[Environmental Health and Preventive Medicine 12, 251–257, November 2007]

Original Article

Effects of Methanolic Extracts from Broad Beans on Cellular Growth and Antioxidant Enzyme Activity

Mizue OKADA[1] and Yoshinori OKADA[2]

[1]*Nutrition Section, Yms Laboratory, Gifu, Japan*
[2]*Laboratory on Ageing and Health Management, Aichi Prefectural College of Nursing and Health, Nagoya, Japan*

Abstract

Objective: There are several reports of cellular-aging-dependent alterations in the antioxidant capacity of human fibroblasts. Fibroblasts show slower the growth rate at late passages (referred to hereafter as old cells) than at early passages (referred to hereafter as young cells). Antioxidants may control cellular growth by modulating reactive oxygen species (ROS). Methanolic extracts from broad beans (MEBB) contain phenolic compounds and have ROS-scavenging activities. In this study, we investigated the effects of MEBB on cellular growth and antioxidant levels in normal human lung fibroblasts.

Methods: To determine cytosolic superoxide dismutase (SOD) activities, cytosolic glutathione peroxidase (GSH-Px) activities, catalase activities, reduced glutathione (GSH) concentrations, and growth rate, MEBB treatments were performed on young and old cells.

Results: In young and old cells treated with 120 μg/ml MEBB, the growth rates increased by 28.1 and 15.2%, respectively, compared with controls. The MEBB treatment of young cells caused a 62.5% increase in SOD activity, but the treatment of old cells caused a 39.5% decrease. The catalase activities of the young and old cells treated with MEBB were equal to those of control cells. Young and old cells treated with MEBB were equal to the control cells in terms of GSH-Px activity. The GSH concentrations in the young and old cells treated with 120 μg/ml MEBB increased by 22.1 and 45.9%, respectively.

Conclusion: These studies elucidated a new cellular growth mechanism whereby human lung fibroblasts modulate intracellular GSH levels via the action of MEBB.

Key words: cellular growth, broad beans, methanolic extracts, antioxidant enzymes, reduced glutathione

Introduction

Normal human diploid fibroblasts have been used as a model to study cellular senescence (1, 2). The *in vitro* aging of these cells is characterized by a gradual and progressive decrease in growth rates (2).

There is considerable evidence that antioxidants, which include phenolic compounds, contribute to the induction of c-fos and c-jun proto-oncogenes (3–5). The production of the c-fos gene is also known to play a critical role in cell cycle progression in human fibroblasts (6). Some investigators (7, 8) reported that reactive oxygen species (ROS) induce cell proliferation at low doses. It was also shown that ROS affects the human c-fos promoter. The importance of ROS modulation in cell culture experiments is concluded from the fact that the alteration of ROS concentration modulates cellular signaling (9, 10), and/or cellular growth (11). Laurent et al. (12) indicated that antioxidant molecules, such as superoxide dismutase (SOD) mimics, increase hydrogen peroxide (H_2O_2) levels through superoxide anion dismutation, as well as *in vitro* proliferation of normal cells. Antioxidants may control cellular growth by modulating ROS concentration.

We have previously reported that methanolic extracts from broad beans (*Vicia faba*) contain phenolic compounds as active ingredients, and they scavenge free radicals such as superoxide anions and H_2O_2 (13). Moreover, methanolic extracts from broad beans (MEBB) have antioxidant activities similar to other phenolic compounds (13).

Received Jun. 5, 2007/Accepted Sep. 18, 2007
Reprint requests to: Yoshinori OKADA
Laboratory on Ageing and Health Management, Aichi Prefectural College of Nursing and Health, Tohgoku, Kamishidami, Moriyama, Nagoya 463-8502, Japan
TEL: +81(52)736-1401, FAX: +81(52)736-1415
E-mail: yolage@aichi-nurs.ac.jp

There are several reports of cellular-aging-dependent alterations in the antioxidant capacity of human diploid fibroblasts, including the activities of SOD (14), catalase (15), glutathione peroxidase (GSH-Px) (16), and the concentration of reduced glutathione (GSH) (14, 16).

