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An Antioxidant, Probucol, Induces Anti-angiogenesis and Apoptosis in Athymic Nude Mouse Xenografted Human Head and Neck Squamous Carcinoma Cells

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Probucol is a very strong synthetic antioxidant that was been safely used for the treatment of hyperlipidemia in Japan since 1985. It has been reported that lipid oxidation products can alter growth factor production, which could influence smooth muscle cell proliferation. Oxidized low density lipoprotein can influence smooth muscle cell proliferation by enhancing the expression of platelet-derived growth factor (*PDGF*)-*AA* gene and PDGF receptor in vascular smooth muscle cell. Further, free radical reactions can cause irreversible alterations of genomic constituents during the initiation phase of carcinogenesis. Antioxidant is considered to protect lipids and low density lipoprotein (LDL) from oxidation, which potentially inhibits angiogenesis, and rapid removal of free radicals by antioxidants could have an anti-carcinogenic effect. In the present study, we investigated whether antioxidant treatment with probucol had an antitumor effect on KB cells, a human head and neck squamous carcinoma cell line. Probucol did not have any effect on KB cells *in vitro*, but probucol treatment of KB cells xenografts in mice had a significant antitumor effect through anti-angiogenic and apoptosis-inducing actions. These results support the idea that probucol is useful for preventing and/or treating cancer.

Key words: Anti-oxidant — Head and neck squamous cell carcinoma — Anti-angiogenesis — Apoptosis

One approach to control the growth of cancer is chemoprevention, in which the disease is prevented, slowed, or reversed by administration of one or more naturally occurring or synthetic compounds. Several studies have shown that microchemicals in the diet, as well as some herbs and plants, can be used for the prevention and/or treatment of various carcinomas.[1–7] Among them, polyphenolic antioxidant,[4–7] a naturally occurring antioxidant that has attracted increasing attention in recent years, can prevent, slow, and/or reverse the induction of cancer and its subsequent development.[1–6, 8]

Angiogenesis is activated during multistage tumorigenesis and tumors cannot grow to more than 2–3 mm in diameter without forming new blood vessels.[9] Thus, one may infer that the growth and metastasis of solid tumors can be suppressed by inhibiting angiogenesis. It has been reported that lipid oxidation products can alter growth factor production, which could influence smooth muscle cell proliferation.[10] Oxidized low density lipoprotein (LDL) can influence smooth muscle cell proliferation by enhancing the expression of platelet-derived growth factor (*PDGF*)-*AA* gene and PDGF receptor in vascular smooth muscle cell.[11, 12] Antioxidant is considered to protect lipids and LDL from oxidation, which would potentially inhibit angiogenesis.

Free radical reactions can lead to irreversible alterations of genomic constituents during the initiation phase of carcinogenesis.[13, 14] Two experimental approaches have provided evidence of the involvement of free radicals in neoplasia. One is the modulation of cellular antioxidant status and the second is suppression of tumorigenesis by antioxidants.[15] If radicals are instrumental in mediating cell damage, their rapid removal by antioxidants should provide cytoprotection.[13, 14] Very strong antioxidant compounds are capable of scavenging both free radicals and reactive oxygen species (ROS·, O_2^-, -OH and H_2O_2), and thus increase the antioxidant potential of cells by ameliorating the deleterious effects of free radical reactions.[16, 17]

Probucol is a very strong synthetic antioxidant, more than three times stronger than vitamin E,[18] and has been used for the treatment of hyperlipidemia in Japan since 1985. Some antioxidants induced apoptosis in Yoshida Sarcoma cells, e.g., Tempicol-2,[19] inhibited cell cycle proliferation in human breast carcinoma cells, e.g., Silymarin,[20] and reduced cyclooxgenase-2 expression and prostaglandin production in human colorectal cancer cells.[21] To investigate whether antioxidants can have an antitumor effect, we studied a head and neck carcinoma cell line (KB,[22] which is frequently used for head and

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neck carcinoma investigations) xenografted into mice, and assessed the effects of probucol on tumor growth.

