyanin conjugates[20,21] exhibit growth-inhibitory properties and pro-apoptotic effects against cancer cells. However, our data did not support these results.

Grape seed extracts

Growth inhibition. Cell proliferation rates were recorded every 24 h, until 96 h. GSE did not induce any detectable growth-inhibitory effect on cultured human dermal fibroblasts (data not shown). Proliferation rates of Caco-2 cells are otherwise significantly reduced by GSE in a dose-dependent manner (Fig. 1). Growth rates display a similar trend in cell populations treated with GSE obtained from the *Italia*, *Palieri* and *Red Globe* cultivars. Nevertheless, focusing on the lowest

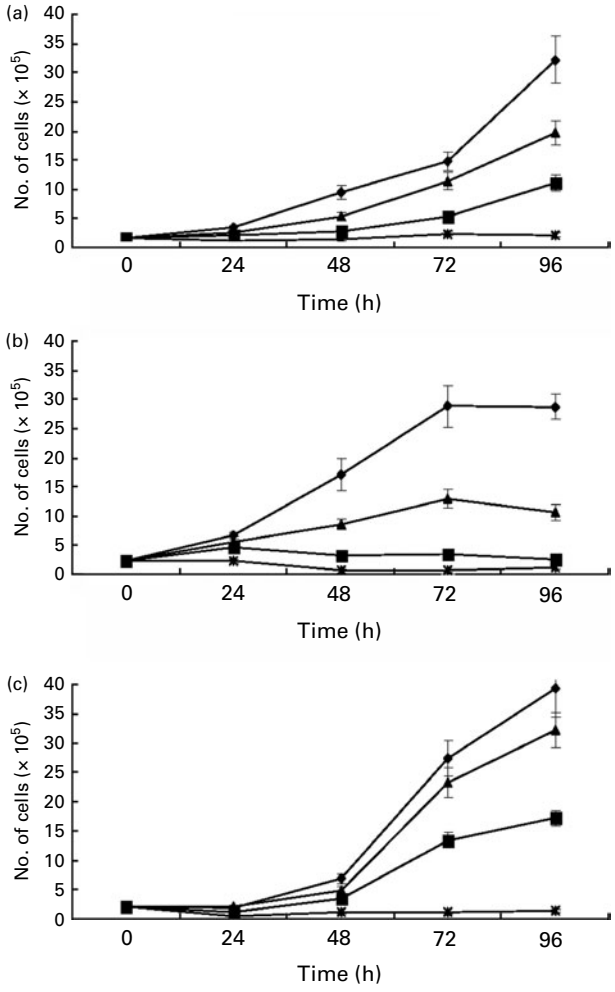

Fig. 1. Effects of grape seed extracts (GSE) compared with control treatment (–◆–) on proliferation of Caco-2 cells after 24, 48, 72 and 96 h. The cells were stimulated with *Italia* (a), *Palieri* (b) or *Red Globe* (c) GSE at 25 (–▲–), 50 (–■–) or 100 (–✳–) μg/ml. Values are means of six independent experiments performed in duplicate, with standard deviations represented by vertical bars. Data are shown in the Supplementary Tables, available online.

active concentration (25 μg/ml), it clearly emerges that the most active inhibition is attained by GSE from the *Italia* and *Palieri* cultivars, after only 24 h. In *Italia* GSE-treated cells, the growth inhibition is statistically significant at each GSE concentration and at all the times considered ($P<0{\cdot}01$), reaching the most relevant inhibition after 96 h at 100 μg/ml (93 %; $P<0{\cdot}001$). In *Palieri* GSE-treated samples, the growth inhibition is statistically significant at every GSE concentration and at all the times studied ($P<0{\cdot}001$), reaching high inhibitory effects at 50 and 100 μg/ml (91 and 96 %, respectively; $P<0{\cdot}001$). In *Red Globe* GSE-treated cells, the growth inhibition is statistically significant at all the times studied only at 50 and 100 μg/ml ($P<0{\cdot}001$), reaching the most relevant inhibition at 100 μg/ml (96 %; $P<0{\cdot}001$). At 25 μg/ml, the growth inhibition reaches significant values only at 48 h ($P<0{\cdot}01$) and 96 h ($P<0{\cdot}05$). At the highest GSE concentration (100 μg/ml), differences between the three cultivars disappear and Caco-2 growth is almost completely inhibited after the first 48 h.