Therefore, we examined whether the cultivation of human diploid fibroblasts in media supplemented with MEBB can affect cellular growth. Moreover, to unravel the mechanisms of cellular growth mediated by MEBB containing phenolic compounds, we examined the effects of MEBB on the levels of antioxidants such as SOD, catalase, and GSH-Px, and the GSH concentration. We observed elevated levels of GSH in cells at early passages (referred to hereafter as young cells) and at late passages (referred to hereafter as old cells). These studies are the first to show the effects of MEBB on the growth of human fibroblasts, and our results suggest that increases in GSH concentrations may stimulate cellular growth. The MEBB treatment may be related to the delay in cellular-aging-dependent degenerative phenomena.

Materials and Methods

MEBB preparation

MEBB (13) was prepared as follows. Briefly, broad beans were hand-shelled and then stored at −20°C. Frozen broad beans (100 g) were minced into 5-mm fragments and extracts were obtained with methanol (300 ml) overnight at room temperature. The extracts were filtered; the filtrates were evaporated to dryness in a vacuum slightly below 40°C using a rotary evaporator. Dimethyl sulfoxide (DMSO) was used to dilute MEBB. Ten microliters of DMSO (0.02% final concentration) was added to the control sample. MEBB was decolorized using cartridges (Sep-Pak C_{18}, Waters) to avoid interference in spectrometrical analysis. The content of total phenolic compounds in MEBB was 2.13 mg/g of broad beans. Also, the content of total phenolic compounds in MEBB was 0.70 mg/ml of methanol-extracted solution.

Cell culture

TIG-1-20 (human lung fibroblasts; Population Doubling Levels (PDL) 20; JCRB0501; referred to hereafter as young) cells and TIG-1-50 (human lung fibroblasts; PDL 50; JCRB0504; referred to hereafter as old) cells were obtained from the Tokyo Metropolitan Institute of Gerontology. Human lung fibroblasts were cultured by methods previously described (17). Briefly, the cells were cultured in an atmosphere of air containing 5% CO_2 at 37°C in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (FBS; Flow Laboratories), 28 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes; Sigma Chemical) and 1.5 μg/ml Fungizone (Sigma Chemical) (referred to hereafter as MFHF medium). Subculture at 1×10^5 cells/dish was performed at about 1 week intervals for young cells (PDL 20, 25% of maximum life span), or at about 10 day intervals for the old cells (PDL 50, 62.5% of maximum life span). We investigated the growth properties of human lung fibroblasts. TIG-1-20 exhibited a doubling time of about 30 hours compared to about 40 hours for the TIG-1-50. Similarly, the TIG-1-50 ceased proliferating after about 30 cumulative population doublings (30 PDL). The treatment with MEBB was performed during the cellular aging from PDL 20 to PDL 39 (48.75% of maximum life span) in young cells, and from PDL 50 to PDL 61 (76.25% of maximum life span) in old cells. To determine cellular growth, the cell numbers were counted using a hemocytometer. Cellular growth was also assessed by the number of cells cultured for 72 hours, as shown in Fig. 1. MEBB concentrations of 120 and 200 μg/ml (final concentration) were chosen for the supplementation experiments because 120 μg/ml MEBB caused a maximum increase in cellular growth as determined by a concentration-dependent pilot experiment using MEBB concentrations between 0 and 520 μg/ml (Fig. 1).