MATERIALS AND METHODS

Materials Probucol was a kind gift from Daiichi Pharmaceutical Industries (Tokyo). Female 5-week-old athymic nude mice (BALB/c *nu*/*nu*) were purchased from Charles River Japan, Inc. (Atsugi). The animals were housed in plastic cages (five mice per cage) with wood chip bedding in a room kept at 24±2°C with a humidity of 40–70% and a 12-h light/dark cycle.

Cell line KB cells, a human oral floor squamous carcinoma cell line, were grown in RPMI-1640 medium (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal calf serum (FCS), 2 m*M* glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 atmosphere.

Assay of cell proliferation KB cells were seeded into a 96-well plate (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) at 1×10^3 cells/well in RPMI-1640 medium containing 10% FCS. After 48, 72, and 96 h of treatment with probucol (10^{-4}–10^{-8} *M*), the number of cells was quantitated by an assay using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO).[23)]

Assay of cell viability KB cells were seeded into a 10-cm plastic dish (Falcon) at 1×10^6 cells/well in RPMI-1640 medium containing 10% FCS. After 48 and 72 h of treatment with probucol (10^{-5} and 10^{-6} *M*), cell viability was determined by counting with a hemocytometer after staining with trypan blue. Viable cells were expressed as a percentage of the total cell population.

Cell cycle analysis KB cells at 80–90% confluency were left untreated, treated with ethanol alone, or treated with 10^{-5} and 10^{-6} *M* probucol in ethanol. After 48 and 72 h, the medium was removed and the cell monolayers were quickly washed twice with cold phosphate-buffered saline (PBS). Then the cells were trypsinized and cell pellets were collected. The cells were washed twice with PBS, fixed in cold methanol, and rewashed with PBS to remove methanol. They were suspended in 500 μl of PBS, then digested with 20 μg/ml RNase at 37°C for 30 min and chilled on ice for 10 min. Then cellular DNA was stained with 50 μg/ml propidium iodide by incubation for 1 h at room temperature in the dark. The cell cycle distribution was analyzed by flow cytometry using a Becton Dickinson FACScan.

***In vivo* tumor growth assay** Because probucol was effective for vasculature disorders, e.g., arterial sclerosis, we thought that it might have some effect on tumor peripheral vessels, and hence on tumor growth. KB cells (5×10^6) were injected subcutaneously into the left flanks of mice. The tumor size was measured once a week by determining two perpendicular dimensions with calipers, and the volume in mm^3 was calculated from the formula $(a\times b^2)/2$, where *a* is the longer and *b* is the smaller dimension. Two weeks after inoculation, the tumors had grown to 600 mm^3 in volume. Then the mice were randomly divided into three groups of 5 animals, which received 0, 50, or 100 mg/kg of probucol, suspended in a 0.5% (w/v) hydroxypropylmethylcellulose (Shinetsu Chemical Co., Nagano) solution, five times per week orally. Because the tumors of control mice were so large at 8 weeks, the animals were killed under anesthesia. At the termination of the investigation, the tumor weight was determined.

Immunohistochemistry Paraffin-embedded tumor sections were deparaffinized in xylene and rinsed in absolute ethanol. After treatment with 0.3% hydrogen peroxide, the sections were incubated in 1% *Bandeiraea simplicifolia* agglutinin for 20 min, and then incubated with an anti-factor VIII rabbit monoclonal antibody (Nichirei, Tokyo) and an anti-Ki 67 rabbit monoclonal antibody (Immunoteck, Marseille, France). Next, the sections were incubated with biotinylated goat antirabbit immunoglobulin, followed by peroxidase-conjugated streptavidin (ABC kit; Nichirei). Reaction products were visualized by treatment with diaminobenzidine and the sections were examined under a microscope after counterstaining with hematoxylin.