In order to obtain a global comparison among the studied cultivars as for growth inhibition along both exposure time and dose dependency, an ANOVA approach was undertaken.

First, on the entire dataset made by thirty-six independent samples (three doses × four times × three cultivars), a statistically significant Pearson correlation (r 0·782; $P<0·0001$) with dose was observed, pointing to a global effect of all the cultivars.

The presence of a statistically significant dose–inhibition relationship is observed for each single cultivar (*Italia*, r 0·868, $P<0·0003$; *Palieri*, r 0·625, $P<0·03$; *Red Globe*, r 0·959; $P<0·001$).

It is worth noting that the Pearson correlation simply measures the goodness of fit of the scattering of the points in the dose–inhibition plane to a straight line without being explicitly linked to the slope of the line, which is analysed by a general linear model (ANOVA) where effects of time are inserted as well. The whole set gives the results showed in Table 1 as for the significance of both time and dose effects.

Both time and dose enter with a statistically significant load into the model, so demonstrating the dependency of growth inhibition on both time of exposure and dose. A similar pattern was recorded for each cultivar (data not shown).

When the cultivars were compared with each other by an ANOVA model, we show that they are different as for general inhibition effect while they do not reach statistical significance as for the slope of their dose–inhibition relationship that is present in all the cultivars (Table 2).

The *Palieri* cultivar was the most effective (average inhibition over all the conditions 70·85), while the *Italia* and *Red Globe* cultivars were markedly lower (57·80 and 51·25, respectively).

The order of effect scales with the amount of procyanidins, with the highest procyanidin content paralleled by the most marked effect. In our experimental setting we cannot statistically separate the effect of procyanidins from the general effect of cultivar; nevertheless we can hypothesise a possible involvement of this class of substances in growth inhibition.

Apoptosis. The possible apoptotic effect of GSE on the colorectal cancer cells was next examined by annexin V and 7-aminoactinomycine-D (7-AAD) staining, where cells were treated with GSE (25, 50, 100 μg/ml) for 24 h under similar conditions as in cell growth studies. Above the threshold value of 50 μg/ml, GSE treatment showed a roughly significant dose-dependent increase in apoptotic cell population (Fig. 2(a) and (b)). Normal human fibroblasts cultured with GSE did not show any significant modification in programmed cell death levels (data not shown). In *Italia*, *Palieri* and *Red Globe* GSE-treated cells apoptosis is higher in respect to both control and camptothecin-treated control samples at 50 and 100 μg/ml ($P<0·001$). At 25 μg/ml, only *Palieri* GSE-treated cells showed a statistically significant increase in apoptotic rate with respect to control. At the highest GSE concentration (100 μg/ml), in all experimental samples the highest apoptotic values were reached, but without statistically significant differences between the three cultivars. At the highest concentrations of GSE, an increase in late apoptosis was observed (Fig. 2(b)). In both *Italia*- and *Red Globe*-treated cells, the rate of late apoptosis was noteworthy at a concentration of 100 μg/ml, while in *Palieri*-treated samples, it became evidently high at 50 μg/ml.

Table 1. ANOVA of time- and dose-related growth inhibition

	F	P	R^2
General effects			
Dependent variable: growth inhibition	43·84	<0·0001	0·7266
Single effects			
Source			
Time	3·75	0·0007	
Dose	8·58	0·0001	

Table 2. ANOVA of cultivar and dose-related growth inhibition

	F	P	R^2
General effects			
Dependent variable: growth inhibition	17·44	<0·0001	0·663
Single effects			
Source			
Cultivar	5·92	0·0068	
Dose	71·53	<0·0001	

Molecular parameters. Molecular parameters were evaluated only on samples treated with GSE at 50 μg/ml. This choice was suggested by the fact that Caco-2 cells treated with GSE at 100 μg/ml gave paradoxical results, i.e. reduced values of the overall apoptotic markers, despite an increased apoptotic rate, were observed in these conditions. As previously observed in DU145 human prostate carcinoma cells treated with GSE fractions[22], one possible explanation could be that the 100 μg/ml dose of GSE produces massive apoptotic cell death (already after 24 h), making the analyses of caspases and PARP cleavages impractical in total cell lysates.

