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Effects of Antioxidants on the Gamma-Radiation Damage of the Cultured Vascular Smooth Mucle Cells of Rat Aorta

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To study the protective effects of antioxidants on the radiation damages of the cells, vascular smooth muscle cells (VSMC) from thoracic aorta of Sprague-Dawley rats were cultured and irradiated with gamma-ray. Cell viability was measured by direct cell counting and MTT assay, and flow cytometry was performed to measure fractional distributions of the cells. Gamma-ray irradiation inhibited cell proliferations accompanied with decreased G1 phase and increased S- and G2/M phases, and the maximum effects were observed at 1500 or 2000 cGy. Submaximal concentrations of antioxidants, such as allopurinol, vitamin C, N-acetylcycteine (NAC), lipoic acid, dihydrolipoic acid and rebamipide tended to increase the cell viability suppressed by low dose of radiation (500 cGy), and enalapril and vitamin E increased it significantly. Allopurinol, vitamin E, NAC, lipoic acid, captopril and enalapril significantly increased G1 phase. Allopurinol and vitamin E tended to increase c-Myc expression, detected by Western blot, that was reduced by the radiation, and enalapril increased it significantly. The cell viability and c-Myc expression were highly correlated (r=0.97) with each other. These results suggest that antioxidants, especially enalapril and vitamin E, recover the viability of VSMC from gamma-radiation injury, through a mechanism which includes increase of c-Myc protein expression.

Key Words: Antioxidant, Vascular smooth muscle cell, Proliferation, Cell cycle, Gamma ray, c-Myc

INTRODUCTION

Ionizing radiations produce reactive ions or radicals, resulting in damages of tissues, cells or nucleic acids. Breen & Murphy (1995) studied on the DNA damage by reactive oxygen-centered radicals, including hydroxyl radical, produced by γ-rays or X-rays. Tice & Strauss (1995) reviewed the single-strand DNA breaks and DNA: DNA cross linking at the level of individual cell exposed to ionizing radiation. There is at present no approved drug available for the prevention or treatment of acute radiation syndrome, neverthless, novel products that are shelf-stable for several years and provide prophylactic protection have been designed, and one promising investigational drug candidate for the indication of radioprotection, BIO 300, has a powerful antioxidant property (Zenk, 2007). Greenstock (1993) suggested that appropriate antioxidant intervention by inhibiting or reducing free radical toxicity may offer protection against radiation.

It is well known that vitamins C and E exert antioxidant effects. Vitamin C and a vitamin E analog decrease hydroxylation of guanosine in DNA, induced by ultraviolet B radiation (Stewart et al, 1996), vitamin C protects nucleated bone marrow cells against X irradiation (Harapanhalli et al, 1996), and vitamin E protects bone marrow of mice against gamma ray (Konopacka et al, 1998).

Lipoic acid and dihydrolipoic acid have powerful antioxidant properties (Scott et al, 1994; Packer et al, 1995). Lipoic acid is effective on protecting cultured human epithelial cells against gamma radiation-induced oxidative stress (Wan et al, 2006).

N-Acetylcysteine (NAC) has been suggested to act by increasing the concentration of cysteine, and hence glutathione, and by scavenging oxidant species (Aruoma et al, 1989; Cotgreave et al, 1991). It is actually effective in protecting rats from the damages caused by gamma-ray radiations (Sridharan & Shyamaladevi, 2002).

Faure et al (1990) suggested that allopurinol may have an alternative pathway of antioxidant action in biological systems, probably through scavenging hydroxyl radicals, Simko & Walker (1996) proved that administration of allopurinol as well as vitamin E and vitamin C were able to reduce reperfusion-induced injury in clinical trial, and Srivastava et al (2002) suggested that allopurinol had the ability to inhibit the radiation-induced increase of xanthine oxidoreductase activities that are major sources of free radicals.

