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Effects of the Radical Scavenger, Water Soluble Protein from Broad Beans on Lipofuscin, Cathepsin B, Cell Growth in Human Lung Fibroblasts

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ABSTRACT—We purified the free-radical scavenger "water soluble protein (WSP)" from broad beans. Oxidative stress, as well as diminished activity of lysosomal proteolytic enzymes, are known to induce lipofuscin accumulation in a variety of cell types. In order to clarify the roles of oxidative stress and lysosomal proteolysis in lipofuscin accumulation, human lung fibroblasts were treated with WSP. Hydrocortisone (HC) which is known to inhibit superoxide generation, was compared with WSP. Cathepsin B activity in young cells showed a decrease during cell culture from 0.203±0.013 mU/ μg protein (4 weeks) to 0.067±0.018 (6 weeks) with an unparalleled increase in lipofuscin (as autofluorescence intensity, AFI/ μg protein) from 1.46±0.05 (4 weeks) to 1.55±0.04 (6 weeks). Similarly, the activity in old cells exhibited a decrease from 0.208±0.013 mU/ μg protein (4 weeks) to 0.043±0.005 (6 weeks) with an unparalleled increase in lipofuscin from 2.11±0.06 AFI/ μg protein (4 weeks) to 2.27±0.05 (6 weeks). Young cells treated with WSP (1.25 μg/ml) for 6 weeks showed increased cathepsin B activity (0.118±0.009 mU/ μg protein) as compared to the control, but the activity in WSP treatment for 4 weeks decreased by 89%. Young cells treated with HC (5 μg/ml) were equal in cathepsin B activity to the control. On the other hand, old cells treated with WSP for 4 weeks exhibited the decreased activity (0.179±0.008 mU/ μg protein). In contrast, HC treatment for 4 weeks increased the activity (36%). WSP treatment for 6 weeks was effective in reducing lipofuscin accumulation in young (25% decrease) and old (57%) cells as compared to the control. Similarly, HC treatments were more effective in reducing lipofuscin accumulation in young cells for 4weeks (31% decrease) and old cells (63%). The present results show that the free-radical scavenging in the cultured fibroblasts reduced the lipofuscin accumulation.

INTRODUCTION

Human diploid fibroblast cultures undergo an aging process which may be related to certain aging mechanisms *in vivo* (Hayflick, 1965). One potential contribution to cell aging may be peroxidation damage to cell structures. Specifically, damage accumulates beyond the cell's capacity to deal with it. A prediction of this model is that fluorescent damage products accumulate in aging cell populations (Jongkind *et al*., 1982; Devi *et al*., 1990). WI-38 cells were examined by fluorescence microscopy (Deamer, 1973). In three experimental series, distinct particulate structures with yellow fluorescence were observed in many cells. In experiments comparing younger (20 to 30 passages) and older (40 to 42 passages) cell cultures, the fluorescence was generally abundant in cells from older cultures and sparse in cells from younger cultures. Some older cells were entirely filled with fluorescence, but the majority had 10 to 100 discernible particles. Most cells from younger cultures had negligible fluorescence. It is possible that the particles represent lysosomes which have accumulated lipofuscin as peroxidation damage products and certain flavin compounds in a time-dependent process. Their appearance in older cultures may be related to aging damage.

Cell biological data clearly indicated that the lipofuscin in the lysosomes contains diverse proteins (30–70%), lipids (20–50%), carbohydrates (4–7%) and some metals (traces), and that these products are derived from the physiological decay of the cells' own constituents. The precise mechanisms involved in the incremental formation of this pigment (lipofuscin) are still unknown. In another words, the reasons why the multiple lysosomal enzymes cannot completely digest this polymeric product are not yet clear (Porta, 1991). There are, of course, some attractive theories on this intriguing and important problem. Some investigators, for example, have proposed that peroxidative damage to the lipofuscin precursors before and/or after their entering the lysosomes may play a fundamental role in the indigestibility of the ageing pigment