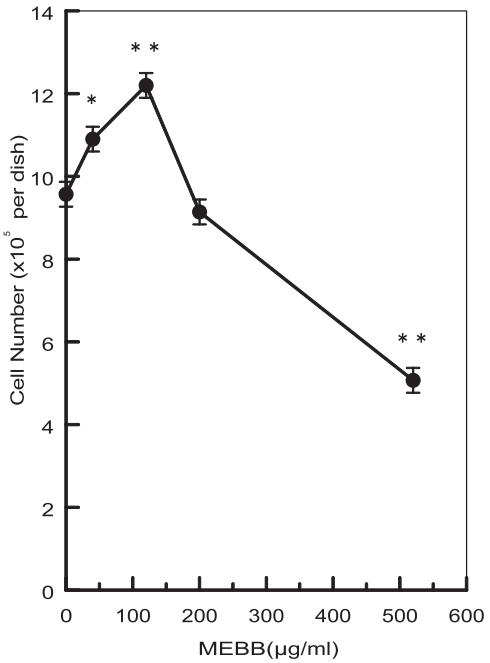

Fig. 1 Effect of MEBB on growth of human lung fibroblasts. TIG-1 cells were seeded into 60-mm culture dishes at 1×10^5 cells/dish in the culture medium containing MEBB at concentrations between 0 and 520 μg/ml. The cell number was assessed after 3 days. Each end of the vertical bar represents the standard error of multiple (4) samples. * $p<0.05$ ** $p<0.01$ compared with control.

Effect of MEBB on antioxidant enzyme activities and GSH concentrations

To determine the effects of MEBB on cytosolic antioxidant enzyme activities, the treated cells were prepared according to a modification of the method of Rikans et al. (18). Briefly, the effect of MEBB on cytosolic antioxidant enzymes was examined using TIG-1 cells. The cells were cultured in MFHF medium supplemented with MEBB. After treatment culture, the medium was rapidly aspirated to prepare the cell homogenates for enzymatic analysis. The cells were washed with Hanks' buffered saline (pH 7.4) and then removed from the dishes by scraping, placed on ice and subsequently homogenized with 5 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA at 4°C. The homogenates were centrifuged at 1,000×g for 10 min and the supernatant fractions were centrifuged at 10,000×g for 20 min at 4°C. Post-mitochondrial supernatant fractions were used for the determination of cytosolic antioxi-

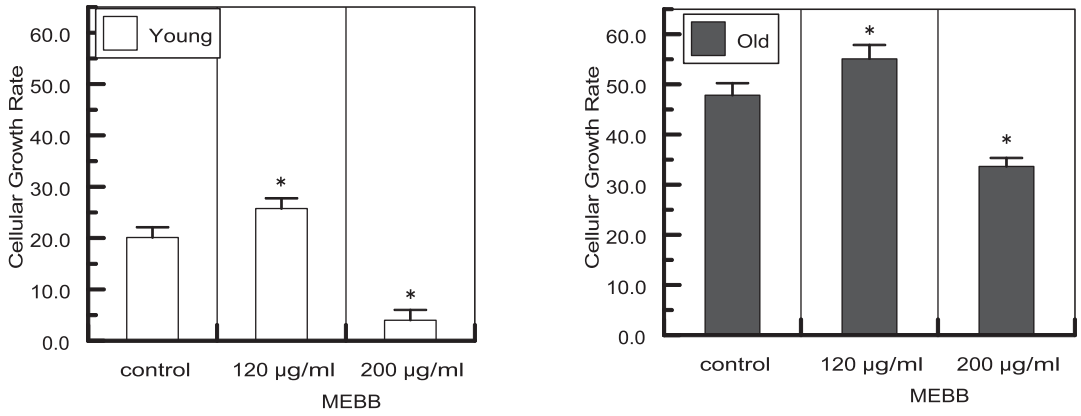

Fig. 2 Effects of MEBB on growth of young and old human lung fibroblasts. Young (early passages, PDL 20, 25% of maximum life span) and old (late passages, PDL 50, 62.5% of maximum life span) TIG-1 cells were seeded into 60-mm culture dishes at 1×10^5 cells/dish in the culture medium containing MEBB at concentrations of 120 and 200 μg/ml. The growth rate of young cells was measured after 7 days, and that of old cells was measured after 10 days. The growth rates obtained after cell culture were calculated as follows: number of cells obtained upon subculture divided by the number of cells inoculated. Each end of the vertical bar represents the standard error of multiple (4) samples. * $p<0.05$ compared with control.

dant enzymes. To determine the effects of MEBB on catalase activities and GSH concentrations, the treated cells were prepared according to a modification of the method of Leist et al. (19).