Captopril, an angiotensin converting enzyme (ACE) in-

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ABBREVIATIONS: VSMC, vascular smooth muscle cell; NAC, n-acetylcystein; ACE, angiotensin converting enzyme; UV, ultra violet; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; DMSO, dimethylsulfoxide; Ab, antibody.

hibitor, was found to protect rat heart against ischemia-reperfusion injury, and the protection may involve an anti-free radical mechanism independent of its ACE inhibition property (Anderson et al, 1996). Hosseinimehr et al (2007) also observed that captopril, due to its free radical scavenging properties, protects mice bone marrow cells from gamma radiation-induced DNA damage and genotoxicity. On the other hand, another ACE inhibitor enalapril enhanced antioxidant defenses in mouse tissue without showing any free radical scavenger activity (de Cavanagh et al, 1997).

Rebamipide, a gastric mucosal protector against HCl-ethanol induced injury has been shown to exert antioxidant properties as hydroxyl radical scavenger (Kawano et al, 1991), and inhibit superoxide production in neutrophils (Yoshikawa et al, 1993). Antoku et al (1997) reported that rebamipide exhibits the protective effect on cultured mammalian cells by its hydroxyl radical scavenging capability against ionizing X-ray irradiation, and Sakurai et al (2004) also reported the scavenging activity of rebamipide against hydroxyl radical generated by UV.

During cell proliferation, the proto-oncogene c-myc encodes a transcription factor c-Myc, which is very important in controlling cell growth and vitality (Thompson, 1998). The c-myc gene is activated through several mechanisms in human cancers, and c-Myc protein plays an important role in cell cycle as a transcription factor (Dang, 1999).

In the present study, we investigated suppressive effect of gamma radiation on the survival and proliferation of cultured vascular smooth muscle cells and protective effects of the above mentioned various antioxidants against the ionizing radiation-induced injury.

METHODS

Culture and identification of vascular smooth muscle cells

Vascular smooth muscle cells (VSMC) were isolated from thoracic aorta of Sprague-Dawley rats. The primary culture cells were subcultured every 3 or 4 days.

To identify the VSMC, the cells embeded on a coverglass were fixed with para-formaldehyde. 0.5% H_2O_2/methanol was added to remove peroxidase, and 0.5% bovine serum was added to prevent any nonspecific reaction. Anti-smooth muscle myosine antibody was added as the primary Ab, then the cells were detained in the incubator of 37°C/100% humidity for 1 hour. Anti-rabbit IgG (Fab)2 peroxidase conjugate was added to the cells as the secondary Ab, and rabbit PAP complex as the tertiary Ab, and they were reacted for 1 hour. The cells were stained with the mixture of 3,3'-diaminobenzidine tetrahydrochloride and H_2O_2 in Tris buffer for 10 minutes and observed under microscope.

Irradiation and administration of antioxidants

Confluently cultured cells were detached, and 5×10^4 cells/ml were transferred to each wells of 6 well culture plates to incubate overnight. Culture media for the ebedded cells were replaced with a new DMEM medium containing 10% FBS, and then the experimental antioxidant drugs were administered. one hour later, the cells were irradiated with gamma ray with the C19 cobalt Teletherapy unit. Doses of gamma ray were 500, 1000, 1500, 2000 and 2500 cGy for different wells.

Measurement of proliferation and viability

To measure the proliferation, cell culture was treated with trypsin, and the suspension was mounted on hemocytometer to be manually counted under light microscope.

To measure the viability of cells, MTT assay was employed. Cells were washed with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4), detached with trypsin and diluted with culture medium. The cells were then centrifuged for 3 minutes at 1,000 rpm. The cells were diluted again with culture media; 200 μl of blank media and 180 μl of cell suspension containing 10,000 cells were transferred into each wells of 96 well culture plate. After equilibrium for one hour, the cells were treated with experimental drugs diluted in 20 μl of culture media. After 96 hours of incubation at 37°C, 50 μl aliquots of 1 mg/ml MTT solution was added to each well (0.1 mg/ml) and incubated for 4 hours. Supernatants were aspirated and the formazan crystals in each well were dissolved in 200 ml of dimethyl sulfoxide (DMSO). Absorbance at 570 nm was read on a Microplate Reader (Bio-Rad).

