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Amelioration of doxorubicin-induced myocardial oxidative stress and immunosuppression by grape seed proanthocyanidins in tumour-bearing mice

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Abstract

We have investigated the protective effects of grape seed proanthocyanidins on doxorubicin-induced toxicity in tumour-bearing mice. The intraperitoneal administration of doxorubicin (2 mg kg^{-1} every other day, cumulative dosage for 18 mg kg^{-1}) significantly inhibited the growth of sarcoma 180, and induced myocardial oxidative stress with decreased superoxide dismutase and glutathione peroxidase activity while increasing malondialdehyde formation in the heart or serum. Doxorubicin-induced myocardial oxidative stress also reduced lactate dehydrogenase and creatine kinase activity in the heart and elevated their levels in the serum. Doxorubicin also affected immune functions of tumour-bearing mice with significantly decreased interleukin-2 (IL-2) and interferon-γ (INF-γ) production, and slightly decreased natural killer (NK) cell cytotoxicity, lymphocyte proliferation and CD4+/CD8+ ratio. It markedly increased the percentages of cytotoxic T cells (CD3+CD8+), helper T cells (CD3+CD4+), IL-2R+CD4+, and IL-2R+ cells as compared with untreated tumour-bearing mice. The intragastric administration of proanthocyanidin (200 mg kg^{-1} daily) significantly inhibited tumour growth, and increased NK cell cytotoxicity, lymphocyte proliferation, CD4+/CD8+ ratio, IL-2 and INF-γ production. Moreover, proanthocyanidin strongly enhanced the anti-tumour effect of doxorubicin and the above immune responses, and completely eliminated myocardial oxidative stress induced by doxorubicin. In conclusion, intragastric administration of proanthocyanidin could enhance the anti-tumour activity of doxorubicin and ameliorate doxorubicin-induced myocardial oxidative stress and immunosuppression in tumour-bearing mice.

Introduction

Doxorubicin, an anthracycline antibiotic, is widely used either alone or in combination with other agents in the treatment of leukaemia and solid tumours (Weiss et al 1986; Chabner & Myers 1989). Nevertheless, its clinical effectiveness is restricted due to dose-limiting toxic effects on normal cells, including cardiotoxicity, myelotoxicity and haematological toxicity (Steinherz et al 1991; Richardson & Johnson 1997). Generally, these toxic effects of doxorubicin are known to be closely related to lipid peroxidation caused by the generation of reactive oxygen species (ROS). Doxorubicin has been shown to be a potent generator of ROS either by an enzymatic pathway or a formation of doxorubicin-Fe^{3+} complex (Goodman & Hochstein 1977; Nakano et al 1984; Zweier 1984). These ROS can then attack membranes or macromolecules and cause lipid peroxidation, which can lead to serious acute and chronic side effects. Acute side effects include myelotoxicity and haematological toxicity, which can cause dysfunction of immune responses, but the major chronic side effect is cardiotoxicity, which leads to critical and life-threatening congestive heart failure. It is clear that doxorubicin-induced cardiotoxicity is due to an increase in oxidative stress caused by free radical overproduction and a decrease in endogenous antioxidant reserve. Ferraro et al (2000) reported that doxorubicin triggers apoptosis of both G_0–G_1 and cycling peripheral blood lymphocytes and induces massive deletion of mature T and B cells in the spleen, lymph nodes and thymus. More attention has been paid to the protective effects of natural antioxidants against toxicity induced by chemotherapeutic agents, especially whenever free radical generations are involved.

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Proanthocyanidins, a group of naturally occurring polyphenolic bioflavonoids, are present in fruits, vegetables, nuts, seeds, flowers, bark, and particularly in grape seeds. Grape seed proanthocyanidins have been reported to have a wide range of biological and pharmacological activities including antioxidative, cardio-protective, anti-tumour, antibacterial, antiviral, and anti-inflammatory actions (Ye et al 1999; Li et al 2001; Bagchi et al 2003; Singh et al 2004). Among these diverse pharmacological actions of proanthocyanidins, the antioxidative and anti-tumour activities are remarkable. Bagchi et al (1997, 1998) showed it to be one of the most potent free radical scavengers and antioxidants in-vitro and in-vivo. It is also a potential chemopreventive agent possessing anti-tumour activity and enhancing the activity of chemotherapeutic agents and ameliorating normal cell toxicity associated with chemotherapeutic agents used in the treatment of cancer (Bagchi et al 2001, 2002, 2003; Sharma et al 2004). It is commonly accepted that the underlying mechanism is partially due to its strong antioxidative properties. Previous studies have shown that proanthocyanidins have anti-tumour activity in-vitro and in-vivo. They enhanced doxorubicin-induced anti-tumour effects via the promotion of doxorubicin-induced apoptosis due to an increase in intracellular doxorubicin, Ca^{2+}, and Mg^{2+} concentrations, and a reduction in pH value, mitochondrial membrane potential and cytochrome *c* release (Agarwal et al 2002; Zhang et al 2005). However, few studies have focused on the immunopotentiating activity of proanthocyanidins.

This study was designed to investigate the effects of proanthocyanidins (intragastric administration) on doxorubicin-induced myocardial oxidative stress and immune functions in tumour-bearing mice.

Materials and Methods

Reagents and antibodies

Proanthocyanidin, a standardized water–methanol extract from grape seeds, was provided by the Laboratory of Applied Organic Chemistry, Lanzhou University (Lanzhou, China). Grape seeds were extracted twice by 70% acetone–water, and the acetone removed by vacuum distillation. The residual water-solution was extracted by n-butanol, and the final extract was separated by gradient elution with methanol–water in a polyethylene gel column. Lamella scanning studies demonstrated that the extract contained 80% dimeric and 16% other polymer and flavonoids.

The following reagents were used: doxorubicin (Meiji Pharmaceutical Co. Ltd, Tokyo, Japan); MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), sodium dodecyl sulfate (SDS) and concanavalin A (Con A) from Sigma Chemical Co. (St Louis, MO); RPMI-1640 medium (GIBCO BRL, Grand Island, NY); bovine serum (Hangzhou Sijiqing Biotechnology Co., Hangzhou, China). Other reagents were of analytical purity.