Investigation of apoptosis Tumors were minced in cold Tris-buffered saline and homogenized. Then the homogenates were filtered and washed with cold Tris-buffered saline, followed by resuspension in 500 μl of lysis buffer

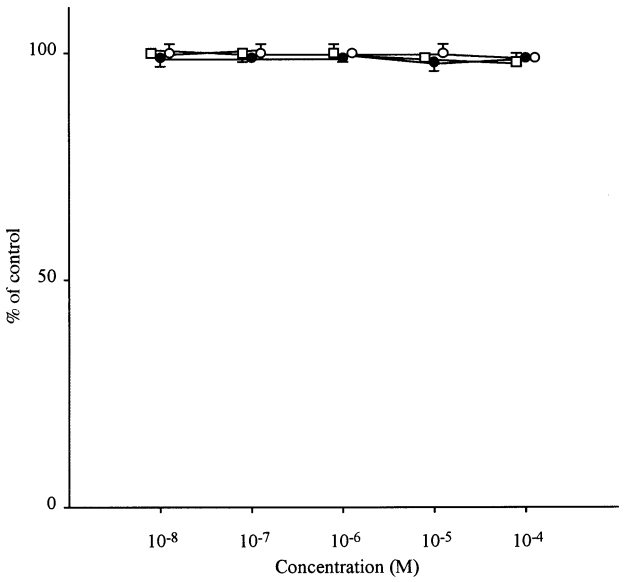

Fig. 1. Probucol treatment showed no effect on cell proliferation in either a dose- or time-dependent manner. □, 48 h; ○, 72 h; ●, 96 h.