Cell cycle analysis

For cell cycle analysis, cells treated with trypsin were washed with PBS containing Ca^{++} (0.1 g/L) and 2% serum, fixed overnight in 95% ethanol at 4°C. Cells were washed again twice with PBS containing Ca^{++} (0.1 g/L) and 2% serum, then diluted in 250 μl of 1.12% sodium citrate buffer containing 50 μg/ml of RNase A. Cell suspension was mixed with 250 μl of 1.12% sodium citrate buffer containing 50 μg/ml of propidium iodide, incubated at 37°C for 20 minutes, and then stored at 4°C. Fluorescent labeling was evaluated using a FACsort (Becton Dickinson, SanJose, CA). Data shown are representative of three independent experiments.

Quantitation of c-Myc protein

Fully confluent cells in culture plates were washed with PBS, detached by spatula, collected in a test tube, and then sonicated with Vibra-Cell™ (Sonics & Materials, Inc. USA). Homogenates were centrifuged at 4°C and 12,000 g, and the supernatants were taken for protein assay (Protein Assay Kit, BIO-RAD, USA). The mixture containing 80 μl protein solution and same amount of twice diluted reagent buffer was loaded on the SDS-PAGE gel that was consisted of resolving and stacking gels. They were electrophoresized, and then transfered to nitrocellulose membrane (NC membrane, Protran, Scheicher & Schuell, Germany). To suppress any nonspecific reaction, the membrane was soaked in 5% non-fat dry milk for 1 hour, and then washed 3 times for 10 minutes each. The membrane was reacted with primary antibody solution, which was diluted in 0.1% BSA and 0.05% Tween 20 PBS, for 1 hour, and then washed 3 times for 10 minutes each. Anti-rabbit HRP (horse radish peroxidase) as the secondary antibody was diluted (in 0.1% BSA, 0.05% Tween 20 PBS) by the ratio of 1 : 4,000, in which the membrane was reacted for 1 hour and then washed 3 times for 10 minutes each. The membrane was reacted with the Renaissance Western Blot Chemiluminescence reagent (NEN life science products, USA) for 1 minute, and then was exposed to X-ray. Densities of c-Myc protein (67 kDa) bends were measured with NIH Image (v. 1.61) program

on Macintosh computer.

Materials

Allopurinol, N-acetylcysteine, Vitamin E, lipoic acid, dihydrolipoic cid, bovine serum albumin, HBSS, ribonuclease A and propidium iodide were purchased from SIGMA-Aldrich. Fetal bovine serum, antibiotics-antimycotics and trypsin-EDTA were obtained from GibcoBRL. Vitamin C was purchased from Hayashi Cemicals, and rebamipide was kindly donated by Otsuka Pharmaceuticals. c-Myc antibody was purchased from Santa Cruz Biotechnology. Protein Assay Kit, protein size marker, nitrocellulose membrane, filter paper, non fat dry milk, SDS, electrophoresis unit, electrotransfer unit and power supply were purchased from BIO-RAD.

Statistics

The correlation coefficient of the percentile data of cell viability and c-Myc expression of all the experimental groups relative to the radiation only group was calculated by StatWorks (v. 1.2) program on Macintosh computer.

Data were evaluated by Student's t-test (unpaired). Values were considered as significantly different from control when p-value was less than 0.05.

RESULTS

Effects of gamma irradiation on cell proliferation and cell cycle

Cell population of non-irradiated control was ($\times 10^4$) 41.9±4.2, and irradiation group with 500 cGy 30.2±1.3, 1000 cGy 14.7±0.7, 1,500 cGy 4.4±0.5, 2,000 cGy 5.0±0.2, and 2,500 cGy 5.5±0.3 (Fig. 1)

Fig. 2 shows typical DNA histograms of control (left) and irradiated (right) group, which indicated that gamma irradiatin reduced G1 phase and increases G2/M phase. The data of cell cycle changes are shown on Fig. 3.