The Cyto Tox 96 non-radioactive cytotoxicity assay kit was purchased from Promega Co. (Madison, WI). Mouse interferon-γ (IFN-γ) and interleukin-2 (IL-2) enzyme-linked immunosorbent assay (ELISA) kits were obtained from Jingmei Biotechnology Co. Ltd (Beijing, China). Malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), lactate dehydrogenase (LDH) and creatine kinase assay kits were from Nanjing Jiancheng Biotechnology Institute (Nanjing, China). Fluorescein isothiocyanate (FITC)-anti mouse CD3e, FITC-anti mouse CD4, phycoerythrin (PE)-anti mouse CD8a, and appropriate isotype controls (FITC-hamster IgG, FITC-rat IgG2b and PE-rat IgG2a) were purchased from *e*Bioscience Co. (San Diego, CA). PE-anti mouse CD25/IL-2R and its isotype control (PE-rat IgM) were from Southern Biotechnology Associates Inc. (Birmingham, AL).

Cell lines

Mouse lymphoma YAC-1 and mouse sarcoma 180 (S180) cell lines were purchased from the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). YAC-1 cells were grown in complete RPMI-1640 medium supplemented with 10% heat-inactivated bovine serum, 2 mM L-glutamine, 100 U mL^{-1} penicillin and 100 $\mu g\,mL^{-1}$ streptomycin at 37°C in a humidified atmosphere of 5% CO_2, and routinely passaged every other day.

Mice

Specific pathogen-free female BALB/c mice (6–8-weeks-old) were purchased from the Experimental Animal Center of the Medical College, Lanzhou University and provided with mouse pellets and water which were freely available. All animal experiments were approved by the Institutional Animal Care and Use Committee.

Tumour transplantation

S180 cells were maintained in BALB/c mice by weekly transplantation of the tumour cells into the peritoneal cavity. Tumour cells were obtained from mice inoculated seven days before, washed twice by centrifugation at 500 *g* and resuspended in 0.9% NaCl solution to a final concentration of 1.5×10^7 viable cells mL^{-1}. According to the protocols of the mouse tumour xenograft model, female BALB/c mice were inoculated subcutaneously (s.c.) into their right axillary fossa with 0.2 mL liquid containing 3×10^6 viable S180 cells. Viability, assessed by the trypan blue dye exclusion, was always found to be 95% or more.

Drug treatments

Proanthocyanidin and doxorubicin were dissolved in 0.9% NaCl. Tumour-bearing mice were randomly divided into four groups (14 mice each group) one day after inoculation. The animals were treated with either proanthocyanidin (200 mg kg^{-1}) intragastrically (i.g.) alone daily,

doxorubicin ($2\,mg\,kg^{-1}$) intraperitoneally (i.p.) alone every other day, or proanthocyanidin ($200\,mg\,kg^{-1}$ i.g. daily) combined with doxorubicin ($2\,mg\,kg^{-1}$ i.p. every other day). The control mice were injected with 0.9% NaCl. Ten days after tumour inoculation, serum and heparinized blood samples were collected for biochemical analysis and analysing peripheral blood lymphocyte subsets after the animals were anaesthetized with pentobarbital sodium ($40\,mg\,kg^{-1}$, i.p.). Spleens were isolated to obtain splenic lymphocytes. Hearts were immediately removed to prepare 10% homogenates in 0.15 M KCl solution and the supernatants were kept frozen at −70°C until biochemical analysis. Additionally, the tumours were removed and weighed.

Biochemical analysis

MDA content was determined using a MDA assay kit according to the manufacturer's instructions for the thiobarbituric acid (TBA) assay (Ohkawa et al 1979). Protein was estimated by the method of Lowry et al (1951). The enzyme activities of SOD, GSH-Px, LDH, and creatine kinase in heart homogenates and serum were carried out using respective assay kits according to the manufacturer's instructions.

Preparation of splenic lymphocytes

To determine the effects of proanthocyanidin and doxorubicin on mouse splenic lymphocyte proliferation, cytokine production and NK cell cytotoxicity, splenic lymphocytes were prepared after in-vivo treatment as described above. Following anaesthetization, spleens (eight mice from each group) were immediately isolated and passed through a double layer of stainless-steel mesh using a syringe plunger to obtain single cell suspensions. After the cells were washed twice in ice-cold (4°C) RPMI-1640 medium, lymphocytes were separated from the erythrocytes by lysing for 30 s in ice-cold sterilized distilled water. Then they were washed twice in fresh RPMI-1640 medium and counted using a haemocytometer. Cell viability was determined by Trypan blue exclusion. The final suspension of splenic lymphocytes was adjusted to a concentration of 5×10^6 cells mL^{-1} in complete RPMI-1640 medium for subsequent experiments.

MTT assay of lymphocyte proliferation

Con A, a well-known T-cell mitogen, markedly stimulated splenic lymphocyte proliferation as measured using the microculture tetrazolium (MTT) assay (Mosmann 1983). Mouse splenic lymphocytes (5×10^5 cells/100 μL) were incubated in the absence or presence of Con A ($1.25\,\mu g\,mL^{-1}$) in a 96-well flat-bottom microculture plate (Costar, Corning, USA) in triplicate for 72 h. At the end of the incubation period, MTT ($5\,g\,L^{-1}$, w/v, in PBS) 10 μL was added to each well and further cultured for the last 4 h. SDS 100 μL (10%, w/v, in 0.01 M HCl) was then added and mixed thoroughly to dissolve formazan crystals. The optical density (OD) was read at 570 nm on a Microplate Reader (Elx800, Bio-Tek Instruments, Inc., USA). The results were expressed as the stimulation index (SI) using the formula:

$$SI = OD_{\text{Con A-stimulated lymphocyte proliferation}} / OD_{\text{spontaneous lymphocyte proliferation without Con A}}$$

SI > 2 was regarded as a positive result provided that lymphoblasts were present in the stimulated cultures (Cederbrant et al 2003).

Cytokine production assay of mouse splenic lymphocytes

Splenic lymphocytes (5×10^5 cells/100 μL) were co-cultured with or without Con A ($1.25\,\mu g\,mL^{-1}$) in 96-well round-bottom microculture plates in triplicate for 48 h. After centrifugation at 250 *g* for 5 min, the culture supernatant from each culture was collected and kept frozen at −70°C until assay. Cytokine (IFN-γ and IL-2) levels were measured using mouse ELISA kits according to the manufacturer's instructions (Ho et al 2004). The OD was read at 450 nm using a Microplate Reader. The mean cytokine concentration of each sample triplicate was calculated using the standard curve.

