Grape extracts suppress the formation of preneoplastic foci and activity of fatty acid synthase in rat liver

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Accepted 22 August 2003

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DEN, diethylnitrosamine; FAS, fatty acid synthase; GPx, glutathione peroxidase; GSH, glutathione; GST, glutathione S-transferase; GST-P⁺ foci, glutathione S-transferase positive foci; PNPH, *p*-nitrophenol hydroxylase; TBARS, thiobarbituric acid reactive substances

Abstract

This study was conducted to examine the effects of dietary grape extracts on preneoplastic foci formation in rat hepatocarcinogenesis, and related hepatic enzymes. Male Sprague-Dawley rats were fed basal diet or grape diet containing 15% concentrated grape extracts (68 bricks). The grape diet groups were divided into whole-period grape diet group (DEN-GW; grape diet group fed throughout experimental period) and postinitiation grape diet group (DEN-GP; grape diet group fed from post initiation stage) according to the starting time point of the grape diet. Hepatocarcinogenesis was induced by diethylnitrosamine (DEN; 200 mg/kg bw) and 2/3 partial hepatectomy (DEN-B; DEN-treated basal diet group, DEN-GW, and DEN-GP groups), while the control group treated with saline and sham operation (Control group). The formation of placental glutathione (GSH) S-transferase positive (GST-P⁺) foci in DEN-GW group was moderately but significantly suppressed, however, not in DEN-GP group. Thiobarbituric acid reactive substances content of DEN-GW group was significantly lower than that of DEN-B group. The activity of fatty acid synthase (FAS) in the grape diet groups was decreased about 1/2 of the DEN-B group. The content of GSH and GSH peroxidase activity were increased by carcinogen treatment, but not modulated by grape diet. The activities of GSH S-trans-

ferase, *p*-nitrophenol hydroxylase, and catalase were not affected by diet or treatment. Conclusively, the grape diet-induced reduction of FAS activity that was expressed highly in neoplastic tissues, might be one of the contributing mechanisms of hepatic cancer prevention.

Keywords: DEN, FAS, Grape, GST-P⁺ foci, Hepatocarcinogenesis

Introduction

Grape has many potent antioxidants, such as anthocyanins, catechin, and resveratrol (Jang *et al.*, 1997). These polyphenolic compounds from grape or the polyphenol extracts have shown vitamin E sparing effect (Simonetti *et al.*, 2002), and have been reported as chemopreventive agents. The polyphenol extracts protected from atherosclerosis progression in high cholesterol-fed rabbit aortic vessels (Yamakoshi *et al.*, 1999), and the solid fraction of red wine delayed the spontaneous onset of tumors in mice (Clifford *et al.*, 1996).

Although grapes have been traditionally known to stimulate blood making, bone firming, and increase the resistance against cancer, there were not enough evidences showing the chemopreventive effects of grape. Recently, some researchers reported the physiological modification by polyphenolic compounds from grape, which include the cancer prevention (Bomser *et al.*, 1999; Carbo *et al.*, 1999; Zhao *et al.*, 1999), the suppression of inflammation, the induction of vasorelaxation (Flesch *et al.*, 1998), the decrease of blood pressure (de Moura *et al.*, 2002), and so on. Resveratrol, the natural compound rich in red grapes, has the possibility of the phytoestrogenic action with the preventive or therapeutic effect for the hormone-related diseases, because its similar structure to estrogen (Jang *et al.*, 1997; Gehm *et al.*, 1999; Lu and Serrero, 1999). Resveratrol or polyphenolic extracts from grape have been reported to alter the amount of nitric oxide by modification of nitric oxide synthase (Flesch *et al.*, 1998). Besides, polyphenolic compounds from grape increased the expression of p53 and p21, inhibited the cell proliferation (Hsieh *et al.*, 1999), induced apoptosis in tumor cell (Agarwal *et al.*, 2002; Katsuzaki *et al.*, 2003; Matito *et al.*, 2003), and acted as protein kinase C inhibitor (Stewart *et al.*, 1999). All of them could be possible mechanisms of the

cancer preventive effect of grapes.