G1 phase of non-irradiation group (control) was 86.1± 0.6%. The irradiation group of 500 cGy was 76.0±3.3%, 1,000 cGy was 68.6±1.6%, 1,500 cGy was 60.8±2.8%, 2,000 cGy was 51.0±0.7%, and 2,500 cGy was 53.8±1.3%. All the data of irradiation group were significantly lower than control ($p<0.05$).

S phase of the control was 5.9±0.7%. The irradiation group of 500 cGy was 9.4±0.6%, 1,000 cGy was 17.0±1.9%, 1,500 cGy was 16.5±1.1%, 2,000 cGy was 13.0±0.3%, and 2,500 cGy was 13.9±0.9%. All the data of irradiation group were significantly higher than the control ($p<0.05$).

G2/M phase of the control was 7.7±0.2%. The irradiation group of 500 cGy tended to increase the G2/M phase, but not significantly, One thousand cGy was 10.5±0.7%, 1,500 cGy was 10.7±1.1%, 2,000 cGy was 20.9±1.6%, and 2,500 cGy was 18.1±1.7%. All the data of irradiation group, except 500 cGy, were significantly higher than the control ($p<0.05$, Fig. 3).

Effects of antioxidants on the viability of gamma-irradiated cells

Cell viability of 500 cGy irradiation group, determined by MTT assay, was about 74% of non-irradiation group, and this data were almost identical with the data acquired by manual counting of living cells by hemocytometer, 75%.

In the present study, we assumed the viability of 500 cGy

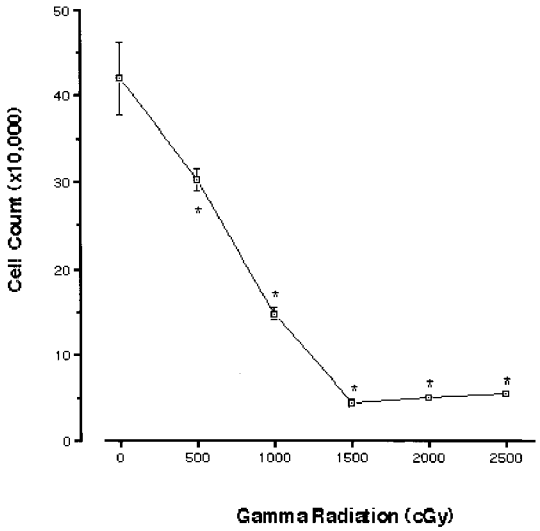

Fig. 1. Dose-Response to gamma radiation of cultured vascular smooth muscle cells isolated from rat aorta. Values express Mean±SE (n=7). *$p<0.05$: significantly different from control.

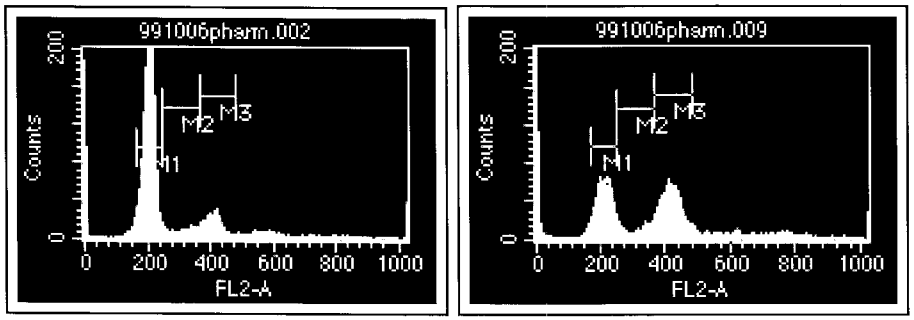

Fig. 2. Typical presentations of flowcytometric analysis of cell cycle. Left panel represents non-radiated vascular smooth muscle cell, right panel irradiated (1000 cGy) vascular smooth muscle cells. M1: G1 phase, M2: S phase, M3: G2/M phase.