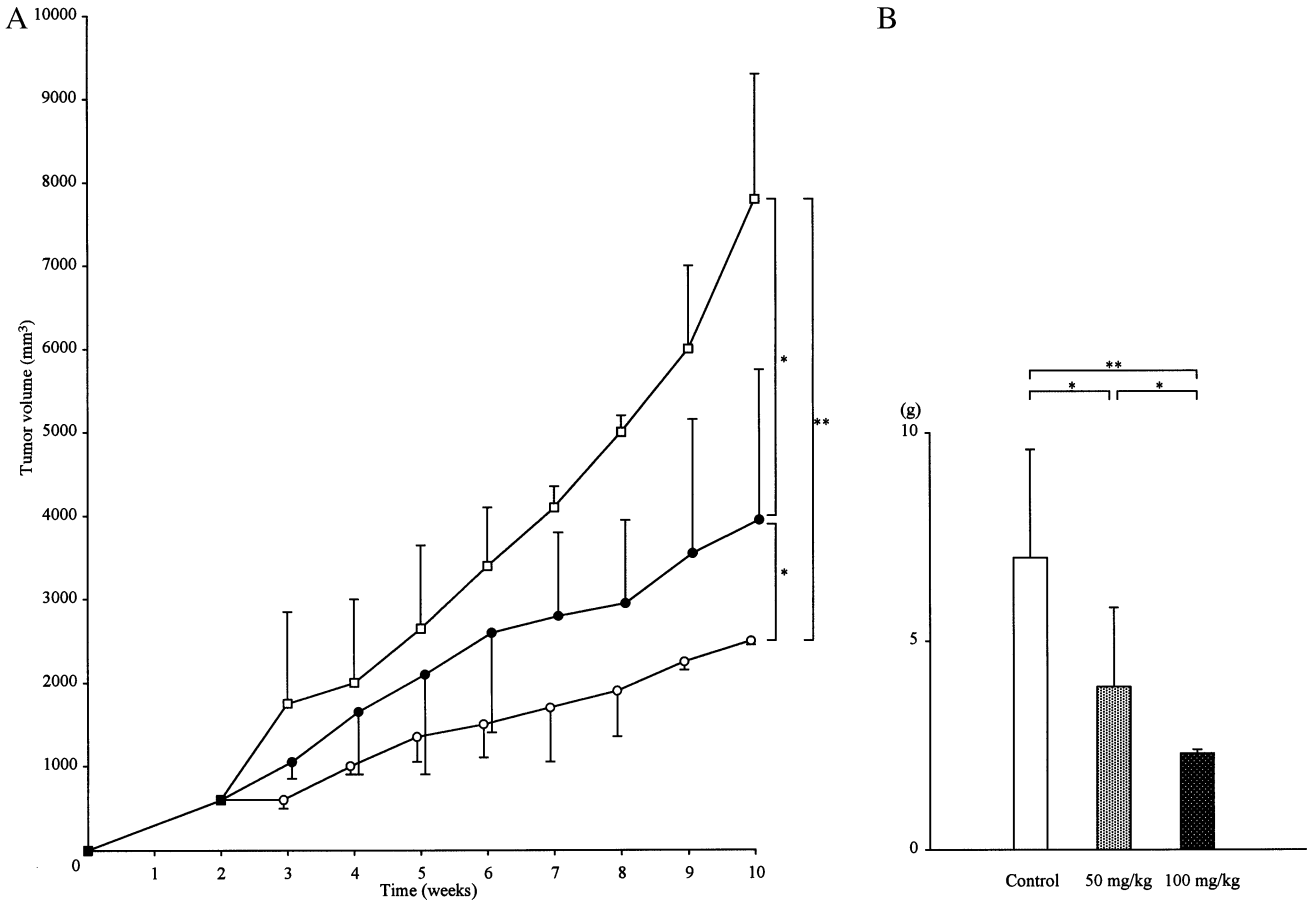

Fig. 2. Effect of probucol on the growth of xenografted KB cells in nude mice; tumor volume (A) and tumor weight (B). Probucol treatment resulted in significant suppression of tumor growth when control mice were compared with those receiving 100 mg/kg. There was no significant difference between control mice and those receiving 50 mg/kg or those given 50 and 100 mg/kg, but the tumor growth of probucol-treated mice tended to be suppressed. *, NS; **, $P<0.05$; bars, SD; □, control; ○, 50 mg/kg; ●, 100 mg/kg.

containing 500 m*M* Tris-HCl (pH 9.0), 2 m*M* EDTA, 10 m*M* NaCl, 1% sodium dodecyl sulfate, and 1 mg/ml proteinase K (Wako Chemical Co., Osaka). After incubation at 50°C for 20 h, samples were extracted twice with phenol, once with phenol-chloroform, and finally with chloroform. Then aliquots (1 μg) were loaded on 1.5% agarose gels and visualized by ethidium bromide staining. The terminal deoxynucleotidyl transferase-mediated cUDP nick end labeling (TUNEL) method was performed using an Apop Taq Plus kit (Oncor, Gaithersburg, MD) for tumor sections. Counting of immunoreactive tumor cells was done in three different fields for each section, and the apoptotic index was expressed as the percentage of TUNEL-positive cells relative to the total number of cells.

Statistical analysis One-way ANOVA, Sigma Stat for Windows, version 1.0, was used to compare different groups at the 95% confidence level. Student's *t*-test was also used to obtain *P* values and to compare different treatment groups, with $P \leq 0.05$ being considered statistically significant.

RESULTS

***In vitro* effect of probucol** Using the MTT assay, we first assessed whether probucol had an antiproliferative effect on KB cells. Probucol treatment showed no dose- or time-dependent effect on cell proliferation (Fig. 1). In addition, cell viability was examined after probucol treatment at 10^{-5} and 10^{-6} *M* for 48 and 72 h. It was found that probucol had no effect on cell viability and that almost all cells (98.1–99.2%) remained alive. The influence of probucol on the cell cycle was assessed using flow cytometry, and again there was no effect of treatment (data not shown). Thus, probucol seemed to have no effect on KB cells *in vitro*.