Caspases. Caspase 8 activity was not significantly modified in GSE-treated cells (data not shown). On the contrary, an increase in caspase 9 was observed already from the first hours in GSE-treated Caco-2 cells and it reached the maximum at 24 h (Fig. 3(a)). Nevertheless, terminal effector caspases (caspases 3 and 7) did not increase until 24 h. It must be emphasised that both caspases 7 and 3 increase up to 5-fold in comparison with the basal values (Fig. 3(b) and (c)). This trend behaved apparently similar for all tested cultivars, even if absolute values differed greatly for each grape type. The highest increase in caspases activity was observed – as expected – in Caco-2 cells treated with GSE obtained from the *Palieri* cultivar. These data suggest that GSE trigger apoptosis through the intrinsic caspase-apoptotic pathways[23].

Apoptosis-inducing factor. In all tested samples, AIF showed the same trend: it increased earlier (at 6 h) than caspases and it reached the highest value at 24 h (Fig. 4(a)). Absolute values were significantly higher in samples treated with GSE obtained from the *Italia* cultivar.

Cleaved poly-ADP-ribose polymerase. A similar trend was observed for cleaved PARP values: an increase of cleaved PARP was observed at 3 h after GSE treatment. The highest levels were reached at 24 h (Fig. 4(b)). As for caspases 3 and 7, the highest absolute concentration was observed in Caco-2 cells treated with *Palieri*-derived GSE. The earliest increase in cleaved PARP seems to suggest that early apoptosis was triggered by AIF – thought to be a caspase-independent apoptotic pathway – and further