LDH release assay for evaluation of NK cell cytotoxicity

NK cells from mouse spleens as described above were used as effector cells. Mouse lymphoma YAC-1 cells sensitive to NK cells were used as target cells. Effector cells (5×10^5 cells/100 μL) and target cells (1×10^4 cells/100 μL) resuspended in RPMI-1640 medium supplemented with 3% heat-inactivated bovine serum were added to each well of a 96-well round-bottom microculture plate in triplicate to obtain an effector:target ratio of 50:1, and incubated for 8 h. The amount of released LDH in the culture supernatant (50 μL/well) was determined using the Cyto Tox 96 non-radioactive cytotoxicity assay kit according to the manufacturer's instructions and the report of Vetvicka & Yvin (2004). The amount of LDH was proportional to the number of lysed cells. The OD was read at 490 nm using a Microplate Reader. To determine the percentage of NK cell cytotoxicity, the following equation was used:

$$\text{cytotoxicity (\%)} = (OD_{\text{experimental release}} - OD_{\text{effector spontaneous release}} - OD_{\text{target spontaneous release}}) / (OD_{\text{target maximum release}} - OD_{\text{target spontaneous release}}) \times 100$$

Where $OD_{\text{experimental release}}$ represents LDH release resulting from co-cultures at an effector:target ratio of 50:1, $OD_{\text{effector spontaneous release}}$ or $OD_{\text{target spontaneous release}}$ represents LDH release from effector or target cells incubated with medium alone, and $OD_{\text{target maximum release}}$ was obtained from target cells lysed with the lysis solution.

Flow cytometry for peripheral blood lymphocyte subsets and expression of IL-2R

The heparinized peripheral blood (10 mice from each group) was collected for analysing lymphocyte subsets and expression of IL-2 receptor (IL-2R) by flow cytometry. The following monoclonal antibodies (mAbs) were used for phenotype analysis of T cells: PE-anti mouse CD25/IL-2R and FITC-anti mouse CD4, FITC-anti mouse CD3e and PE-anti mouse CD8a, or appropriate isotype controls (PE-rat IgM, FITC-rat IgG2b, FITC-hamster IgG, and PE-rat IgG2a). After the heparinized peripheral blood containing mAbs was incubated for 15 min in the dark, erythrocytes were lysed for 35 s using a Coulter Q-Prep immunology workstation (Beckman-Coulter Inc, Fullerton, CA). Five thousand cells were collected for each sample and data obtained were analysed using a Coulter Epics XL flow cytometry (Beckman-Coulter Inc, Fullerton, CA).

Statistical analysis

Results were expressed as mean ± s.d. Data analysis was made by analysis of variance followed by LSD-*t* post-hoc test for multiple comparisons using the computer statistical package SPSS 11.0 (for Windows). Differences were considered to be statistically significant at $P < 0.05$.

Results

Anti-tumour effect in-vivo

The data in Figure 1 show that administration of proanthocyanidin (200 mg kg^{-1} i.g. daily) and doxorubicin (2 mg kg^{-1} i.p. every other day) alone or in combination had a significant anti-tumour effect on the growth of S180 in tumour-bearing mice as compared with the untreated control ($P < 0.01$). Their inhibitory percentages were 39.90%, 41.85%, and 68.82%, respectively. The results indicated that the anti-tumour activity of proanthocyanidin plus doxorubicin was the strongest of the treated groups.

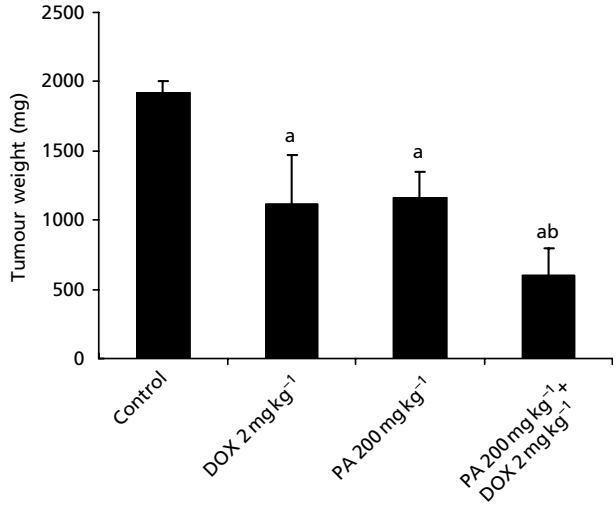

Figure 1 Anti-tumour effects of proanthocyanidin (PA) and doxorubicin (DOX) on the growth of S180 in tumour-bearing mice. Results are expressed as mean ± s.d. (n = 14). [a]$P < 0.01$ compared with untreated control, [b]$P < 0.01$ compared with doxorubicin-treated group (analysis of variance, LSD-*t* test).

Effects on the formation of MDA and enzyme activities of SOD, GSH-Px, LDH and creatine kinase

Doxorubicin treatment significantly decreased SOD and GSH-Px activity while increasing MDA formation in the heart and serum as compared with that of untreated tumour-bearing mice. Doxorubicin reduced LDH and creatine kinase activity in the heart and increased their levels in the serum. Proanthocyanidin by itself had almost no effect on the formation of MDA and enzyme activities of SOD, GSH-Px, LDH and creatine kinase in the heart or serum. However, activity levels completely recovered from the changes induced by doxorubicin when proanthocyanidin and doxorubicin were used combined (Tables 1, 2).

Effects on mouse splenic lymphocyte proliferation

The results indicated that neither proanthocyanidin nor doxorubicin had an influence on lymphocyte proliferation in the absence of Con A. However, splenic lymphocyte proliferation was significantly elevated when stimulated with Con A as compared with their respective spontaneous proliferation. Doxorubicin (2 mg kg^{-1}, i.p.) had almost no effect on the stimulation index (SI), while proanthocyanidin (200 mg kg^{-1}, i.g.) significantly increased the SI (SI > 2) compared with the untreated control. The combination of proanthocyanidin with doxorubicin strongly increased the SI to a level even higher than that of the proanthocyanidin-treated group (Figure 2).