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(peroxidative theory). Another very interesting theory (Ivy *et al*., 1984) was propounded that the progressive lysosomal accumulation of lipofuscin may be due to either an age-wise decline in the activity of thiol proteases, to an increase in the natural inhibitors (cystatins) of these proteases, or to both. This thiol-protease decline theory is based on the demonstration that lipofuscin-like substances accumulated in lysosomes of diverse post-mitotic and slow-dividing cells of experimental animals become infused with synthetic inhibitors of cysteine proteases such as leupeptin (Ivy *et al*., 1984; Ivy and Gurd, 1988; Ivy *et al*., 1990). The strengths and weakness of this theory have already been discussed (Porta, 1991), and it seems to us that determining whether the activities of lysosomal cysteine proteases of normal animals and humans really decrease with age in the cells where lipofuscin increasingly accumulates with cellular age will resolve this question. The object of our study was to clarify this issue.

Cathepsin B is a typical and well-characterized lysosomal cysteine proteinase that is widely but unevenly distributed in a variety of mammalian cell types including fibroblasts (Ivy and Gurd, 1988). Besides its intralysosomal proteolysis for intracellular protein turnover, this enzyme is thought to be involved in a variety of pathological conditions such as inflammation (Ivy *et al*., 1990), neoplastic transformation (Poole *et al*., 1987), and human tumor progression (Yan *et al*., 1998). Cathepsin B is believed to be important in intercellular protein catabolism. On the other hand, growth-related proteolysis has been recognized for many years (Bratt and Scott, 1995; Scott, 1997). Growth-related effects of endogenous proteinases have also been found. These seemed to be largely independent of proteinase-activated receptors. Endogenous proteinase involved in cell growth was identified when antibodies prepared against a human leukocyte membrane proteinase were shown to inhibit the proliferation of human fibroblasts. Recently, Bush *et al* (1998) have identified a hirudin-sensitive proteinase from human fibroblasts. Inhibition of this enzyme results in partial inhibition of cell growth in culture. Similarly, cathepsin B may also be related to cell proliferation.

We purified a new type of free-radical scavenging protein, the water soluble protein (WSP) from broad beans, *Vicia faba* (Okada and Okada, 1998). WSP exhibited a marked scavenging effect on superoxide, and also had an effect on hydrogen peroxide. WSP, in addition, had a small amount of sulfhydryl groups. Moreover, WSP was able to increase both the cytosolic antioxidant enzyme activity and cellular growth in the old culture cell. On the other hand, WSP showed the scavenging activity in a *tert*-butyl hydroperoxide (BHP)-induced oxidative stress model system (Okada and Okada, 1999).

Continuous application of hydrocortisone (HC) enhanced the cellular growth and extended the *in vitro* life span (Cristofalo, 1972; Kondo *et al*., 1983). Fluder (1977) also reported that the increase in density resulted from a faster grown rate. In addition, it was shown that HC inhibited superoxide generation (Korchak and Weissmann, 1978).

The aims of this study were (1) to identify changes in lipofuscin accumulation with culture age, (2) to examine the relationship between lipofuscin storage and cathepsin B activity, and (3) to analyze the effects of WSP on lipofuscin accumulation and cathepsin B activity.

MATERIALS AND METHODS

WSP preparation

WSP (Okada and Okada, 1998) was prepared as follows: Briefly, the purification procedures included ammonium sulfate precipitation followed by sequential chromatography on Sephadex G-75. The final gel filtration step yielded two peaks of scavenging activity, each containing *M*r values of 70 kDa and 28 kDa. The protein of 28 kDa yielded by the final gel filtration step was WSP, which was used as a free-radical scavenger in the following experiments.

Human lung fibroblasts

Human lung fibroblasts (TIG-1 cells) were obtained from the Tokyo Metropolitan Institute of Gerontology (Tokyo, Japan). TIG-1 cells were cultured by methods previously described (Okada *et al*., 1986). Briefly, the cells were cultured in an atmosphere of air containing 5% CO_2 at 37°C in Eagle's minimum essential medium (MEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% FBS (Flow Laboratories, North Ryde, N. S. W., Australia), 28 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES) and 1.5 μg/ml Fungizone (Sigma, St. Louis, MO)(refer this medium to MFHF medium).