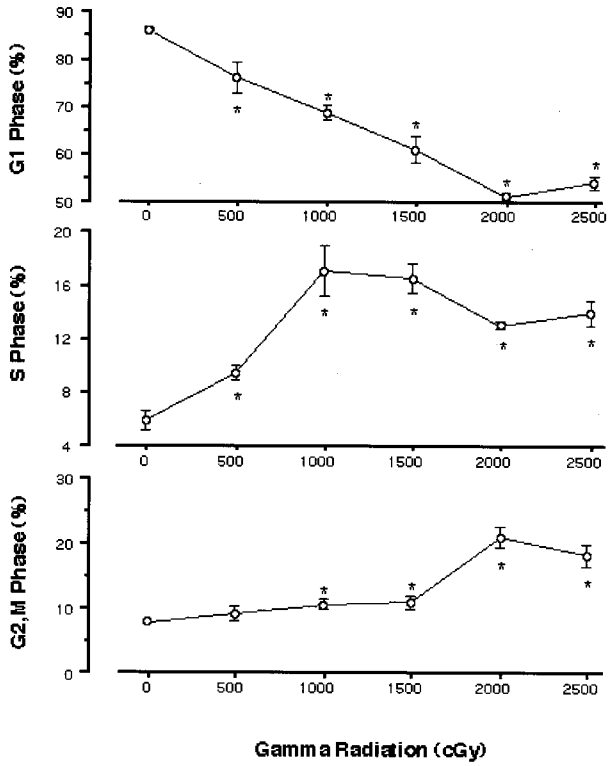

Fig. 3. Effect of gamma radiation on the cell cycle of cultured vascular smooth muscle cells isolated from rat aorta. Values express Mean±SE (n=7). *$p<0.05$: significantly different from control.

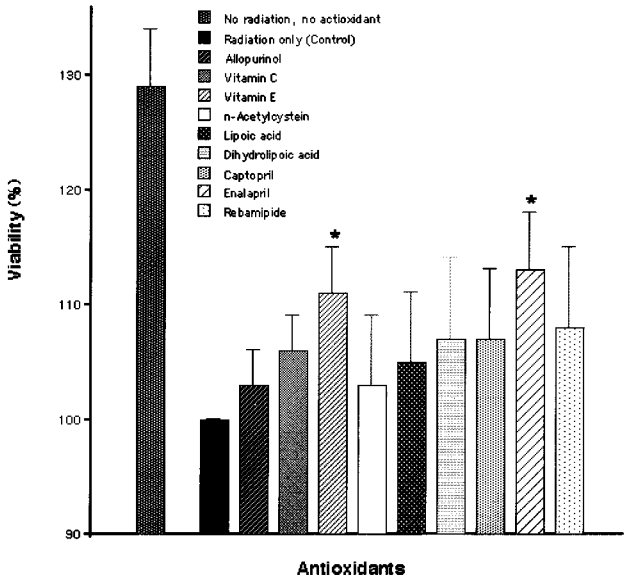

Fig. 4. Effects of antioxidants on the viability of gamma-irradiated (500 cGy) cultured vascular smooth muscle cells isolated from rat aorta. Drug concentrations: Allopurinol, vitamin C, n-acetylcysteine, lipoic acid, dihydrlipoic acid and rebamipide at 10^{-6} M, Vitamine E at 10^{1} IU. Values express Mean±SE (n=9). *$p<0.05$: significantly different from control.

irradiation group as 100% (control), and the viabilities induced by antioxidants were compared to it. The viability of vitamin E and enalapril groups were significantly higher than control (111±4% and 113±5%, respectively ($p<0.05$)). The other antioxidants showed slightly increasing tendencies of viability, but did not show any statistically significant improvement (allopurinol, 103±3%; vitamin C, 106±3%; n-acetylcysteine, 103±7%; lipoic acid, 105±6%; dihydrolipoic acid, 107±7%; captopril, 107±6%; and rebamipide, 108±7%) (Fig. 4).