Effects on cytokine productions

The levels of IFN-γ and IL-2 induced by Con A were significantly decreased in splenocytes of the doxorubicin-treated group, while they were significantly increased in those of the proanthocyanidin-treated group as compared with the control (Figure 3). In particular, proanthocyanidin completely reversed the reduction of IFN-γ and IL-2 induced by doxorubicin treatment, and strongly enhanced IFN-γ and IL-2 productions to a level even higher than those of proanthocyanidin alone. IFN-γ and IL-2 productions were undetectable in splenocytes without Con A stimulation (data not shown).

Effects on NK cell cytotoxicity

As shown in Figure 4, proanthocyanidin (200 mg kg^{-1}) significantly increased NK cell cytotoxicity (28.13%), while doxorubicin (2 mg kg^{-1}) slightly inhibited NK cell

Table 1 Effects of proanthocyanidin (PA) and doxorubicin (DOX) on the formation of MDA and enzyme activities of SOD, GSH-Px, LDH and creatine kinase in the heart homogenates of tumour-bearing mice

Treatment	Dose (mg kg^{-1})		MDA (nmoL (mg protein)$^{-1}$)	SOD (U (mg protein)$^{-1}$)	GSH-Px (U (g protein)$^{-1}$)	LDH (U (g protein)$^{-1}$)	Creatine kinase (U (mg protein)$^{-1}$)
	PA	DOX					
Control	–	–	9.25 ± 2.02	480.32 ± 87.08	10.70 ± 2.20	3232.62 ± 600.01	129.82 ± 34.09
DOX	–	2	12.25 ± 2.61^{a}	330.36 ± 32.23^{b}	7.05 ± 1.98^{b}	1798.01 ± 546.99^{b}	89.96 ± 23.45^{a}
PA	200	–	8.80 ± 2.48^{d}	469.29 ± 84.82^{d}	9.99 ± 1.48^{d}	2988.96 ± 467.80^{d}	137.61 ± 30.16^{b}
PA + DOX	200	2	9.59 ± 1.59^{c}	420.31 ± 73.05^{c}	9.48 ± 1.63^{c}	2673.27 ± 611.86^{d}	120.74 ± 17.85^{c}

Results are expressed as mean ± s.d. (n = 8). $^{a}P < 0.05$, $^{b}P < 0.01$ compared with untreated control, $^{c}P < 0.05$, $^{d}P < 0.01$ compared with doxorubicin alone (analysis of variance, LSD-*t* test).

Table 2 Effects of proanthocyanidin (PA) and doxorubicin (DOX) on the formation of MDA and enzyme activities of SOD, GSH-Px, LDH and creatine kinase in the serum of tumour-bearing mice

Treatment	Dose (mg kg^{-1})		MDA (nmoL mL^{-1})	SOD (U mL^{-1})	GSH-Px (U mL^{-1})	LDH (U mL^{-1})	Creatine kinase (U mL^{-1})
	PA	DOX					
Control	–	–	7.57 ± 1.84	386.49 ± 12.06	390.45 ± 28.53	8514.01 ± 416.70	10.20 ± 1.98
DOX	–	2	10.31 ± 1.89^{b}	323.10 ± 18.35^{b}	308.21 ± 25.48^{b}	9442.58 ± 723.38^{b}	23.93 ± 4.86^{b}
PA	200	–	7.63 ± 1.24^{d}	375.62 ± 30.12^{d}	415.83 ± 26.46^{d}	8543.42 ± 580.07^{d}	10.20 ± 2.31^{d}
PA + DOX	200	2	8.21 ± 0.94^{c}	366.37 ± 34.30^{d}	364.53 ± 14.81^{d}	8667.37 ± 435.31^{c}	16.35 ± 4.39^{d}

Results are expressed as mean ± s.d. (n = 8). $^{a}P < 0.05$, $^{b}P < 0.01$ compared with untreated control, $^{c}P < 0.05$, $^{d}P < 0.01$ compared with doxorubicin alone (analysis of variance, LSD-*t* test).

cytotoxicity (16.03%) as compared with the untreated control (19.35%). A combination of proanthocyanidin with doxorubicin strongly enhanced NK cell cytotoxicity (31.26%) to a level even higher than that of the proanthocyanidin-treated group.

Effects on peripheral blood lymphocyte subsets and expression of IL-2R

The mean percentages of helper T cells (CD3+CD4+), cytotoxic T cells (CD3+CD8+) lymphocyte subsets and expression of IL-2R in peripheral blood of experimental mice are shown in Table 3. Doxorubicin treatment markedly increased the percentages of CD3+CD8+, CD3+CD4+, IL-2R+CD4+, and IL-2R+ cells as compared with the untreated control ($P < 0.01$) and led to a decrease in the CD4+/CD8+ ratio. Proanthocyanidin treatment significantly increased the above percentages also, and slightly increased the CD4+/CD8+ ratio. More importantly, proanthocyanidin plus doxorubicin further enhanced the percentages of CD3+CD4+, IL-2R+CD4+, IL-2R+ cells, and the ratio of CD4+/CD8+ to a level even higher than that of the proanthocyanidin-treated group.

Discussion

In recent years, a great deal of evidence has linked reactive oxygen species (ROS) with many types of cancer. ROS play a crucial role in human cancer development, especially in the promotion and progression phases. Cullen et al (2003) showed that pancreatic cancer cells had decreased levels of Mn-SOD immunoreactive protein as well as activity, and decreases in Mn-SOD levels correlated well with increased rates of tumour cell proliferation. This suggested that antioxidants might prevent or delay the onset of some types of cancer and possess anticancer activity. Therefore, the treatments with antioxidants might hold promise in cancer therapeutics. In agreement with our previous observations (Zhang et al 2005), the results of this study demonstrated that intragastric administration of proanthocyanidin exhibited a significant inhibitory effect on the growth of S180, while proanthocyanidin combined with doxorubicin strongly inhibited the tumour growth when compared with doxorubicin alone ($P < 0.01$). The data showed that intragastric administration of proanthocyanidin had anti-tumour effects and enhanced the anti-tumour effects