To study the effect of WSP on cellular growth, TIG-1 cells were seeded into 60 mm culture dishes at 1×10^5 cells/dish (28 cm^2) in MFHF medium with or without 1.25 μg/ml WSP. This medium was replaced every 3 or 4 days by fresh medium, with or without WSP. The cell number was counted at regular intervals. Similarly, the hydrocortisone (HC) treatment was examined by the addition of 5 μg/ml HC to the growth medium. Subculture at 1×10^5 cells/dish was performed at approximately 1-week intervals for young cells (TIG-1-20, population doubling level (PDL) 20, 25% of the maximum life span), or at approximately 10 day intervals for the old cells (TIG-1-50, PDL 50, 62.5% of the maximum life span). Treatment with WSP or HC was performed for 4 and 6 weeks in young cells, and in old cells by the addition of this substance to the medium. A WSP concentration of 1.25 μg/ml (final concentration) was chosen for the supplementation experiments because this resulted in a maximum increase of cellular growth as determined by a concentration finding pilot experiment using WSP concentrations between 0 and 25 μg/ml (data not shown). An HC concentration of 5 μg/ml (final concentration) was also chosen for the supplementation experiments according to Fluder (1977). The growth rate achieved between subcultures was calculated by number of cells obtained at subculture/number of cells inoculated.

Lipofuscin

Lipofuscin was extracted from the harvest cells in 4 ml $CHCl_3$/CH_3OH (1:2, v/v) solvent (Koster and Slee, 1980) at cell cultures of young (4 and 6 weeks) or old (4 and 6 weeks) cells. After centrifugation, 3 ml $CHCl_3$ and 3 ml H_2O were added to the supernatant. The $CHCl_3$ layer was withdrawn and CH_3OH added to clarify the sample. The autofluorescence intensity (AFI) was measured by a spectrofluorophotometer at the excitation wavelength of 353 nm and an emission wavelength of 453 nm, with quinine hydrobromide in 0.05 M H_2SO_4 as standard.

Assay of cathepsin B activity

Cells cultured by the above method were homogenized according to the method of Chang *et al.* (1981). Cathepsin B activity was measured according to Barrett (1980). Briefly, the reaction mixture for the measurement of cathepsin B activity was composed of 0.7 ml of 400 mM potassium phosphate buffer, pH 5.5, containing 4.0 mM ethylenediaminetetraacetic acid (EDTA), 8.0 mM cysteine, 0.05 ml of

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the enzyme solution and 0.75 ml of 0.02 mM substrate. The substrate, carbobenzoxy-arginyl-arginine-4-methyl-coumaryl-7-amide (Z-Arg-Arg-MCA) was dissolved in dimethyl sulfoxide as a 10 mM solution and stored in the dark at –20°C. When the substrate stock was used, it was freshly diluted to 0.02 mM with 0.1% Triton X-100. The reaction was started by the addition of diluted substrate and stopped by adding 1.5 ml of 100 mM sodium acetate buffer, pH 4.3, containing 100 mM sodium chloroacetate. The reaction mixture was incubated at 40°C for 10 min. One unit of enzyme activity was defined as the amount of enzyme to release 1.0 nmol of aminomethylcoumarin from Z-Arg-Arg-MCA/min. Cathepsin B activity was shown as mU/ μg protein.

Determination of protein

Protein content was measured by the method of Bradford (1976) using bovine serum albumin as the standard.

Statistical analysis

Data are expressed as mean values with standard deviations. Mean values were assessed for significance by ANOVA. *p*-Values<0.05 were considered significant.

RESULTS

Figs. 1 and 2 show the effects of WSP or HC on the growth of young and old TIG-1 cells. The growth rate in young cells was measured after 7 days, and the growth rate in old cells was measured after 10 days. When WSP at 1.25 μg/ml was added to the young cells for 4 weeks, statistically significant inhibitions were observed ($p < 0.05$). The growth rate of young cells incubated with WSP for 6 weeks was decreased by 47% as compared with the control. Similarly, the decrease of growth in young cells treated with HC for 6 weeks was 63%, but the growth rate in young cells treated with HC for 4 weeks increased by 41%.