Effects of antioxidants on proliferation and cycles of irradiated cells

With a high dose of radiation, 1,000 cGy, non-antioxidant group proliferated only 19.6±2.7% relative to non-irradiated control (100%). Allopurinol, vitamin C, n-acetylcysteine, lipoic acid, dihydrolipoic acid and rebamipide were administered at 10^{-11} M to 10^{-7} M concentrations, and vitamin E was administered at 10^{-4} IU to 10^{0} IU concentrations. All the antioxidant drugs in this experiment showed cell proliferations, ranging from 13.4±2.9% to 25.3±7.6%, indicating that they are not significantly different from that of radiation-only group (Data not shown).

Cell cycles of 1000 cGy group were as follows: 60.6±1.3% was in G1 phase, 8.0±1.1% was in S phase, and 28.9±1.7% was in G2/M phase. 10^{-4} IU of vitamin E showed G1 populations of 57.3±1.2% and 10^{-4} M dihydrolipoic acid showed 62.2±2.9%. These data indicated that antioxidants generally tended to increase (non-significant) the cell population in G1 phase. As for S phase, all the antioxidants showed various populations, ranging from 7.1±1.0% to 9.9±2.0%, which were not significantly different from irradiation-only control group. G2/M population tended to be slightly reduced by antioxidants, showing 31.2±1.3% by 10^{-10} M n-acetylcycteine and 25.2±3.3% by 10^{-7} M lipoic acid. They were not significantly (Data not shown).

With low dose of gamma ray, 500 cGy, higher dose of antioxidants were tested. Allopurinol, vitamin C, n-acetylcysteine, lipoic acid, dihydrlipoic acid and rebamipide were administered by the concentration of 10-6 M, whereas 10^{1} IU vitamin E was administered. Radiation-only control group showed 83.13±0.24% of G1, 6.40±0.38% of S, and 10.15±0.36% of G2/M phase. Allopurinol increased G1 to 84.22±0.28% ($p<0.05$) and decreased S to 5.20±0.27% ($p<0.05$); vitamin E increased G1 to 84.78±0.36% ($p<0.05$) and decreased S to 5.06±0.33% ($p<0.05$); lipoic acid increased G1 to 84.35±0.35% ($p<0.05$) and decreased S to 5.16±0.31% ($p<0.05$); n-Acetylcystein, captopril and enalapril significantly ($p<0.05$) increased G1 to 84.57±0.28%, 84.44±0.33% and 84.33±0.35%, respectively, and these drugs showed decreasing tendencies of G2/M population (Fig. 5).

Effects of antioxidants on the c-Myc protein expression of gamma-irradiated cells

Cells irradiated with 500 cGy of gamma ray expressed about 83% of c-Myc protein compared to non-irradiated cells. For convenience, we assumed the c-Myc expresson of 500 cGy irradiation group as 100% (control). In this experiment, only enalapril significantly increased c-Myc expression [146±17% ($p<0.05$)]. Lipoic acid, captopril, vitamin E, and allopurinol tended to increase the c-Myc expression (108±9%, 113±16% 138±18% and 136±19%, re-