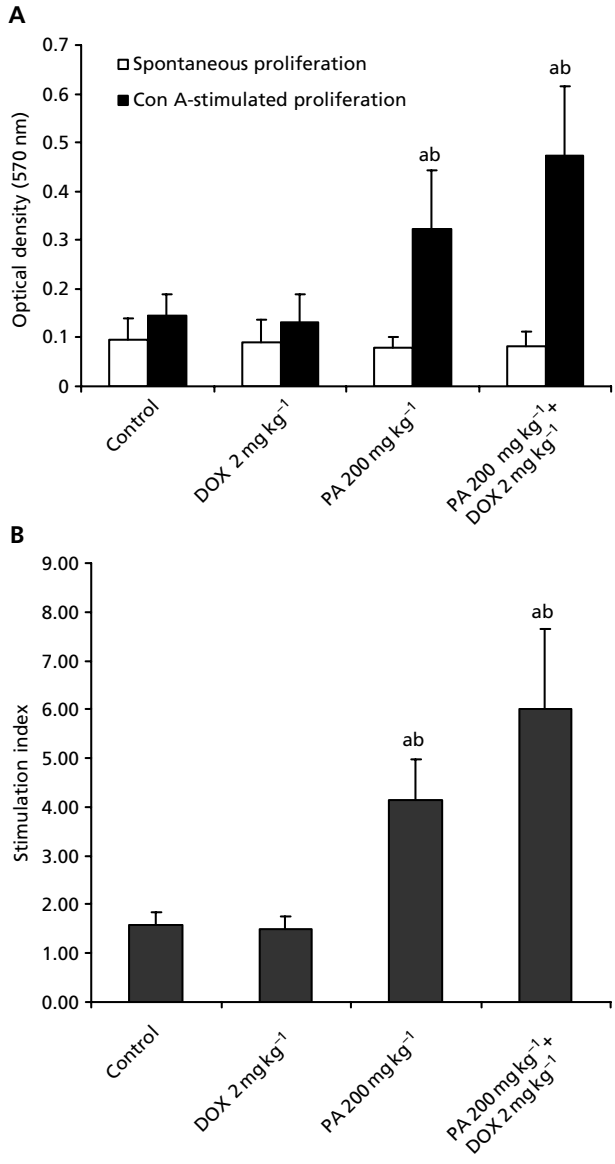

Figure 2 Effects of doxorubicin (DOX) and proanthocyanidin (PA) on mouse splenic lymphocyte proliferation using the MTT assay. A. Spontaneous or Con A-stimulated splenic lymphocyte proliferation. B. Stimulation index (SI) = $OD_{\text{Con A stimulated proliferation}}$/$OD_{\text{spontaneous proliferation without Con A}}$. Results are expressed as mean ± s.d. (n = 8). $^{a}P < 0.01$ compared with untreated control, $^{b}P < 0.01$ compared with doxorubicin-treated group (analysis of variance, LSD-*t* test).

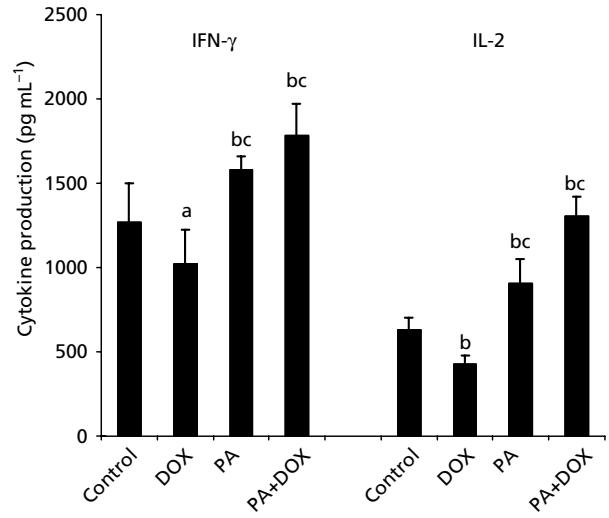

Figure 3 Effects of proanthocyanidin (PA, 200 mg kg^{-1}) and doxorubicin (DOX, 2 mg kg^{-1}) on the levels of IFN-γ and IL-2 in culture supernatants of splenocytes. Results are expressed as mean ± s.d. (n = 8). $^{a}P < 0.05$, $^{b}P < 0.01$ compared with untreated control, $^{c}P < 0.01$ compared with doxorubicin-treated group (analysis of variance, LSD-*t* test).

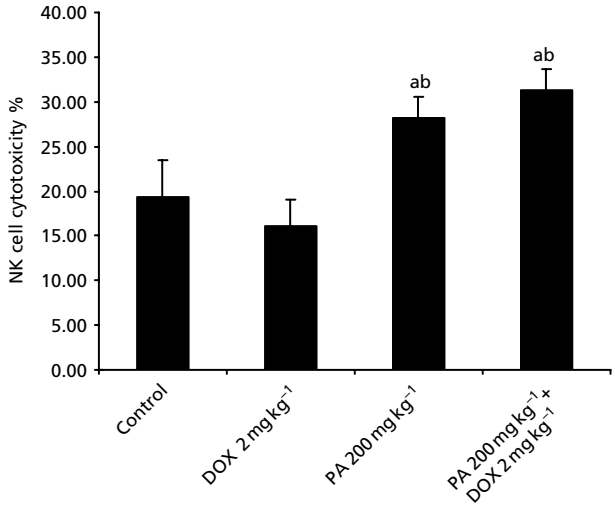

Figure 4 Effects of proanthocyanidin (PA) and doxorubicin (DOX) on NK cell cytotoxicity. Results are expressed as mean ± s.d. (n = 8). $^{a}P < 0.01$ compared with untreated control, $^{b}P < 0.01$ compared with doxorubicin-treated group (analysis of variance, LSD-*t* test).

of doxorubicin in tumour-bearing mice. Meanwhile, doxorubicin treatment significantly decreased SOD and GSH-Px activity while increasing MDA formation in the heart or serum. It also reduced LDH and creatine kinase activity in the heart and increased their levels in the serum. This indicated that doxorubicin induced oxidative stress, which led to heart lipid peroxidation accompanied by LDH and creatine kinase increase in the serum. However, there was no evident myocardial oxidative stress of proanthocyanidin. It is suggested that proanthocyanidins could potentiate the anti-tumour activity of doxorubicin and decrease doxorubicin-induced myocardial oxidative stress, and its antioxidative properties contributed to the actions.