On the other hand, the growth rate of old cells treated with WSP for 4 weeks increased by 11%, whereas the growth rate in old cells treated for 6 weeks decreased by 60% (Fig. 1and 2). The old cells treated with HC for 4 or 6 weeks exhibited growth rate decreases of 31 or 49%, respectively.

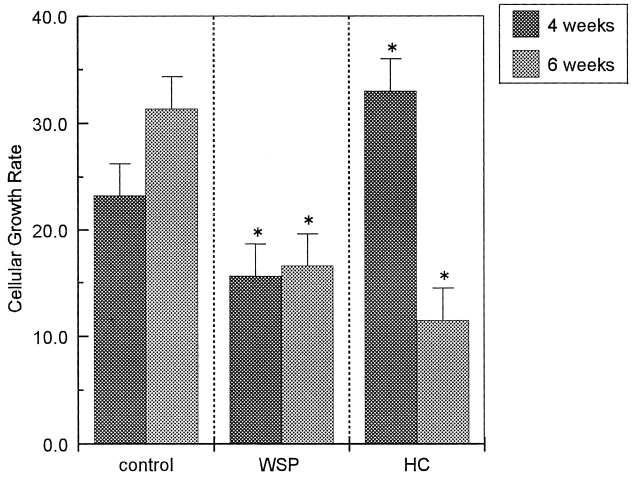

Fig. 1. Effects of WSP on the cellular growth of young human lung fibroblasts. Young TIG-1 cells (PDL 20, 25% of the maximum life span) were seeded into 60 mm culture dishes at 1×10^5 cells/dish in the culture medium containing 1.25 μg/ml WSP or 5 μg/ml HC. The cell number was counted on day 7, as described in Materials and Methods. WSP or HC was supplemented for 4 or 6 weeks for young cells. The growth rate achieved between subcultures was calculated by number of cells obtained at subculture/number of cells inoculated. Values represent the mean of triplicate determination. *p<0.05 compared with the control.

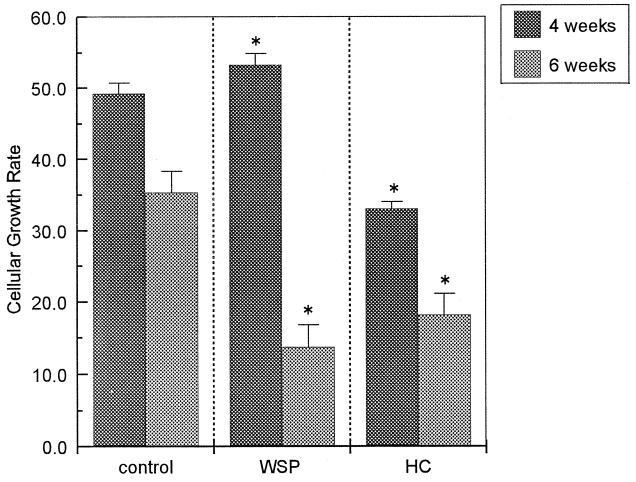

Fig. 2. Effects of WSP on the cellular growth of old human lung fibroblasts. Old TIG-1 cells (PDL 50, 62.5% of the maximum life span) were seeded into 60 mm culture dishes at 1×10^5 cells/dish in a culture medium containing 1.25 μg/ml WSP or 5 μg/ml HC. The cell number was counted on day 10, as described in Materials and Methods. WSP or HC was supplemented for 4 or 6 weeks for old cells. The growth rate achieved between subcultures was calculated by number of cells obtained at subculture/number of cells inoculated. Values represent the mean of triplicate determination. *p<0.05 compared with the control.