Biochemical analysis

All enzyme assays were carried out on freshly prepared cell protein extracts. The cytosolic SOD activity was measured by the method of Crapo et al. (20) using xanthine-xanthine oxidase. The cytosolic GSH-Px activity was assayed according to the procedure of Tappel (21). The catalase activity was determined by direct measurement of the decrease in absorption at 250 nm caused by the decomposition of H_2O_2 by catalase (22). The GSH concentration was determined in cell homogenates using a modification of the cycling method of Tietze (23). The protein content was measured by the method of Bradford (24) using bovine serum albumin as the standard.

Statistics

Data are expressed as means with standard deviations. Means were assessed for significance by ANOVA. p-Values less than 0.05 were considered significant.

Results

To determine the sensitivity of human lung fibroblasts to MEBB toxicity, we incubated human fibroblasts with MEBB at concentrations ranging from 0 to 520 μg/ml (Fig. 1). After 72 hours, a significant increase in cellular growth was observed at MEBB concentrations below 120 μg/ml (Fig. 1). At high concentrations, 200 to 520 μg/ml, MEBB decreased cellular growth in a dose-dependent manner from 10 to 53% (Fig. 1). Surprisingly, we observed a significant maximum peak in the dose-response curve when the incubation concentration was increased from 0 to 520 μg/ml. In other words, the maximum peak in cellular growth was observed at 120 μg/ml MEBB. The onset of fibroblast injury was observed at MEBB concentrations as high as 200 μg/ml (Fig. 1). Namely, cellular growth did not decrease in a classic dose-dependent manner. Although cellular growth decreased to 10% with 200 μg/ml MEBB, it was consistently higher in the presence of 120 μg/ml (28%) and decreased only after the MEBB concentration was increased to 200 μg/ml. Our data indicate that MEBB induces both cell injury and cell growth pathways in human fibroblasts.

Figure 2 shows the effect of MEBB on the growth of young and old cells. The growth rate of young cells was measured after 7 days, while that of old cells was measured after 10 days. When 120 μg/ml MEBB was added to the young and old cells, statistically significant stimulations were observed ($p<0.05$). The growth of young cells incubated with 120 μg/ml MEBB increased by 28.1% compared with that of the control. On the other hand, the increase in growth of old cells was 15.2%.

The cytosolic SOD activity of young cells treated with 120 μg/ml MEBB increased by 62.5% compared with that of the control, but that of young cells treated with 200 μg/ml MEBB was similar to that of the control. On the other hand, the SOD activities of the old cells treated with MEBB at 120 and 200 μg/ml decreased by 39.5% compared with that of the control (Fig. 3). The catalase activities of the young and old cells treated with 120 μg/ml MEBB were virtually equal to that of the control, but that of the young cells treated with 200 μg/ml MEBB increased by 135% compared with that of the control (Fig. 4). On the other hand, the catalase activities of the old cells treated with 200 μg/ml MEBB were virtually equal to that of the control (Fig. 4). The cytosolic GSH-Px activity of young cells treated with 200 μg/ml MEBB increased by 84.2% compared with that of the control (Fig. 5), but those of young and old cells treated with 120 μg/ml MEBB were essentially identical to that of the control (Fig. 5).

The GSH concentration in young cells treated with 120 μg/ml MEBB increased by 22.1% compared with that in the control, but that in the young cells treated with 200 μg/ml MEBB decreased by 25.2% compared with that in the control (Fig. 6). Similarly, the GSH concentration in old cells treated with MEBB at 120 μg/ml increased by 45.9% compared with that in the control, whereas that in old cells treated with 200 μg/ml MEBB was virtually equal to that in the control (Fig. 6).