On the other hand, immune cells are particularly sensitive to oxidative stress because of higher production of ROS, which play a pivotal role in their normal function (Meydani et al 1995). Sharmanov et al (1990) reported that a deficiency of vitamin E facilitated the increase in ROS concentration leading to functional changes in the

Table 3 Peripheral blood lymphocyte subsets in tumour-bearing mice

Treatment	Dose (mg kg^{-1})		CD3+CD8+ (%)	IL-2R+CD4+ (%)	CD3+CD4+ (%)	IL-2R+ (%)	CD4+/CD8+ ratio
	PA	DOX					
Control	–	–	19.47 ± 8.70	27.29 ± 6.79	29.24 ± 6.99	55.33 ± 5.10	1.82 ± 0.95
DOX	–	2	27.91 ± 6.08^{b}	42.27 ± 5.45^{b}	46.63 ± 7.28^{b}	64.96 ± 3.38^{b}	1.76 ± 0.55
PA	200	–	27.25 ± 5.17^{b}	48.07 ± 8.26^{bc}	54.12 ± 8.22^{bc}	63.64 ± 6.36^{b}	2.07 ± 0.49
PA+DOX	200	2	23.83 ± 3.95	54.06 ± 4.02^{bd}	59.66 ± 3.40^{bd}	65.87 ± 3.93^{b}	2.56 ± 0.40^{ad}

PA, proanthocyanidin; DOX, doxorubicin. Results are expressed as mean ± s.d. (n = 10). $^{a}P < 0.05$, $^{b}P < 0.01$ compared with untreated control, $^{c}P < 0.05$, $^{d}P < 0.01$ compared with doxorubicin alone (analysis of variance, LSD-*t* test).

immune system, and antioxidants improved the proliferative capacity of lymphocytes, increased host defense and immunoglobulin synthesis (Bendich 1990). Thus, the oxidant–antioxidant balance was critical for immune cell function (Knight 2000). Many experimental studies and clinical trials have observed systemic immunodeficiency in patients and mice with advanced cancer (Dix et al 1999; Mashino et al 2002; Hadden 2003). Moreover, it is becoming increasingly clear that, in addition to their anti-tumour action, several pro-oxidant anti-tumour agents, such as doxorubicin, also affect host immune responses (Rapozzi et al 1998; Ferraro et al 2000). Therefore, certain types of immunotherapy support the possibility that treatments with immunomodulatory agents may hold promise in cancer therapeutics (Brenner et al 2000; Muller et al 2003). Extensive studies in the last few years have revealed that phytochemicals derived from fruits and vegetables, referred to as chemopreventive agents, can reduce the risk of acquiring specific cancers and suppress cancer cell proliferation by their immunomodulatory actions (Mao et al 2000; Aziz et al 2003). Our results showed that doxorubicin treatment suppressed cytokine production (IL-2 and IFN-γ), while it increased the percentages of CD3+CD4+, CD3+CD8+ lymphocyte subsets and IL-2R expression as compared with the control. Unexpectedly, there was a discrepancy between cytokine productions and percentages of CD3+CD4+, CD3+CD8+ lymphocytes in doxorubicin treatment. Although it has been reported that under certain circumstances doxorubicin can stimulate or suppress immune responses, either directly or indirectly (Rapozzi et al 1998; Ferraro et al 2000; Mihich 2000), it is difficult to explain the difference between the decrease in cytokine levels and the increase in percentages of CD3+CD4+, CD3+CD8+ cells in the doxorubicin-treated group.

Adaptive tumour immunity is provided by helper T cells (CD3+CD4+), cytotoxic T cells (CD3+CD8+) and NK cells which can independently defend against cancer cells (Miller 2001; Xiang et al 2001; Adam et al 2003; Chikamatsu et al 2003; Shiku 2003). It has been assumed that the predominant tumoricidal effector mechanism involved in rejecting tumours is direct cytotoxic killing by CD3+CD8+ cells, and in many models CD3+CD8+ cells have been effective in eliminating tumours in the absence of CD3+CD4+ cells. However, CD3+CD4+ cells are required to provide help for CD3+CD8+ cells' anti-tumour function (Bennett et al 1997; Shiku 2003). In fact, CD3+CD4+ cells are the major regulator of all immune system activity. These cells form a series of protein mediators called cytokines such as IL-2 and IFN-γ that act on other cells of the immune system (Tham et al 2002; Adam et al 2003). The cytokines can initiate B-cells to produce antibodies or enhance production of CD3+CD8+ cells. Without these cytokines, the remainder of the immune system does not function as effectively as it would with the appropriate cytokine environment. CD3+CD4+ cells amplify themselves by secreting cytokines, particularly IL-2 which induces the expression of IL-2R by T cells, results in T-cell proliferation by the subsequent interaction of IL-2 with IL-2R, regulates immune response and enhances the helper cell response as well as the entire immune system's response to foreign antigens (Sun et al 2000). CD3+CD4+ cells are the primary, initiating and organizing component of host immunoresponsiveness against grafts, and CD3+CD8+ cells are recruited secondarily to the site to complete the acute rejection process. Consequently, the CD4+/CD8+ ratio and cytokine levels are major indicators for assessing the function of cell-mediated immunity. In this study, doxorubicin alone suppressed IL-2 and IFN-γ production and CD4+/CD8+ ratio, but increased CD3+CD4+ and CD3+CD8+ cells which might have been deficient in immune function. This indicated that even though it could increase the percentages of CD3+CD4+, CD3+CD8+ cells and IL-2R expression, doxorubicin was still an immunosuppressant as demonstrated in many reports (Rapozzi et al 1998; Ferraro et al 2000). However, in agreement with the findings of Nair et al (2002), intragastric administration of proanthocyanidin significantly enhanced Con A-induced lymphocyte proliferation, IL-2 and IFN-γ production, NK cell cytotoxicity, CD3+CD4+ and CD3+CD8+ lymphocyte subsets, IL-2R expression, and the CD4+/CD8+ ratio. Meanwhile, proanthocyanidin enhanced all the immune responses induced by doxorubicin. The results indicated that proanthocyanidin was an immunostimulant that could stimulate doxorubicin-induced immune responses. Therefore, intragastric administration of proanthocyanidin

could enhance the anti-tumour activity of doxorubicin and ameliorate doxorubicin-induced myocardial oxidative stress and immunosuppression in tumour-bearing mice.