Fat has an important role as the membrane component, together with energy source in cancer cells. Already, there are many reports showing the excessive biosynthesis of lipids in highly proliferative cells, including cancer cells (Pizer *et al.*, 1996). Fatty acid synthase (FAS) is a key enzyme regulating fatty acid synthesis, and it is also expressed highly in cancer cells, regenerative epithelium, and fetal tissues (Kusakabe *et al.*, 2002). The high expression of FAS at cancer cells was examined in many tissues, such as ovary (Pizer *et al.*, 1996), prostate (Swinnen *et al.*, 2002), breast (Kuhajda *et al.*, 2000; Thupari *et al.*, 2001), esophagus (Nemoto *et al.*, 2001), colon (Huang *et al.*, 2000), and stomach (Kusakabe *et al.*, 2002), but there are few reports in hepatic cancer. Some researchers have reported FAS inhibition induced apoptosis in human breast, colon, prostate, and ovarian cancer cells (Thupari *et al.*, 2001). If we could find the naturally occurring modulator of FAS expression among dietary factors, these will have the potency of chemopreventive agents by the alteration of lipid metabolism.

This study was designed to show the anticarcinogenic effect of grape extracts on rat hepatocarcinogenesis, and to evaluate the potent mechanism including the modulation of drug-metabolizing enzyme systems, antioxidative enzymes, and FAS. This study would contribute to the discussion of the relationships between chemopreventive activity of grapes and the modification of lipid metabolism.

Materials and Methods

Animals and diet

Weanling male Sprague-Dawley rats weighing 40-60 g were supplied from Animal Care Facility of Seoul National University (Seoul, Korea), and they were kept at polycarbonate cages in a room with controlled temperature ($23\pm 2^\circ\text{C}$), humidity ($55\pm 5\%$), and light-dark-cycle (0700-1900). According to the experimental diets and treatment, they were divided into 4 groups (Figure 1), and fed one of the experimental diets (basal or grape diets) for 11 weeks. All of them, except the control group, were given intraperitoneal injection of diethylnitrosamine (DEN; 200 mg/kg body weight; Sigma Chemical Co., St. Louis, MO) at 6-week old, and 2/3 partial hepatectomy (PH) were performed after 3 weeks (Ito *et al.*, 1989). The control group was treated with saline and sham operation. The grape diet groups were divided into two groups, whole-period grape diet group (DEN-GW) and postinitiation grape diet group (DEN-GP). Animals of the DEN-GW group was fed the grape diet for whole experimental period, while those in the DEN-GP

Figure 1. Experimental protocol. Hepatocarcinogenesis was induced by i.p. injection of DEN (200 mg/kg body weight; ●), and 2/3 PH (✂). Instead of DEN and PH, control group was treated with saline (○) and sham operation (✓). All animals were fed with one of the experimental diets, basal diet (□) or 15% grape diet (■) for 11 weeks, and sacrificed (✚).

Table 1. The composition of experimental diets.

Components	Basal diet	Grape diet
Cornstarch	54.7	41.2
Casein	20.0	19.3
Corn Oil	15.0	14.5
α-Cellulose	5.0	4.8
Mineral mixture	4.0	3.9
Vitamin mixture	1.0	1.0
DL-methionine	0.3	0.3
Grape extract (68 brix)	-	15.0

In consideration of moisture, and carbohydrate contents (approximately 32 and 68% respectively; w/w) of grape extracts, both of the diet were designed to give out the same amounts of calorie nutrients, carbohydrate, protein, and fat.

group were fed from post initiation stage (beginning at 2 weeks after DEN injection) succeeded from the basal diet. They were sacrificed by decapitation at the 11th week, and their livers were used for assay. Liver sections were fixed in cold acetone and residual liver was homogenized in Tris-HCl buffered solution (pH 7.4). Microsomal, cytosolic and 600 g supernatant fractions were prepared by differential centrifugation and were stored in small aliquots at -70°C until used.