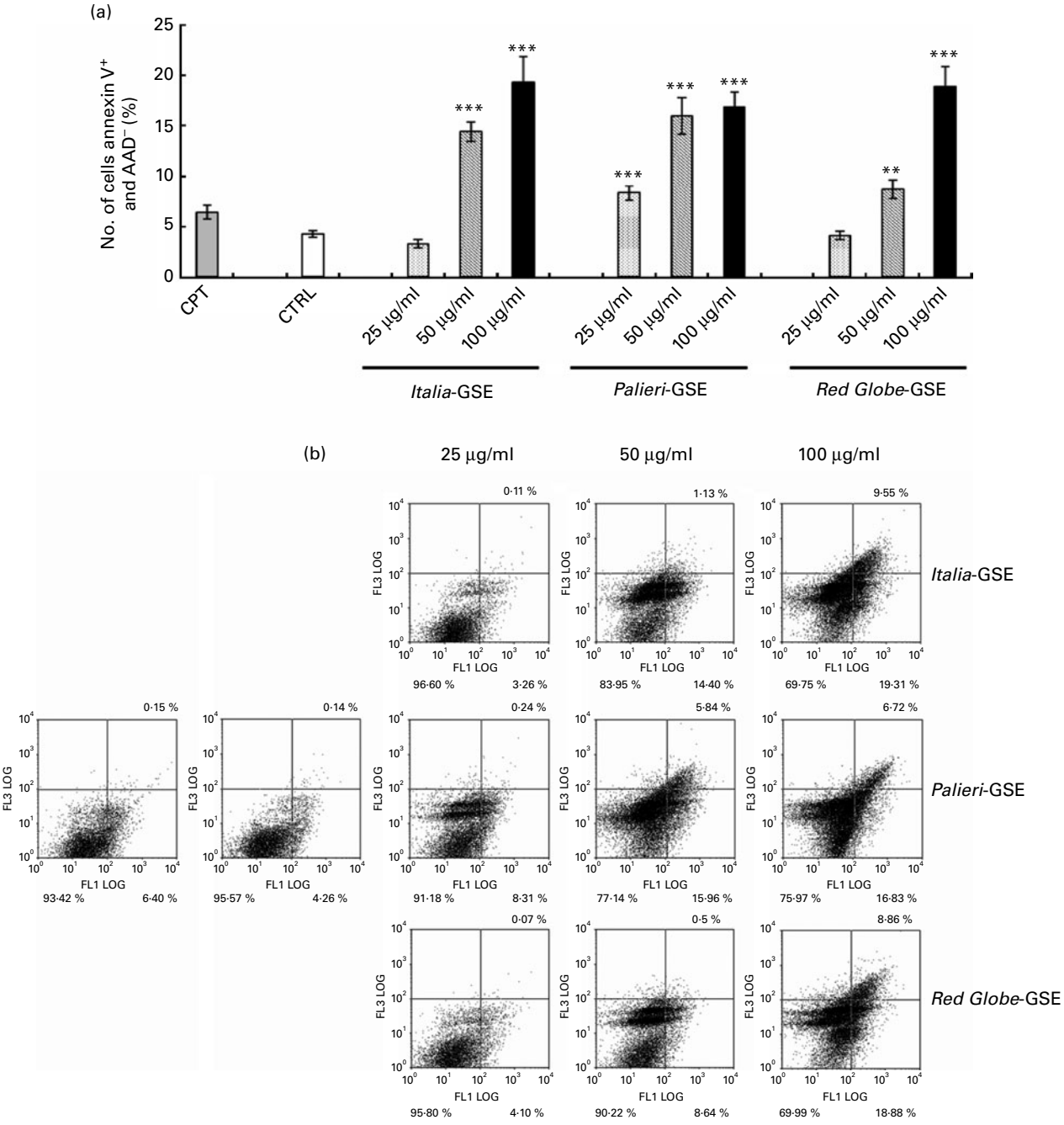

Fig. 2. (a) Effects of grape seed extracts (GSE) on apoptosis of Caco-2 cells after 24 h. Values are means of three independent experiments, with standard deviations represented by vertical bars. Mean value was significantly different from that for the control (CTRL) treatment: ** $P<0{\cdot}01$, *** $P<0{\cdot}001$. 7-AAD, 7-aminoactinomycine-D; CPT, camptothecin. (b) Dual-parameter flow cytometric density dot plots for GSE-treated Caco-2 cells. Fluorescence intensity for annexin V–fluorescein isothiocyanate is plotted on the *x*-axis and 7-AAD is plotted on the *y*-axis. The lower left quadrant cells (annexin $V^-/7\text{-}AAD^-$) were defined as viable cells, the lower right quadrant cells (annexin $V^+/7\text{-}AAD^-$) as apoptotic cells, and the upper right quadrant cells (annexin $V^+/7\text{-}AAD^+$) as late apoptotic cells.

enhanced by increased activation of the terminal caspase effectors, i.e. caspases 3 and 7.

Discussion

In the last 10 years, several studies have convincingly documented the anticancer and cancer-chemopreventive efficacy of GSE against various cancers[24–26]; however, only few studies have investigated the anticancer effects exerted by GSE on human colon tumours[11,15,27,28]. Moreover, a major caveat has been the composition of various GSE preparations being marketed under different names, and those being used under laboratory conditions. The lack of standardised preparations has limited the validity and translational potential of the research findings obtained in the laboratory setting using different preparations or sources of GSE. As previously shown by our laboratory, GSE obtained from different cultivars, and even GSE provided by the same cultivars, grown