Conclusions

Besides potent anti-tumour activity, doxorubicin could induce myocardial oxidative stress, and resulted in immunosuppression in tumour-bearing mice. Proanthocyanidin could enhance the anti-tumour activity of doxorubicin and ameliorate doxorubicin-induced myocardial oxidative stress and immunosuppression in tumour-bearing mice via its antioxidative and immunomodulatory properties.

References

Adam, J. K., Odhav, B., Bhool, K. D. (2003) Immune responses in cancer. *Pharmacol. Ther*. **99**: 113–132

Agarwal, C., Singh, R. P., Agarwal, R. (2002) Grape seed extract induces apoptotic death of human prostate carcinoma DU145 cells via caspases activation accompanied by dissipation of mitochondrial membrane potential and cytochrome *c* release. *Carcinogenesis* **23**: 1869–1876

Aziz, M. H., Kumar, R., Ahmad, N. (2003) Cancer chemoprevention by resveratrol: in vitro and in vivo studies and the underlying mechanisms (Review). *Int. J. Oncol*. **23**: 17–28

Bagchi, D., Garg, A., Krohn, R. L., Bagchi, M., Tran, M. X., Stohs, S. J. (1997) Oxygen free radical scavenging abilities of vitamins C and E and grape seed proanthocyanidin extract in vitro. *Res. Commun. Mol. Pathol. Pharmacol*. **95**: 179–189

Bagchi, D., Garg, A., Krohn, R. L., Bagchi, M., Bagchi, D. J., Balmoori, J., Stohs, S. J. (1998) Protective effects of grape seed proanthocyanidins and selected antioxidants against TPA-induced hepatic and brain lipid peroxidation and DNA fragmentation, and peritoneal macrophage activation in mice. *Gen. Pharmacol*. **30**: 771–776

Bagchi, D., Ray, S. D., Patel, D., Bagchi, M. (2001) Protection against drug- and chemical-induced multiorgan toxicity by a novel IH636 grape seed proanthocyanidin extract. *Drugs Exp. Clin. Res*. **27**: 3–15

Bagchi, D., Bagchi, M., Stohs, S. J., Ray, S. D., Sen, C. K., Preuss, H. G. (2002) Cellular protection with proanthocyanidins derived from grape seeds. *Ann. N. Y. Acad. Sci*. **957**: 260–270

Bagchi, D., Sen, C. K., Ray, S. D., Das, D. K., Bagchi, M., Preuss, H. G., Vinson, J. A. (2003) Molecular mechanisms of cardioprotection by a novel grape seed proanthocyanidin extract. *Mutat. Res*. **523–524**: 87–97

Bendich, A. (1990) Antioxidant nutrients and immune functions. In: Bendich, A., Phillips, M., Tergerdy, R. P. (eds) *Advances in experimental medicine and biology*. Plenum Press, New York and London, pp. 1–12

Bennett, S. R. M., Carbone, F. R., Karamalis, F., Miller, J. F. A. P., Heath, W. R. (1997) Induction of a CD8+ cytotoxic T lymphocyte response by cross-priming requires cognate CD4+ T cell help. *J. Exp. Med*. **186**: 65–70

Brenner, M., Rossig, C., Sili, U., Young, J. W., Goulmy, E. (2000) Transfusion medicine: new clinical applications of cellular immunotherapy. *Hematology* **2000**: 356–375

Cederbrant, K., Anderson, C., Andersson, T., Marcusson-Stahl, M., Hultman, P. (2003) Cytokine production, lymphocyte proliferation and T-cell receptor Vß expression in primary peripheral blood mononuclear cell cultures from nickel-allergic individuals. *Int. Arch. Allergy Immunol*. **132**: 373–379

Chabner, B. A., Myers, C. E. (1989) Clinical pharmacology of cancer chemotherapy. In: De Vitta, V. T. J., Hellman, S., Rosenberg, S. A. (eds) *Cancer: principles and practice of oncology*. J. B. Lippincott Co., Philadelphia, pp 349–395

Chikamatsu, K., Albers, A., Sanson, J., Kwok, W. W., Appella, E., Whiteside, T. L., Deleo, A. B. (2003) P53(110–124)-specific human CD4+ T-helper cells enhance in vitro generation and antitumor function of tumor-reactive CD8+ T cells. *Cancer Res*. **63**: 3675–3681

Cullen, J. J., Weydert, C., Hinkhouse, M. M., Ritchie, J., Domann, F. E., Spitz, D., Oberley, L. W. (2003) The role of manganese superoxide dismutase in the growth of pancreatic adenocarcinoma. *Cancer Res*. **163**: 1297–1303

Dix, A. R., Brooks, W. H., Roszman, T. L., Morford, L. A. (1999) Immune defects observed in patients with primary malignant brain tumors. *J. Neuroimmunol*. **100**: 216–232

Ferraro, C., Quemeneur, L., Prigent, A. F., Taverne, C., Revillard, J. P., Bonnefoy-Berard, N. (2000) Anthracyclines trigger apoptosis of both G_0–G_1 and cycling peripheral blood lymphocytes and induce massive deletion of mature T and B cells. *Cancer Res*. **60**: 1901–1907

Goodman, J., Hochstein, P. (1977) Generation of free radicals and lipid peroxidation by redox cycling of adriamycin and daunomycin. *Biochem. Biophys. Res. Commun*. **77**: 797–803

Hadden, J. W. (2003) Immunodeficiency and cancer: prospects for correction. *Int. Immunopharmacol*. **3**: 1061–1071

Ho, C. Y., Lau, C. B. S., Kim, C. F., Leung, K. N., Fung, K. P., Tse, T. F., Chan, H. H. L., Chow, M. S. S. (2004) Differential effect of *Coriolus versicolor* (Yunzhi) extract on cytokine production by murine lymphocytes in vitro. *Int. Immnopharmacol*. **4**: 1549–1557