The basal diet was composed of 20% casein protein (Sigma Chemical Co.), 54.7% corn starch, 15% corn oil, 5% α-cellulose, 4.0% AIN-76 mineral mixture (ICN Biochemicals, Cleveland, OH), 1% AIN-76 fortified vitamin mixture (ICN Biochemicals), and 0.3% DL-methionine. The grape extract was substituted for a part of cornstarch, and the level of supplementation was 15 g per 100 g diet (15%). The consideration of moisture, and carbohydrate contents (approximately 32 and 68% w/w, respectively) of grape extract, both of the diets were designed to provide the same amounts of calorie nutrients, car-

bohydrate, protein, and fat.

Immunohistochemical staining for placental glutathione S-transferase positive foci (GST-P⁺ foci)

Liver sections from acetone-fixed tissues were stained for GST-P⁺ foci. It was carried out using the avidin-biotin-peroxidase complex method (Vectastain ABC kit, Vector Lab. Inc., Burlingame CA; GST-P antibody, MBL Co., Japan; Hsu *et al.*, 1981). The area of foci was measured by means of an image analyzer with a microscope (Image-Pro Plus v.4, Media Cybernetics, L.P., Silver Spring, MD).

Determination of lipid peroxidation

Microsomal lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) according to the method of Buege and Aust (1978). The absorbance of the resulting chromophore was determined at 535 nm, and the extinction coefficient for TBARS was taken to be $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

Fatty acid synthase (FAS) activity

FAS activity was determined using the method of Linn (1981). The assay mixture contained acetyl-CoA, NADPH, and cytosolic fraction in potassium phosphate buffer. Following preincubation at 37°C in a cuvette, the reaction was initiated by the addition of malonyl-CoA. The change of absorbance at 340 nm was monitored over a period of 10 min, and enzyme activity was expressed using a molar extinction coefficient of NADPH, $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$.

Catalase activity

Catalase activity was measured by the method of Aebi (1984). Diluted 600×g supernatant was reacted with hydrogen peroxide, and the decomposition of hydrogen peroxide was determined at 240 nm spectrophotometrically. To avoid the intervention by bubbling, the reaction time was controlled with not exceeding 30 s.

p-Nitrophenol hydroxylase (PNPH) activity

PNPH activity was assayed according to Reinke *et al.* (1985), and the reaction was accomplished with ascorbic acid, NADPH, and hepatic microsomes in phosphate buffer. A p-nitrocatechol, the product of the reaction, was colored by NaOH, and determined spectrophotometrically.

Determination of total GSH content

A piece of liver (0.5 g) was quick-frozen immediately on separating from carcass, and used for assay in 12 h. Total GSH content was measured by the 5,5'-dithiobis (2-nitrobenzoic acid)-GSH reductase recycling procedure (Anderson, 1985). The rate of 5-thio-2-nitrobenzoic acid formation was followed at 412 nm, and the total GSH content was expressed as mole of reduced GSH equivalents per g liver.

Glutathione (GSH)-dependent enzyme assay and protein concentration determination

Glutathione S-transferase (GST) activity was assayed in the hepatic cytosolic fraction using the method of Habig *et al.* (1974). The conjugate of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) was measured at 340 nm using a dual beam spectrophotometer. Calculation was made using a molar extinction coefficient of $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$. The activity of GSH peroxidase (GPx) in the hepatic cytosolic fraction was measured by monitoring the oxidation of NADPH at 340 nm according to the method of Tappel (1978). Protein concentration was determined by modified Lowry method (Bollag *et al.*, 1996).

Statistical analysis

All statistical analyses were carried out using SAS program with Duncan's multiple range tests. Differences were considered statistically significant at $P < 0.05$.