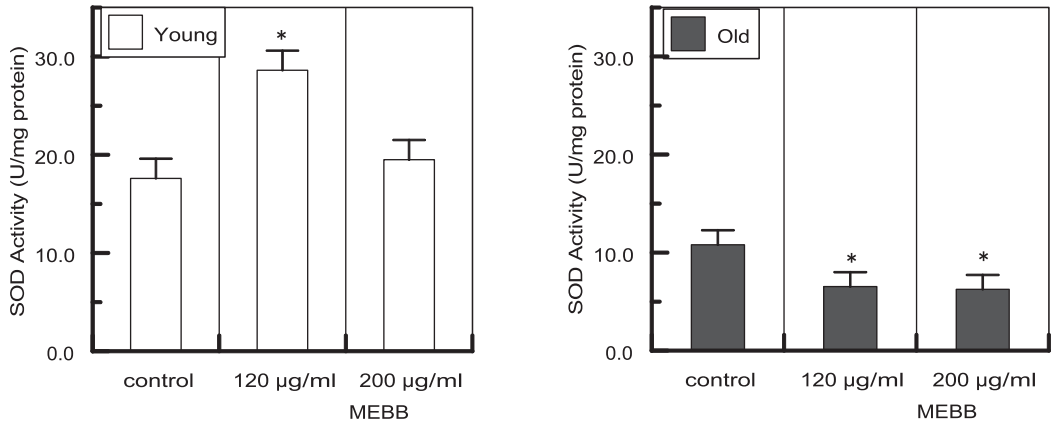

Fig. 3 Effects of MEBB on cytosolic SOD activities of young (early passages, PDL 20, 25% of maximum life span) and old (late passages, PDL 50, 62.5% of maximum life span) TIG-1 cells. The enzyme activities of young and old cells are indicated by unshaded and shaded columns, respectively. Data are expressed as mean±SD of three replicate analyses. N=4 for all treatments. * $p<0.05$ compared with controls.

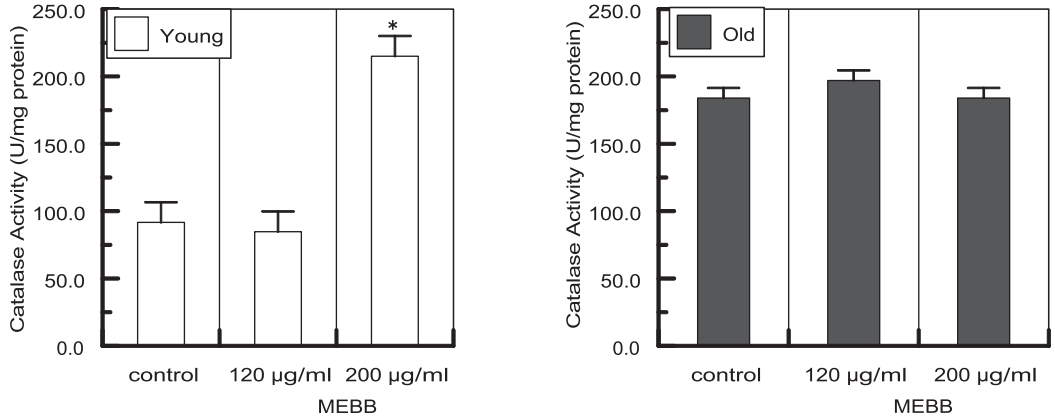

Fig. 4 Effects of MEBB on catalase activities of young (early passages, PDL 20, 25% of maximum life span) and old (late passages, PDL 50, 62.5% of maximum life span) TIG-1 cells. The enzyme activities of young and old cells are indicated by unshaded and shaded columns, respectively. Data are expressed as mean±SD of three replicate analyses. N=4 for all treatments. * $p<0.05$ compared with controls.