Knight, J. A. (2000) Free radicals, antioxidants, and the immune system. *Ann. Clin. Lab. Sci*. **30**: 145–158

Li, W. G., Zhang, X. Y., Wu, Y. J., Tian, X. (2001) Anti-inflammatory effect and mechanism of proanthocyanidins from grape seeds. *Acta Pharmacol. Sin*. **22**: 1117–1120

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. **193**: 265–275

Mao, T. K., Powell, J., Water, J. V., Keen, C. L., Schmitz, H. H., Hammerstone, J. F., Gershwin, M. E. (2000) The effect of cocoa procyanidins on the transcription and secretion of interleukin 1 β in peripheral blood mononuclear cells. *Life Sci*. **66**: 1377–1386

Mashino, K., Sadanaga, N., Tanaka, F., Ohta, M., Yamaguchi, H., Mori, M. (2002) Effective strategy of dendritic cell-based immunotherapy for advanced tumor-bearing hosts: the critical role of Th1-dominant immunity. *Mol. Cancer Ther*. **1**: 785–794

Meydani, S. N., Wu, D., Santos, M. S., Hayek, M. G. (1995) Antioxidants and immune response in aged persons: overview of present evidence. *Am. Clin. Nutr*. **62**: 1462–1476

Mihich, E. (2000) On the immunomodulating effects of anticancer drugs and their therapeutic exploitation. *Jap. J. Clin. Oncol*. **30**: 469–471

Miller, J. S. (2001) The biology of natural killer cells in cancer, infection, and pregnancy. *Exp. Hematol*. **29**: 1157–1168

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**: 55–63

Muller, M. R., Grunebach, F., Nencioni, A., Brossart, P. (2003) Transfection of dendritic cells with RNA induces CD4- and CD8-mediated T cell immunity against breast carcinomas and reveals the immunodominance of presented T cell epitopes. *J. Immunol*. **170**: 5892–5896

Nair, N., Mahajan, S., Chawda, R., Kandaswami, C., Shanahan, T. C., Schwartz, S. A. (2002) Grape seed extract activates Th1 cells in vitro. *Clin. Diagn. Lab. Immunol.* **9**: 470–476

Nakano, H., Ogita, K., Gutteridge, J. G., Nakano, M. (1984) Inhibition by the protein ceruloplasmin of lipid peroxidation stimulated by an $Fe3^{+}$–ADP–adriamycin complex. *Fed. Eur. Biochem. Soc. Lett.* **166**: 232–236

Ohkawa, H., Ohishi, N., Yaki, K. (1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem.* **95**: 351–358

Rapozzi, V., Zorzet, S., Comelli, M., Mavelli, I., Perissin. L., Giraldi, T. (1998) Melatonin decreases bone marrow and lymphatic toxicity of adriamycin in mice bearing TLX5 lymphoma. *Life Sci.* **63**: 1701–1713

Richardson, D. S., Johnson, S. A. (1997) Anthracyclines in haematology: preclinical studies, toxicity and delivery systems. *Blood Rev.* **11**: 201–223

Sharma, G., Tyagi, A. K., Singh, R. P., Chan, D. C., Agarwal, R. (2004) Synergistic anti-cancer effects of grape seed extract and conventional cytotoxic agent doxorubicin against human breast carcinoma cells. *Breast Cancer Res. Treat.* **85**: 1–12

Sharmanov, A. T., Aidarkhanov, B. B., Kurmangalinov, S. M. (1990) Effects of vitamin E deficiency on oxidative metabolism and anti-oxidant enzyme activity of macrophage. *Ann. Nutr. Metab.* **34**: 143–146

Shiku, H. (2003) Importance of CD4+ helper T-cells in antitumor immunity. *Int. J. Hematol.* **77**: 435–438

Singh, R. P., Tyagi, A. K., Dhanalakshmi, S., Agarwal, R., Agarwal, C. (2004) Grape seed extract inhibits advanced human prostate tumor growth and angiogenesis and upregulates insulin-like growth factor binding protein-3. *Int. J. Cancer* **108**: 733–740

Steinherz, L. J., Steinherz, P. G., Tan, C. C., Helle, G., Murphy, L. (1991) Cardiac toxicity 4 to 20 years after completing anthracycline therapy. *J. Am. Med. Assoc.* **266**: 1672–1677

Sun, A. D., Chu, C. T., Liu, B. Y., Wang, J. T., Leu, J. S., Chiang, C. P. (2000) Expression of interleukin-2 receptor by activated peripheral blood lymphocytes upregulated by the plasma level of interleukin-2 in patients with recurrent aphthous ulcers. *Proc. Natl. Sci. Counc. ROC (B).* **24**: 116–122

Tham, E. L., Shrikant, P., Mescher, M. F. (2002) Activation-induced nonresponsiveness: a Th-dependent regulatory checkpoint in the CTL response. *J. Immunol.* **168**: 1190–1197

Vetvicka, V., Yvin, J. C. (2004) Effects of marine ß-1,3 glucan on immune reactions. *Int. Immunopharmacol.* **4**: 721–730

Weiss, R. B., Sarosy, G., Clagette-Carr, K., Russo, M., Leyland-Jones, B. (1986) Anthracycline analogs: the past, present, and future. *Cancer Chemother. Pharmacol.* **18**: 185–197

Xiang, J., Chen, Z., Huang, H., Moyana, T. (2001) Regression of engineered myeloma cells secreting interferon-γ-inducing factor is mediated by both CD4+/CD8+ T and natural killer cells. *Leukemia Res.* **25**: 909–915

Ye, X., Krohn, R. L., Liu, W., Joshi, S. S., Kuszynski, C. A., McGinn, T. R., Bagchi, M., Preuss, H. G., Stohs, S. J., Bagchi, D. (1999) The cytotoxic effects of a novel IH636 grape seed proanthocyanidin extract on cultured human cancer cells. *Mol. Cell Biochem.* **196**: 99–108

Zhang, X. Y., Bai, D. C., Wu, Y. J., Li, W. G., Liu, N. F. (2005) Proanthocyanidin from grape seeds enhances anti-tumor effect of doxorubicin both in vitro and in-vivo. *Pharmazie* (In press)

Zweier, J. L. (1984) Reduction of O_2 by iron-adriamycin. *J. Biol. Chem.* **259**: 6056–6058