Results and Discussion

In recent years, grape, grape extracts, and wine have been public interest for chemopreventive effect on human health, such as cancer prevention (Bomser *et al.*, 1999; Carbo *et al.*, 1999; Zhao *et al.*, 1999), the induction of vasorelaxation (Flesch *et al.*, 1998), and the modulation of plasma lipid profiles (Yamakoshi *et al.*, 1999). The most of evidences have been reported on the polyphenolic compounds in some parts of grapes. Matito *et al.* (2003) reported that polyphenolic fraction of grapes seemed to have antiproliferative effect in mouse hepatoma Hepa-1c1c7 cells, and the fraction with the highest degree of polymerization and galloylation showed the most induction of apoptosis and effectiveness on the cell cycle. Some anthocyanins from skin of red grape also inhibited human lymphoid leukemia Molt 4B cell growth, which could be induced by apoptosis (Katsuzaki *et al.*, 2003). In addition, the cancer preventive effect of grape seed extract was examined on the human prostate carcinoma DU145 cells possi-

Table 2. Effect of grape extract supplementation on final body weight, liver weight, and relative liver weight.

Group	No. of rats	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)
Control	5	440.6±16.3	12.7±0.53	2.89±0.11
DEN-B	7	417.9±10.9	12.1±0.59	2.88±0.08
DEN-GW	6	428.7±10.3	12.1±0.43	2.72±0.04
DEN-GP	7	426.1±15.8	12.0±0.96	2.79±0.14

Rats, except control group, were treated with DEN and PH to induce hepatic carcinogenesis. Rats were fed with one of experimental diets for 11 weeks; Control and DEN-B group, basal diet; DEN-GW, grape diet for the whole experimental period; DEN-GP, grape diet 2 weeks later after DEN treatment following the basal diet. Values are mean±SE from final weight of body and liver. Relative liver weight was calculated by % of liver weight to body weight. There was no difference between groups at $P < 0.05$ by Duncan's multiple range test.

Figure 2. Effect of grape extract supplementation on the area of GST-P⁺ foci. Rats, except control group, were treated with DEN and PH to induce hepatic carcinogenesis. Rats were fed with one of experimental diets for 11 weeks; Control and DEN-B group, basal diet; DEN-GW, grape diet for the whole experimental period; DEN-GP, grape diet 2 weeks later after DEN treatment following the basal diet. Values are mean±SE from image reading of stained GST-P⁺ foci. Means with the same superscripts are not significantly different at $P < 0.05$ by Duncan's multiple range test. **Figure 3.** Effect of grape extract supplementation on the content of TBARS. Rats, except control group, were treated with DEN and PH to induce hepatic carcinogenesis. Rats were fed with one of experimental diets for 11 weeks; Control and DEN-B group, basal diet; DEN-GW, grape diet for the whole experimental period; DEN-GP, grape diet 2 weeks later after DEN treatment following the basal diet. Values are mean±SE from absorbance readings of chromophore. Means with the same superscripts are not significantly different at $P < 0.05$ by Duncan's multiple range test.

bly via apoptotic death (Agarwal *et al.*, 2002). Though more rare *in vivo*, the solid fraction of red wine delayed the spontaneous onset of tumors in transgenic mice (Clifford *et al.*, 1996). However, there was no study done on the effect of grape diet on the hepatocarcinogenesis. This study was conducted to show the suppressive effect of grape diet on preneoplastic foci formation in the chemically induced rat hepatocarcinogenesis model.

The area of GST-P⁺ foci was reduced by the grape diet, which might be related the decrease of TBARS content and the activity of FAS. No significant differences in body weight, liver weight, and relative liver

weight (g/100 g of animals) of animals were observed among different groups (Table 2). Also, the energy intake was not modulated by the experimental diets. GST-P⁺ foci, the preneoplastic lesions, appeared in all rats exposed to DEN (Figure 2). The formation of GST-P⁺ foci was inhibited by grape diet, and the foci area of animals fed grape diet for whole experimental period was significantly decreased compared to DEN-treated basal diet (DEN-B) group. TBARS contents were decreased in grape diet groups, and the reduction of TBARS content in group fed grape diet in whole period was significant in comparison with those values of the DEN-treated basal diet group

Table 3. Effect of grape extract supplementation on the activities of catalase, PNPH, and GST.