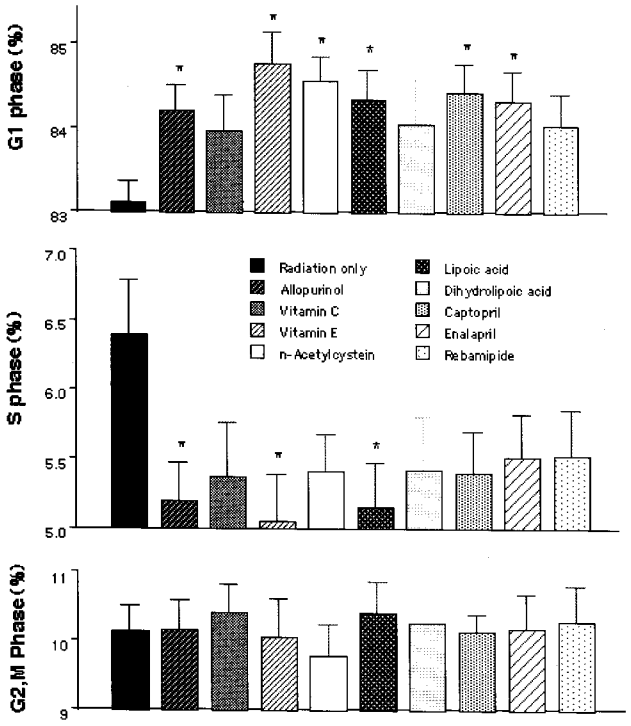

Fig. 5. Effects of antioxidants on the cell cycle of gamma-irradiated (500 cGy) cultured vascular smooth muscle cells isolated from rat aorta. All the antioxidants, except vitamin E (10^1 IU), were added at 10^{-6} M. Values express Mean±SE (n=6). *$p<0.05$: significantly different from control.

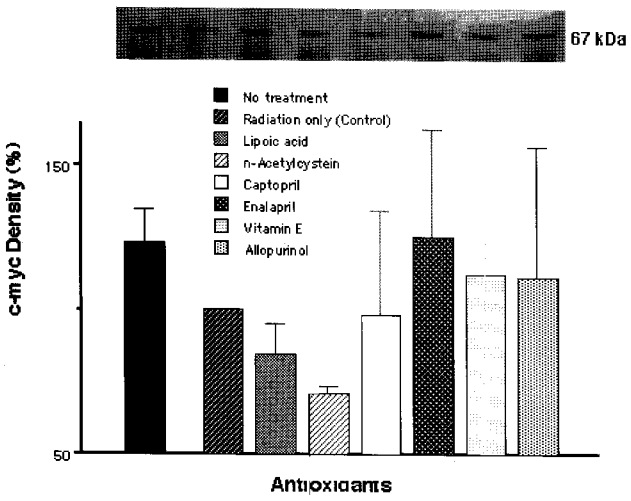

Fig. 6. Effects of antioxidants on the c-Myc protein expression of gamma-irradiated cultured vascular smooth muscle cells isolated from rat aorta. All the antioxidants, except vitamin E (10^1 IU), were added at 10^{-6} M concentration. Values express Mean±SE (n=7). *$p<0.05$: significantly different from control.

spectively). However, they were not statistically significant. In contrast, n-acetylcysteine tended to decrease the c-Myc expression (90±15%) but not significantly (Fig. 6).

The c-Myc expression in gamma irradiated cells was highly correlated to the viability of the cells (Fig. 7).

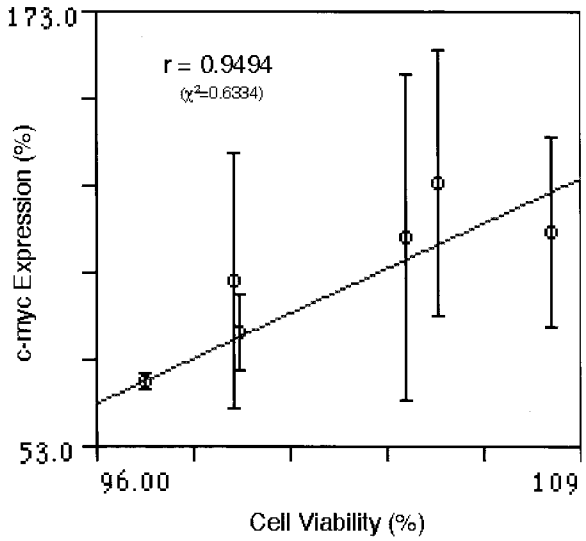

Fig. 7. Correlation between the cell viability and the expression of c-Myc protein in the antioxidants-pretreated gamma-irradiated vascular smooth muscle cells isolated from rat aorta. Data of the experimental groups, relative to the radiation only group, were percentile. Correlation coefficient was calculated by StatWorks (v. 1.2) program on Macintosh computer.