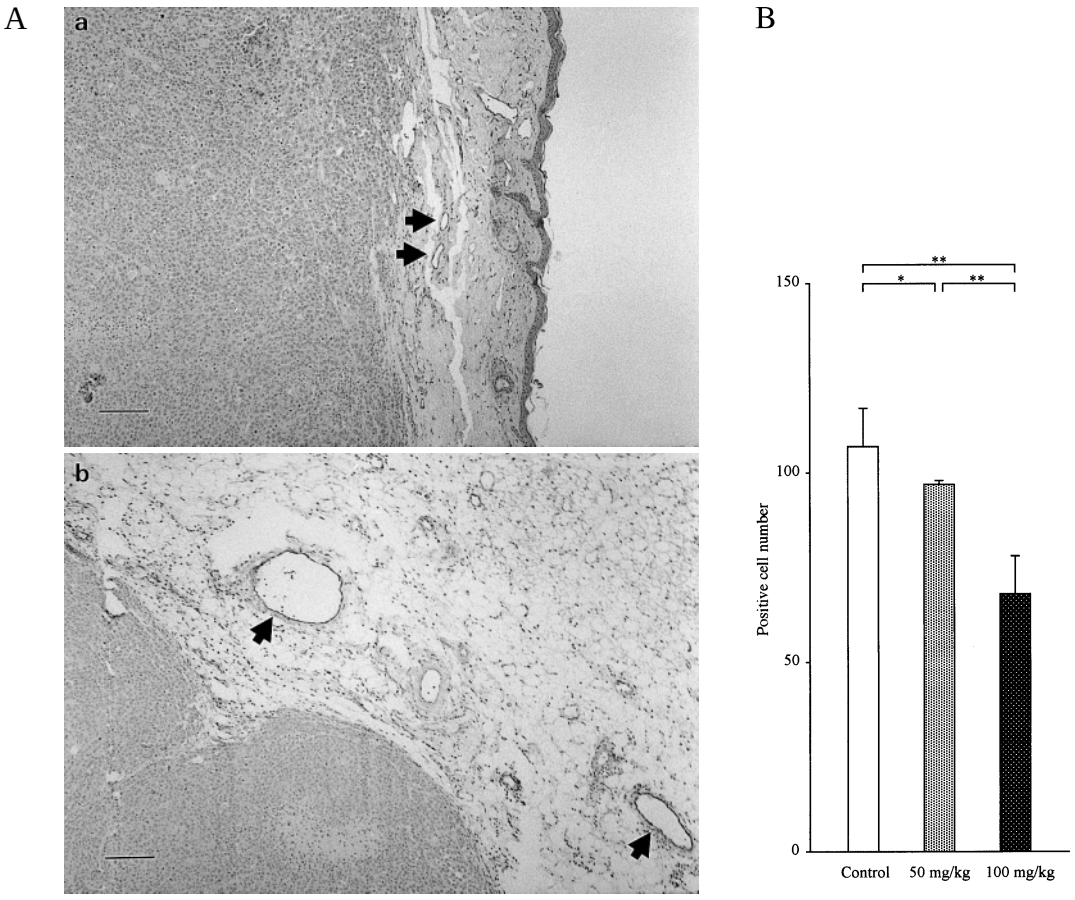

Fig. 3. Staining of tumor microvessels by anti-human factor VIII was observed in every group and the number of positive vessels was significantly less when mice were given 100 mg/kg of probucol. A shows factor VIII-positive vessels in 100 mg/kg treated (a) and control (b) mice tumors. Quantitative data are summarized in B. The number in the tumor treated with 50 mg/kg was less than that of control mice, though the difference was not significant. *, NS; **, $P<0.05$; bars, SD; arrow, factor VIII-positive vessels; scale, 100 μm.

***In vivo* effect of probucol** Female nude mice underwent inoculation with KB cells on day 0 and probucol was administered orally from day 14 (five times/week) at doses of 50 and 100 mg/kg for 8 weeks. Treatment of tumor-bearing nude mice with probucol had no significant effect on the body weight at any dose administered (data not shown). The slight loss of weight observed in the treated animals was a result of the decreased tumor weight caused by the antitumor effect of the drug. The effect of probucol on KB cell xenografts growing in nude mice is shown in Fig. 2A and tumor weights at the termination of treatment are shown in Fig. 2B. Probucol treatment resulted in significant suppression of tumor growth when control mice were compared with those given 100 mg/kg of probucol ($P<0.05$). There was no significant difference between control mice and those given 50 mg/kg of probucol or between 50 mg/kg and 100 mg/kg of probucol, but tumor growth (volume and weight) tended to be suppressed by probucol at 50 mg/kg. Macroscopic evaluation showed that the tumors of treated mice developed ulcers at the beginning of the 6th week and the change was dose-dependent. In contrast, the untreated control mice showed no ulceration of their tumors.