Group	Catalase (mole hydrogen peroxide decomposed/ min/mg protein)	PNPH (mole 4-catechol/ min/mg protein)	GST (nmole CDNB conjugated/min/mg protein)
Control	97.66±5.910	0.4460±0.0552	796.7±40.2
DEN-B	93.41±4.715	0.5197±0.0302	776.3±42.5
DEN-GW	101.04±4.778	0.4496±0.0220	813.3±38.7
DEN-GP	96.99±3.981	0.5284±0.0427	682.4±47.6

Rats, except control group, were treated with DEN and PH to induce hepatic carcinogenesis. Rats were fed with one of experimental diets for 11 weeks; Control and DEN-B group, basal diet; DEN-GW, grape diet for the whole experimental period; DEN-GP, grape diet 2 weeks later after DEN treatment following the basal diet. Values are mean±SE. There was no difference between groups at $P < 0.05$ by Duncan's multiple range test.

Figure 4. Effects of grape extract supplementation on the activity of FAS. Rats, except control group, were treated with DEN and PH to induce hepatic carcinogenesis. Rats were fed with one of experimental diets for 11 weeks; Control and DEN-B group, basal diet; DEN-GW, grape diet for the whole experimental period; DEN-GP, grape diet 2 weeks later after DEN treatment following the basal diet. Values are mean±SE from absorbance readings of the change rate of oxidized NADPH. Means with the same superscripts are not significantly different at $P < 0.05$ by Duncan's multiple range test.

Figure 5. Effects of grape extract supplementation on the content of GSH. Rats, except control group, were treated with DEN and PH to induce hepatic carcinogenesis. Rats were fed with one of experimental diets for 11 weeks; Control and DEN-B group, basal diet; DEN-GW, grape diet for the whole experimental period; DEN-GP, grape diet 2 weeks later after DEN treatment following the basal diet. Values are mean±SE from the rate of 5-thio-2-nitrobenzoic acid formation. Means with the same superscripts are not significantly different at $P < 0.05$ by Duncan's multiple range test.

(Figure 3). The FAS activity was induced in DEN-treated basal diet group, which was suppressed in grape diet groups to half of the activity of basal diet group (Figure 4). There was no difference among groups in catalase and PNPH activities (Table 3). The contents of GSH and the activity of GPx were elevated by DEN treatment, which were not modulated by experimental diets (Figure 5 and 6). GST activities were not different among groups (Table 3).

The suppressive effect of grape diet was shown

differently by the duration of diet feeding (whole-period grape diet group, 11 wk; postinitiation grape diet group, 6 wk) in rat hepatocarcinogenesis. The GST-P⁺ foci formation in both grape diet groups was inhibited, however there was a statistical significance only in group fed grape diet throughout the whole period compared to DEN-treated basal diet group. Hepatic carcinogenesis is induced throughout multi-stages, including initiation and promotion (Moriwaki, 2002). In most cases, foreign toxic chemicals are metabolized quickly in relatively short time within 2

Figure 6. Effects of grape extract supplementation on the activity of GPx. Rats, except control group, were treated with DEN and PH to induce hepatic carcinogenesis. Rats were fed with one of experimental diets for 11 weeks; Control and DEN-B group, basal diet; DEN-GW, grape diet for the whole experimental period; DEN-GP, grape diet 2 weeks later after DEN treatment following the basal diet. Values are mean $\pm$ SE from absorbance readings of the change rate of oxidized NADPH. Means with the same superscripts are not significantly different at $P < 0.05$ by Duncan's multiple range test.

weeks, and the host defense enzyme systems are also modulated promptly and then stabilized (Ip and Lisk, 1997). It is not always possible that we predict when we will be exposed to certain carcinogens, therefore routine consumption of plenty chemopreventive foods could be protective at the safety level to prevent cancer. Proanthocyanidins are natural compounds and widespread in fruits, vegetables, tea, chocolate, etc., and are consumed approximately 460-1,000 mg/day (Scalbert & Williamson, 2000). Bentivegna and Whitney (2002) designed the 90 day grape seed extract or grape skin extract feeding study at the level of 2.5% in the diet, relatively huge amount of grapes, to show the safety of these extracts, and could achieve an evidence of no observed adverse effect of the respective extracts.