Discussion

Many biological functions of antioxidants including phenolic compounds have been studied (3–5). Concerning the proliferation assays, a stimulation effect was observed for labdane diterpene solidagenone compounds on MRC-5 fibroblasts (25). Also, Paduch et al. (26) showed that the extract of *Lamii albi flos* can stimulate the proliferation of human skin fibroblasts. However, there have been relatively few reports on the action of antioxidants on the growth of normal culture cells (27, 28) and on the relationship between cellular growth and phenolic compounds (13). MEBB contains a high amount of phenolic compounds (2.13 mg/g of broad beans) (13). In these studies, we showed that MEBB increased the growth of TIG-1 cells. The growth mechanism of normal lung fibroblasts induced by phenolic compounds is not clear. Antioxidant-responsive transcription factor (AP-1) is important for converting growth factor signals into proliferative responses (29). The phenolic compounds may increase cellular growth by activating AP-1 via the induction of c-fos expression (4) or by modulating free radical concentrations via the action of ROS scavenging (8). ROS have emerged as important signaling molecules in the regulation of various cellular processes. Kim et al. (30) investigated the effect of ROS on Chinese hamster lung fibroblast proliferation. Treatment with H_2O_2 and superoxide anions increased cell proliferation. Moreover, ROS such as H_2O_2 stimulate c-fos, c-jun and c-myc (31). Therefore, an investigation of the antioxidant ability of MEBB in culture cells would be helpful in understanding the mechanism of cellular growth function of phenolic compounds.

We examined the effect of MEBB on the antioxidant activities of the TIG-1 cells of different culture ages. Our results showed that the treatment of young cells with 120 μg/ml MEBB caused increases in the cytosolic SOD activities, but the treatment of old cells with MEBB caused decreases in the activities (Fig. 3). Kong and Fanburg (32) suggested that the extent of cell proliferation did not affect the level of cytosolic SOD (Cu, Zn-SOD). On the other hand, Laurent et al. (12) showed that antioxidant molecules, such as SOD mimics, increase H_2O_2 levels through superoxide anion dismutation, as well as *in vitro* proliferation of normal cells. Because 120 μg/ml MEBB was shown to increase the growth of young cells, the cytosolic SOD seems related to cellular growth.

Catalase is an important enzyme involved in cellular

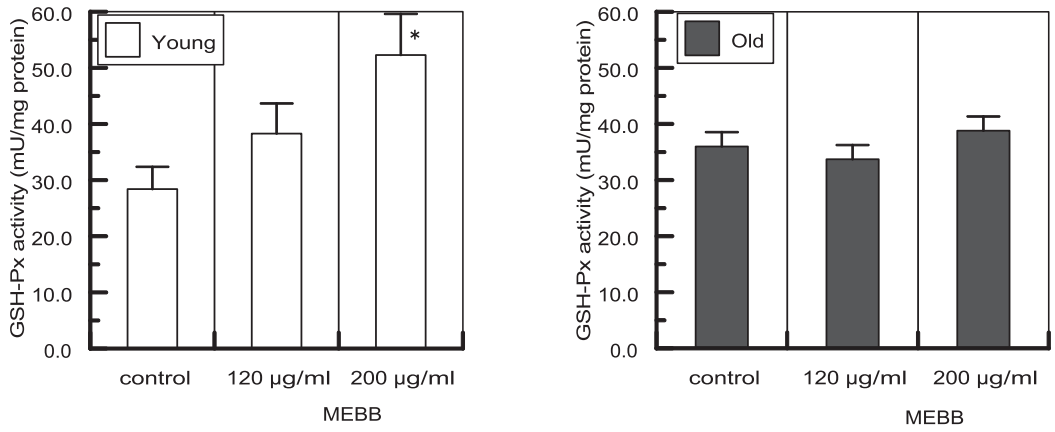

Fig. 5 Effects of MEBB on cytosolic GSH-Px activities of young (early passages, PDL 20, 25% of maximum life span) and old (late passages, PDL 50, 62.5% of maximum life span) TIG-1 cells. The enzyme activities of young and old cells are indicated by unshaded and shaded columns, respectively. Data are expressed as mean±SD of three replicate analyses. N=4 for all treatments. * $p<0.05$ compared with controls.