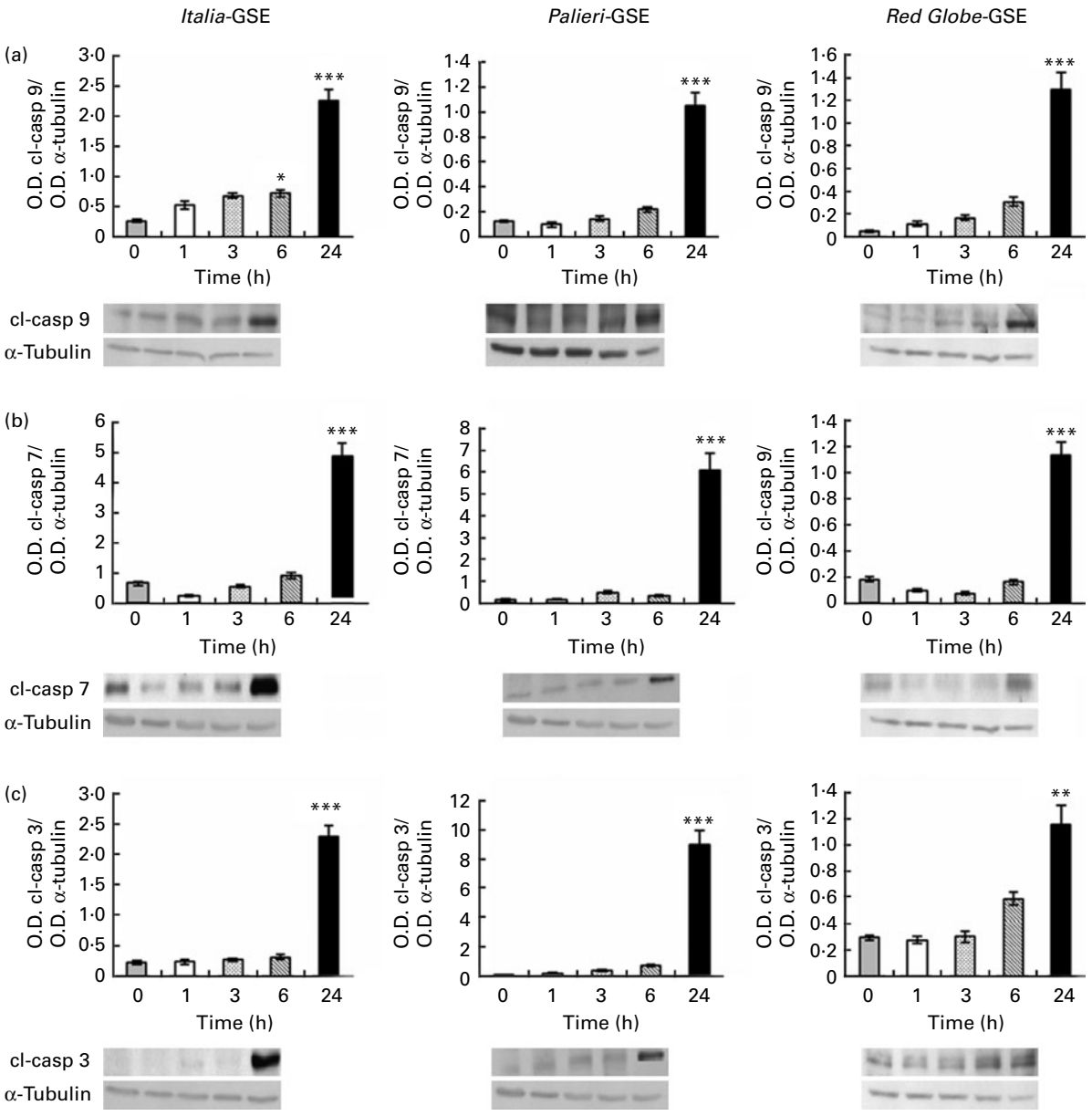

Fig. 3. Immunoblots showing the expression of cleaved caspase (cl-casp) 9 (a), cl-casp 7 (b) and cl-casp 3 (c) in Caco-2 cells treated with *Italia*, *Palieri* and *Red Globe* grape seed extracts (GSE) from 0 to 24 h. Data represent densitometric quantification of optical density (O.D.) of specific protein signal normalised with the O.D. values of α-tubulin, served as a loading control. Values are means (n 3), with standard deviations represented by vertical bars. Mean value was significantly different from that for the control treatment: * $P<0{\cdot}05$, ** $P<0{\cdot}01$, *** $P<0{\cdot}001$.

under different agronomical conditions, display a remarkable diversity in their composition[16]. To address some of these issues, we compared the biological effects of GSE procured from three different cultivars (*Italia*, *Palieri* and *Red Globe*) against the human colon cancer cell line Caco-2.

Irrespective of its source, GSE produce strong biological effects on Caco-2 cells, which include growth inhibition, induction of markers of apoptotic signalling pathways, and programmed cell death. Growth inhibition becomes evident already from the first 24 h of treatment and progressively increases. This trend is quite similar in all experiments and seems to be independent from the type of GSE studied, even if the highest inhibitory effect was recorded in the *Palieri* GSE-treated cells. Growth inhibition is clearly dose-dependent, increasing linearly with the concentration of the grape extract. Independently from the type of cultivar, in samples treated with GSE at 100 μg/ml, cell growth is almost completely abolished after the first 24 h.

Loss of apoptotic function is a major contributor towards the resistance of cancer cells to both metabolic (hypoxic, energetic) stresses and cytotoxic treatments. Therefore, it should be highly desirable to diversify the availability of apoptotic and pro-apoptotic substances that could be used in cancer therapy. As previously outlined, GSE not only exert significant inhibitory effects on cancer growth, but also induce a significant programmed cell death response in colon cancer cells. This effect seems to be clearly dose-dependent, with the highest apoptotic rates observed in Caco-2 cells treated with GSE