Immunohistochemistry To evaluate the anti-angiogenic effect of probucol, the tumor was immunohistochemically stained with anti-human factor VIII. Tumor microvessels in the peripheral region of tumors, positive for anti-human factor VIII, were observed in every group and the number was significantly less in the group given 100 mg/kg of probucol (Fig. 3). The number in the group treated with 50 mg/kg was somewhat less than in the control, but the difference was not significant. To assess the effect on the cell cycle *in vivo*, immunohistochemical staining with anti-human Ki 67 (MIB-1) antibody was done. This antibody

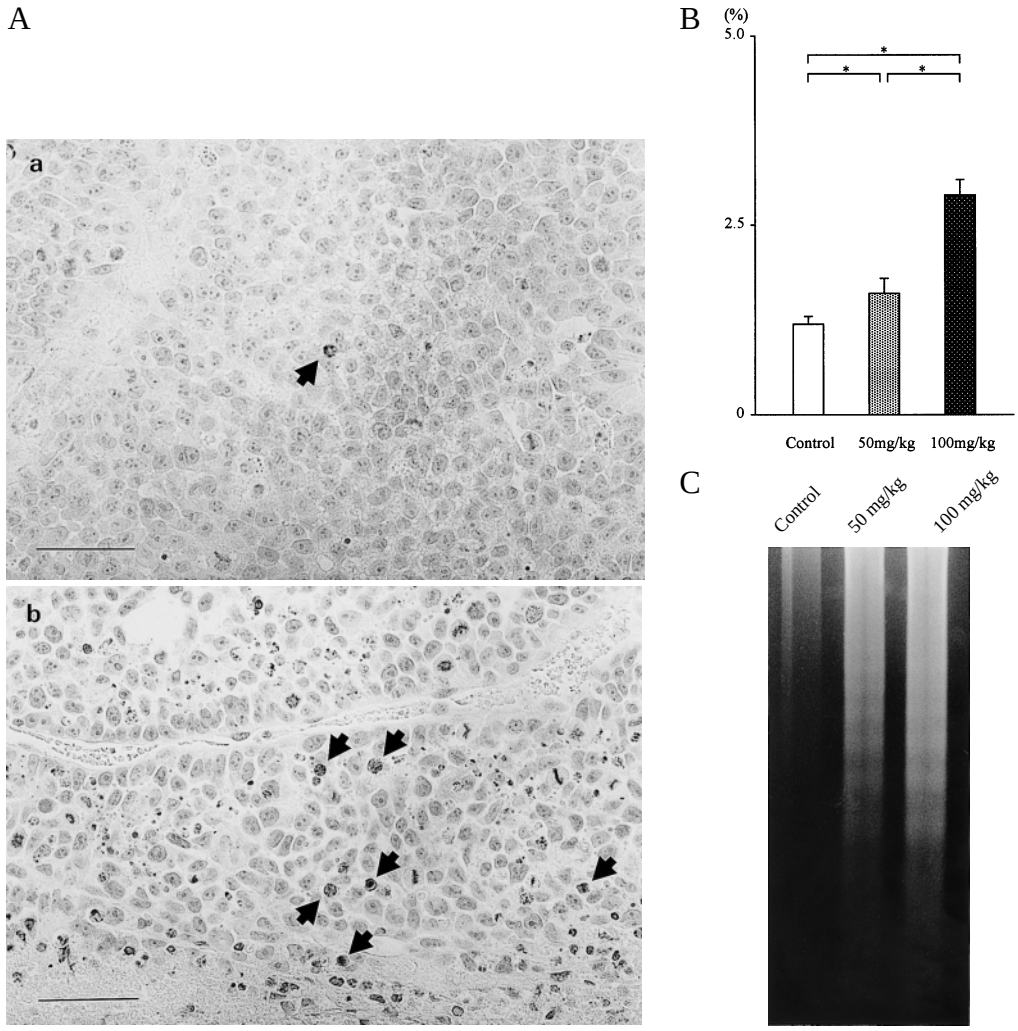

Fig. 4. TUNEL-positive cells were increased in the probucol treatment group (A: a, control; b, 100 mg/kg). Quantitative data are summarized in B. The apoptotic index was significantly higher in tumors from mice that had been treated at a dose of 50 and 100 mg/kg with probucol as compared with untreated control ones. DNA was degraded to oligonucleosomal DNA fragments that showed apoptotic laddering on agarose gel electrophoresis when tumor-bearing mice were treated with 100 mg/kg of probucol, whereas such laddering was not observed in mice given 50 mg/kg or untreated control mice (C). ∗, $P<0.05$; bars, SD; arrow, TUNEL-positive cells; scale, 50 μm.

stains $S+G_2$ and M phase cycle cells. Ki 67-positive cells were observed in every group; about 70% (68.3–71.8%) of viable tumor cells were positive and the positivity rate was not significantly different among the groups. Because KB cells do not express cyclin D1,[24] immunohistochemical staining with anti-cyclin D1 was not done for cell cycle analysis.