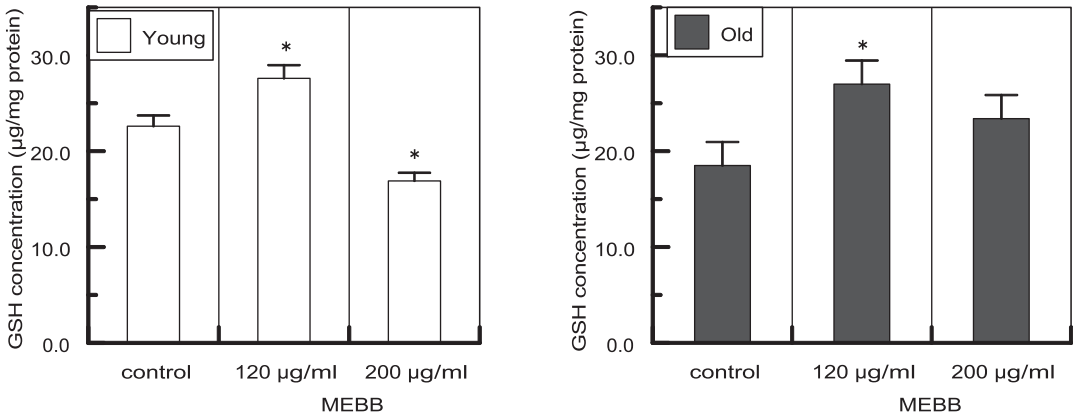

Fig. 6 Effects of MEBB on reduced glutathione (GSH) concentrations in young (early passages, PDL 20, 25% of maximum life span) and old (late passages, PDL 50, 62.5% of maximum life span) TIG-1 cells. The enzyme activities in young and old cells are indicated by unshaded and shaded columns, respectively. Data are expressed as mean±SD of three replicate analyses. N=4 for all treatments. * $p<0.05$ compared with controls.

defense against oxygen-mediated cytotoxicity. The application of MEBB (120 μg/ml) to the TIG-1 cells could not increase the levels of catalase in the young and old cells (Fig. 4). Kim et al. (30) reported that treatment with exogenous catalase suppresses normal cell proliferation. Because the catalase activity of the young cells treated with 200 μg/ml MEBB increased compared with that of the control (Fig. 4), the increased activity might decrease cell proliferation. These results show that the catalase activity seems unrelated to the increase in cellular growth induced by MEBB.

Matsumura et al. (33) indicated that the phenolic compound found in tea, epigallocatechin-3-O-gallate (EGCg), is known to have antioxidant activities, and EGCg also has pro-oxidative activities such as H_2O_2 generation. H_2O_2 was generated from EGCg at concentrations of more than 300 μg/ml. In addition, when L929 cells were treated with 300 μg/ml EGCg and 1 μg/ml catalase, EGCg inhibited cellular growth, and cell proliferation was restarted after EGCg removal. Therefore, our results were similar to theirs in terms of EGCg and catalase activities.

The cytosolic GSH-Px activities of the young and old cells treated with MEBB were equal to those of the controls, whereas those of the young cells treated with 200 μg/ml MEBB were higher than in those of the control cells (Fig. 5). In the control, the GSH-Px activities were higher in old than in young cells. This shows that increases in the activities of GSH-Px were observed during *in vitro* cellular aging at late passage, as described by Kaneko et al. (34). In the case of the young cells, the treatment with 200 μg/ml MEBB increased the GSH-Px activities and inhibited the cell proliferation. Therefore, our data suggested that GSH-Px might play a role against cellular growth inhibition. However, because MEBB increases the growth of both young and old cells, it seems that the GSH-Px activity is not involved in the regulation of cell proliferation.

Decreased total GSH concentration has been associated with both disease states and proliferative senescence (35). The ability of cells to maintain GSH levels has also been suggested to play a role in determining *in vitro* life span (36). In this study, the control obtained from old cells exhibited a lower GSH concentration than the control from young cells (Fig. 6). The fact that GSH declined with culture age may, in part, reflect culture-age-related differences in the responsiveness of cells to serum factors that affect GSH concentration. Yuan et al. (14) indicated that human diploid cells are more susceptible to

oxidative stress at late passages than at early passages, presumably because of decreases in cellular-GSH concentration. More, they suggested that the primary defense against oxidative stress appear to be GSH. Also, Laurent et al. (12) indicated that H_2O_2, controlled by the glutathione system, is pivotal for the modulation of normal cell proliferation. As shown in Fig. 6, the GSH concentrations of young and old cells were increased by 120 μg/ml MEBB. MEBB increased the cellular growth and simultaneously raised GSH levels. The increased GSH concentrations may lead to decreases in H_2O_2 concentrations. Because MEBB increases GSH concentration in the TIG-1 cells, the alterations in oxidative stress parameters elucidated here might reflect internal cellular changes in oxidant defense.