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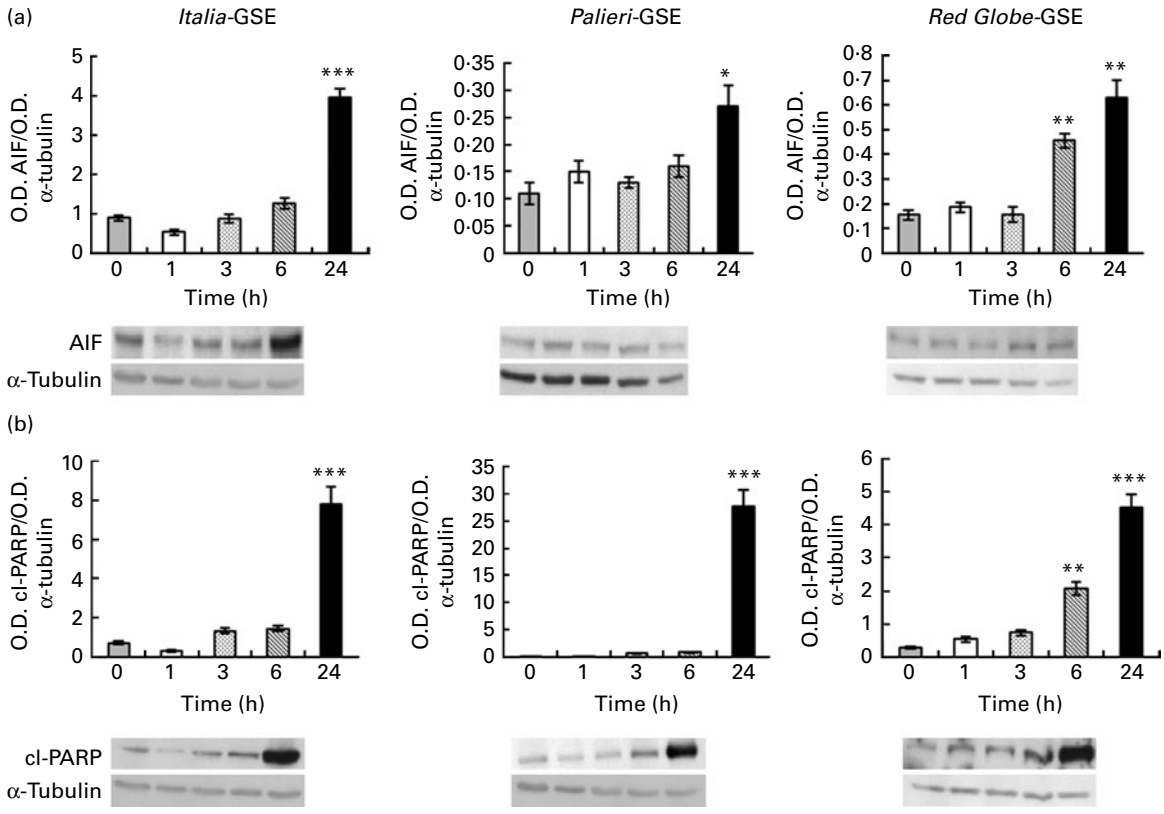

Fig. 4. Immunoblots showing the expression of apoptosis-inducing factor (AIF) (a) and cleaved poly-ADP-ribose polymerase (cl-PARP) (b) in Caco-2 cells treated with *Italia*, *Palieri* and *Red Globe* grape seed extracts (GSE) from 0 to 24 h. Data represent densitometric quantification of optical density (O.D.) of specific protein signal normalised with the O.D. values of α-tubulin, served as a loading control. Values are means (n 3), with standard deviations represented by vertical bars. Mean value was significantly different from that for the control treatment: * $P<0{\cdot}05$, ** $P<0{\cdot}01$, *** $P<0{\cdot}001$.

at a concentration of 100 μg/ml. However, some subtle differences are emerging from the whole picture. In fact, when treating cancer cells with low and intermediate concentrations of GSE (25 and 50 μg/ml), *Palieri*-treated samples showed a significantly higher response compared with the other two cultivars. As expected, both cleaved PARP and caspase 3 activity were significantly enhanced in samples treated with *Palieri*-derived GSE than in both *Red Globe*- and *Italia*-treated cells. It is likely that such differences could be explained by a different chemical composition of GSE obtained from the *Palieri* cultivar.

Indeed, GSE obtained from our different sources showed significant diversities in flavan-3-ol composition, analysed by means of the liquid chromatography–MS technique, as previously reported[17].