DISCUSSION

Ionizing radiation induces DNA damages, resulting in impairments of survival and proliferation of cells. Maity et al (1994) reviewed that the exposure of a wide variety of cells to ionizing (X- or gamma-) irradiation results in division delay which may have several components including G1 block, G2 arrest or S phase delay.

In this study, we examined viability and proliferation of gamma-irradiated VSMCs and the effects of several antioxidants on the radiation-induced impairments of cell growth.

In VSMCs isolated from rat aorta, 500 cGy gamma ray significantly inhibited the proliferation, and 1,500 cGy showed maximal inhibitory effect. We emplyed 1,000 cGy as submaximal high dose and 500 cGy as low dose.

The proliferation of 1,000 cGy group was severely suppressed, and none of the antioxidants even at high concentrations could improve the cell growth. Significant decrease in G1 phase or significant increases in S phase and G2/M phase could not be altered by any of the antioxidants used in this study. Because 1,000 cGy of gamma ray might be too high for medium concentration of antioxidants to reverse the damage, we tried relatively lower dose (500 cGy) of gamma ray and higher concentrations of antioxidants.

In spite of some controversy of its specificity or accuracy, MTT tetrazolium salt colorimetric assay, previously described by Mosmann (1983), has widely been used to measure cytotoxicity, proliferation or activation (Hayon et al, 2003; Berridge et al, 2005). The data obtained by MTT in this study were very consistent with the data acquired by manual counting of living cells by hemocytometer.

The antioxidants, vitamin E and enalapril, increased the

viabilities of irradiated (500 cGy) cells. Vitamin E protected the retina from light injury (Aonuma, 1997), ameliorated the DNA damaging effects of UVA (Lehmann et al, 1998) and protected the rat salivary gland from gamma-irradiation dysfunction (Ramos et al, 2006). In the present study, vitamin E recovered the reduced G1 phase by gamma-irradiation, and significantly decreased the enlarged population of S phase. Such recoveries of cell cycle distribution and the increment of viability indicate that vitamin E has a protective activity against gamma-irradiation damages.

Enalapril showed similar activities on cell cycle and viability as vitamin E. Le Guen et al (1992) observed that enalapril had weaker free radical scavenging effect than captopril. On the other hand, Benzie & Tomlinson (1998) studied the antioxidant effect of captopril, enalapril, fosinopril, perindopril, quinapril and ramipril, and observed that only captopril showed significant antioxidant capability. In the present study, captopril significantly recovered the G1 phase, but tended to recover S phase and increase viability (statistically not significant) at the concentration in which enalapril significantly recovered the G1 phase and viability.

c-Myc is a well known proto-oncogen which was originally known to be expressed in various tumors (Hayward et al, 1981; Zimmerman & Alt, 1990; Fearon & Dang, 1999; Kim et al, 2007). However, Rupnow et al (1998) suggested that the c-Myc transcription factor is involved in the regulation of cellular proliferation and differentiation, Thompson suggested that the proto-oncogene c-myc encodes a transcription factor c-Myc, which plays an important role in controlling cell growth and vitality, and Liu et al (2006) reported that c-Myc plays a critical role in hypoxia-induced proliferation and survival of astrocytes by overcoming injury caused by oxidative stress. In the present study, gamma-irradiation suppressed the expression of c-Myc protein, and some antioxidants tended to restore the production of the protein. Such a recovery was closely related to the improvement of viability of the irradiated cells.

These results suggest that gamma-irradiation suppresses the proliferation of cultured vascular smooth muscle cells isolated from rat aorta. The growth inhibition could be prevented or recovered by high concentration of vitamin E or enalapril. Finally the mechanism of recovery of viability might involve the increase of c-Myc protein expression.

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