Apoptosis We also examined whether treatment of tumor-bearing mice with probucol was able to cause apoptotic cell death *in vivo*. Probucol treatment resulted in an increase of TUNEL-positive cells (Fig. 4A). Quantitative data are summarized in Fig. 4B. The apoptotic index was significantly higher in mice treated with 50 mg/kg and 100 mg/kg of probucol as compared with untreated control mice. The results of DNA fragmentation analysis are shown in Fig. 4C. DNA was degraded to oligonucleosomal DNA fragments that showed apoptotic laddering on agarose gel electrophoresis when tumor-bearing mice were treated with 50 mg/kg and 100 mg/kg of probucol, whereas such laddering was not observed in untreated control mice.

DISCUSSION

The concept of systematic administration of compounds that exhibit antioxidant activity is appealing.[14, 24] Efforts are being made to identify naturally occurring antioxidants, e.g. polyphenolic antioxidants,[4–7] which can prevent, slow, and/or reverse the processes of carcinogenesis and tumor development.[1–6, 8] Agents which possess strong antioxidant properties may be useful for preventing tumorigenesis and tumor growth,[20] because free radical reactions can give rise to irreversible alterations of cellular genomic constituents related to the initiation phase of carcinogenesis.[13, 14] Protection against the initiation of cancer may be provided by antioxidant supplements[25] because tumor cells usually show reduced activity of their primary cell defense system.[14, 26] The involvement of oxidative stress in the induction of cancer and its subsequent development is becoming increasing clear, as are the associated molecular mechanisms,[6, 27] e.g., modulation of the cellular antioxidant status, and suppression of tumorigenesis by antioxidants.[15]

Probucol is a strong antioxidant (more than 3 times stronger than vitamin E[18]) and it has been clinically used for the treatment of hyperlipidemia in Japan since 1985. As a therapeutic agent, it is well tolerated and largely free from side effects. It has been shown to be nontoxic in acute, subacute, and chronic toxicity studies even at high doses, and the 50% lethal dose (LD_{50}) could not be determined. Probucol is effective for inhibiting restenosis after percutaneous transluminal coronary angioplasty (PTCA) in acute myocardial infarction patients.[28] The process of restenosis after PTCA involves smooth muscle cell proliferation, extracellular matrix formation and vascular remodeling.[29] There is considerable evidence to suggest that oxidative stress plays an important role in the restenosis process. It has been demonstrated[10] that lipid oxidation products can alter growth factor production, which could influence smooth muscle cell proliferation. Oxidized LDL has been shown[11] to be a chemoattractant for smooth muscle cells and a stimulant of smooth muscle cell proliferation. It enhances *PDGF-AA* gene expression and PDGF receptor expression in vascular smooth muscle cell.[12] Probucol has a superoxide free radical scavenging effect and inhibits the production of PDGF, and also protects LDL from oxidation, which potentially reduces smooth muscle cell proliferation. In this study, tumor growth was significantly suppressed in probucol-treated mice compared with that in untreated mice. Probucol did not directly act on KB cells *in vitro*, but an antitumor effect was observed in tumor-bearing mice via an anti-angiogenic action, which might induce macroscopic ulceration and cellular apoptosis as a result of decreasing blood supply.

Oxidative stress is an important contributor to carcinogenesis.[30–35] Considering its lack of effect on the cell cycle *in vivo*, probucol should not influence KB cell viability or inhibit carcinogenesis in an advanced model. But as an antioxidant, probucol could scavenge ROS and free radicals by terminating biological reactions that generate them and/or preventing depletion of the antioxidant system[36] in an early-phase model. Additional studies are needed. The significant inhibitory effect of probucol on KB cell xenografts in mice suggests that its strong antioxidant activity may have an anti-angiogenic effect, leading to apoptosis *in vivo*. These results suggest that probucol could be a useful tumor-preventing agent for human head and neck squamous cell carcinoma.

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