Results of this study have shown that MEBB can increase the growth of young and old cells. Despite the respective differences in effect over enzyme activities, the increased GSH concentrations are beneficial to cellular growth only when the cells are exposed to 120 μg/ml MEBB. Thus, these results support the hypothesis that there should be an optimal balance between free radical production and radical scavenging under normal conditions. Future experiments on internal cellular ROS production and scavenging in fibroblasts treated with MEBB will elucidate the more mechanistic details of these responses.

Flavonoids are ubiquitous compounds present in plant extracts. They represent a major active component of the plant extract and are often known for their anti-inflammatory and anti-tumor effects. Kim et al. (37) demonstrated the enhancing effects of Ginkgo biloba extracts, particularly the flavonoids, namely, quercetin, kaempferol, sciadopitysin, ginkgetin, isoginkgetin, on the proliferation of normal human skin fibroblast. Because kaempferol 3-0-galactoside, 7-0-rhamnoside is the major compound in broad beans (38), kaempferol seems related to the increase in cellular growth induced by MEBB.

Cultured human fibroblasts display age-dependent transcriptomic differences. Braam et al. (39) hypothesized that aging-associated oxidative stress affects gene expression, and monitored the transcriptome in confluent fibroblasts from young and old individuals cultured without and with an antioxidant mixture (vitamin E, quercetin, hydroxytyrosol and kaempferol). Antioxidant treatment modulated a similar number of genes in all donors and induced cell cycle regulatory genes. In addition, donor-age-dependent decline in glutathione content and resistance to glutathione depletion were observed. Namely, the gene expression of fibroblasts is affected by donor age and a subset was corrected by antioxidants. These results are consistent with our studies that indicated that cellular growth induced by MEBB is associated with the increase in GSH concentration. Future experiments will elucidate the more mechanistic details of these responses.

Concerning the activities of GSH increased by MEBB, Ochiai et al. (40) demonstrated the effect of gamma-glutamylcysteinyl synthase (gamma-GCS) on GSH synthesis. They demonstrated that crocin, a carotenoid pigment of saffron, can suppress the serum-deprivation-induced death of PC12 cells by increasing GSH synthesis. Moreover, they showed that crocin promotes the expression of gamma-GCS mRNA, which contributes to GSH synthesis. Thus, our findings indicate that MEBB may be responsible for the increased gamma-GCS activity, resulting in the increase in GSH concentration in young and old cells.

In summary, these studies elucidated a new cellular growth mechanism whereby human lung fibroblasts modulate intracellular GSH levels via the action of MEBB. This newly recognized function of MEBB plays a role in cellular growth. For example, vascular oxidative stress increases under numerous conditions other than hypertension, including diabetes, atherosclerosis, and aging. Attention has focused on the sources of ROS such as NADPH oxidase and xanthine oxidase, and antioxidants such as SOD, GSH-Px, catalase, and GSH. Our study indicates that MEBB plays an important role in the modulation of fibroblast oxidative stress and that modulation of its GSH might represent an important therapeutic target for the treatment of aging-related disease.

In addition, vegetables and fruits that are rich in antioxidants are essential for lowering disease risks stemming from ROS in the body. Among the vegetables, broad beans are excellent sources of such beneficial antioxidants of polyphenolic nature, just like cocoa and chocolates. Antioxidants also promote healthy aging and may protect against Alzheimer's and Parkinson's diseases. Nutritional lifestyles can be described for most populations in the world and offer the possibility of a healthy long life.

Acknowledgements

We thank Ms. W. Miyata for her critical review of the manuscript. We also thank Dr. H. Kondo for supplying us with the human diploid fibroblasts, TIG-1.

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