In this study, the TBARS content was decreased by the grape diet. The antioxidative action of some plants is regarded to an important factor to maintain cell viability (Lee *et al.*, 2002). Grapes, especially red grapes, have many potent antioxidants, such as quercetin, resveratrol, proanthocyanidins, catechins, etc (Jang *et al.*, 1997). Grape extracts given orally reduced malondialdehyde content in rat heart, and might be resistant to myocardial ischemia reperfusion injury (Cui *et al.*, 2002). Orally supplied alcohol-free hydroalcoholic grape skin extract from a vinifera grape, significantly reduced systolic and diastolic arterial

pressure in chemically induced Wistar rat hypertension, as well as the decrease of lipid peroxidation (de Moura *et al.*, 2002). The previous antihypertensive effect was seemed to be partly owing to antioxidant actions of grape seed extracts. There is a similar study in healthy human that Concord grape juice, a rich source of flavonoids, was supplied to subjects at the level of 10 ml/kg body weight/day for 2 weeks (O'Byrne *et al.*, 2002). The grape juice induced the increase of serum antioxidant capacity and the protection of low density lipoprotein against oxidation to a similar extent to that achieved by 400 IU α -tocopherol/day.

FAS is a multifunctional enzyme in most tissues, however it is highly expressed at the cells with the high rate of turnover including cancer cells. Kusakabe *et al.* (2002) reported that FAS expression occurred at the early stage of tumorigenesis as well as carcinoma in human stomach, and suggested that could play an important role in the formation of pre-neoplastic foci. In addition, there are several reports that some inhibitors of FAS induced apoptosis in human breast, colon, prostate, and ovarian cancer cells (Thupari *et al.*, 2001). In this study, we did confirm some of the earlier findings (Thupari *et al.*, 2001; Kusakabe *et al.*, 2002) in the rat hepatocarcinoma model. The FAS activity of DEN-treated basal diet group was dramatically higher (almost two-fold) than that of the control group. Whereas, grape-diet fed groups showed even lower values of FAS activity than those values of the control group and one half of the values of DEN-treated basal diet group. Synthesized FAS inhibitor, cerulenin and C75, inhibited the activity of FAS, fatty acid synthesis, and the growth of SKBR3 human breast cancer cells and normal skin fibroblasts *in vitro* (Kuhajda *et al.*, 2000). Because high expression of FAS has been examined in many cancerous tissues, the reduction of FAS by the grape diet may be one of the chemopreventive mechanisms.

PNPH, reflecting cytochrome 2E1 activity, and GST activities were evaluated, however there was no difference among groups. The activity of catalase, an antioxidative enzyme, also was not modulated by any treatment. The content of GSH and the activity of GPx were elevated by DEN and PH treatment, however no change was noticed by the grape diet.

Conclusively, we have shown that the diet with grape extracts at the level of 15% suppressed the formation of GST-P⁺ foci, lipid peroxidation, and the activity of FAS. The 15% grape diet in rats can be converted to approximately 400 g (one medium bunch) of grapes to 70 kg human. The inhibitory effect of grape diet in preneoplastic foci formation might be affected by the duration of diet feeding. The reduction of FAS activity, expressed highly in tumor tis-

sues, might be one of the mechanisms of hepatic cancer prevention.

Acknowledgement

This work was supported by the Brain Korea 21 Project of Food and Nutrition for the Study on Prevention of Chronic Diseases (2001).

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