Both stilbenes and anthocyanin conjugates are represented at high concentrations in skin extracts (with the exception of the white grape cultivar, *Italia*); meanwhile, they are recorded only in traces in extracts obtained from seeds. No appreciable quantities of gallic acid have been found both in skin and seed extracts. On the contrary, flavan-3-ols – procyanidin B1 and B2, procyanidin dimers, catechin, epicatechin and epigallocatechin-gallate – are highly represented only in GSE[17]. However, GSE obtained from the three cultivars greatly differ in their pyrogallol-type structure-containing compounds: catechin and epicatechin gallate, procyanidin dimers, trimers and tetramers, procyanidins gallate, collectively known as proanthocyanidins. Proanthocyanidins are naturally occurring polyphenolic flavan-3-ols with different chemical structure, pharmacology and characteristics, widely distributed in plants[29]. Polymeric and oligomeric proanthocyanidins (also called condensed tannins) are polyphenols composed of chains of flavan-3-ol units, (+)-catechin and (−)-epicatechin linked through C4–C6 and C4–C8 interflavan bonds. Oligomeric proanthocyanidins are the only macromolecular constituents present in GSE, which contain variable amounts of monomeric catechin and epicatechin chains[14,30].

It has been suggested that flavan-3-ols can exert anticancer activity when they are provided by a pyrogallol-type structure[31]. In fact, previous reports have evidenced that epigallocatechin gallate induces apoptosis in colon cancer[32], while this induction was very weak by catechin and epicatechin, which lack a galloyl group[33,34], suggesting a certain structure–function relationship in apoptosis-inducing activity. Therefore, it is likely that a pyrogallol-type structure in a B-ring may contribute to the apoptosis-inducing activity. Indeed, the highest concentration of compounds provided by a pyrogallol-type structure was observed in *Palieri* GSE[17] and, as expected, the most significant apoptotic rate was obtained in *Palieri* GSE-treated samples.

It must be emphasised that these results are strictly dependent on the cancer cell line studied and they should not to be extrapolated to other types of colon cancers.

The apoptotic rate induced by GSE on Caco-2 cells is lower than that recorded in other tumours (prostate, leukaemia), but is comparable with the results obtained by Agarwal and

co-workers on several colon cancer cell lines (SW480, HT29 and LoVo)[12] and by Kim *et al.* on SNU-C4 tumour cells[35]. Even if GSE-induced apoptosis is likely to be p53-independent (though Caco-2 cells are p53 defective), apoptosis is triggered through a caspase-dependent mechanism, leading to the activation of two caspase effectors, caspases 3 and 7. This result confirms previous data[11], indicating that apoptosis induced by GSE belongs to the intrinsic apoptotic pathway, though caspase 8 levels in GSE-treated cancer cells are unmodified. The caspase-dependent pathway might not be the only apoptotic mechanism triggered by GSE, bearing in mind that a rise in cleaved PARP – even if it is not always statistically significant – can be recorded before an increase in caspase activity is observed. Indeed, AIF, known to induce apoptosis via a caspase-independent mechanism, increases early in GSE-treated samples. Even if two previous studies[36,37] demonstrated the involvement of AIF-mediated apoptosis in cancer cells treated with epigallocatechin gallate, until now no data have been published about the possibility that GSE could trigger a similar caspase-independent apoptotic pathway in colon cancer. For the first time we demonstrated that GSE enhance a significant AIF release in colon cancer cells together with an increase in caspase activity. These results suggest that GSE-induced apoptosis in Caco-2 cells can be considered a biphasic process, obtained through both caspase-dependent and caspase-independent pathways.

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S. D. designed and performed the experiments, analysed the data and wrote the manuscript. A. C. contributed to the design of the experiments and gave conceptual advice. A. P., S. P. and F. D'A. contributed to the performance of the experiments and analysis of the data. G. P., A. R. S. and A. L. prepared and chemically characterised the GSE. P. C. performed cytofluorimetric analysis. D. A. provided grape seeds. A. G. statistically analysed output data. M. B. supervised the experiments and wrote the manuscript.

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All authors have no personal or financial conflicts of interest and they have not entered into any agreement that could interfere with our access to the data on the research or on our ability to analyse the data independently, to prepare manuscripts and to publish them.

The Supplementary Tables are available online only at http://journals.cambridge.org/action/displayJournal?jid=bjn

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