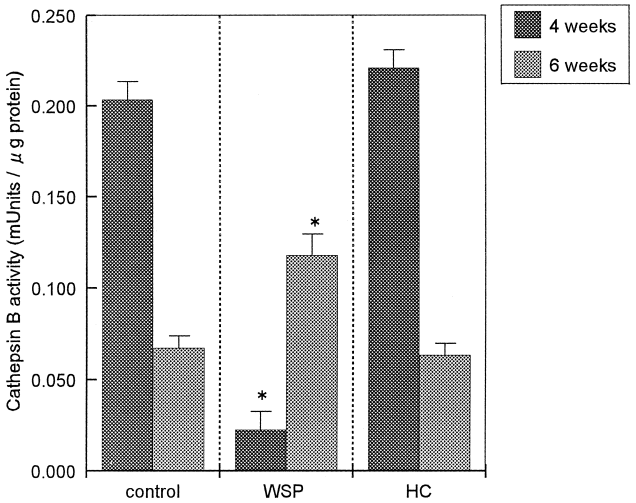

Fig. 3. Effects of WSP on cathepsin B activity as a function of culture age of young TIG-1 cells. Cathepsin B activities in young cells (PDL 20, 25% of the maximum life span). The cells were incubated with WSP or HC for 4 and 6 weeks. Data are expressed as the means ± standard deviation of three replicate analyses. Cathepsin B is expressed as mU/ μg protein. *p<0.05 compared with the control.

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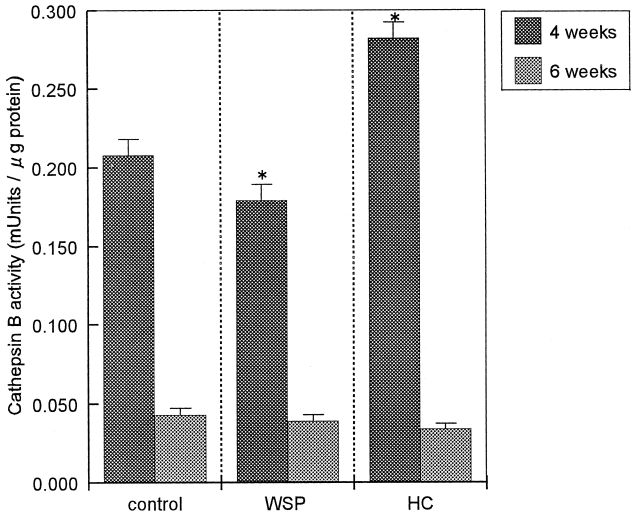

Fig. 4. Effects of WSP on cathepsin B activity as a function of culture age of old TIG-1 cells. Cathepsin B activities in old cells (PDL 50, 62.5% of the maximum life span). The cells were incubated with WSP or HC for 4 and 6 weeks. Data are expressed as the means ± standard deviation of three replicate analyses. Cathepsin B is expressed as mU/ μg protein. *p<0.05 compared with the control.

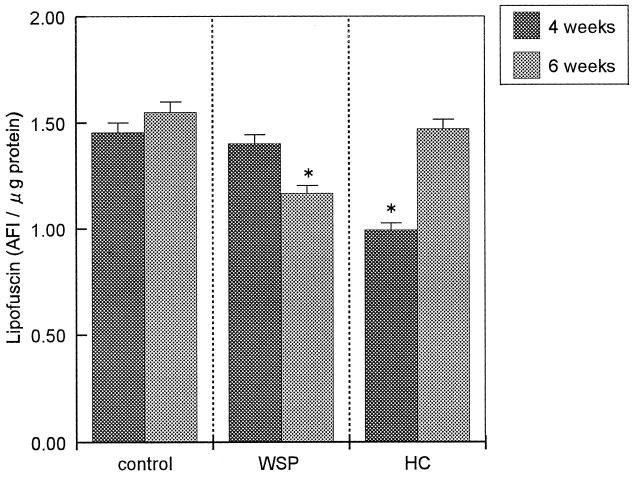

Fig. 5. Effects of WSP on lipofuscin accumulation as a function of culture age of young TIG-1 cells. Lipofuscin accumulation in young cells (PDL 20, 25% of the maximum life span). The cells were incubated with WSP or HC for 4 and 6 weeks. Data are expressed as the means ± standard deviation of three replicate analyses. Lipofuscin accumulation is expressed as AFI/ μg protein. *p<0.05 compared with the control.

Cathepsin B activity

A progressive decrease in cathepsin B activity was observed from 4 weeks to 6 weeks in both young and old cell cultures (Figs. 3 and 4). In young cultures treated for 4 weeks, cells exhibited a decreased cathepsin B activity (89%) in response to 1.25 μg/ml WSP (Fig. 3). A similar but lesser decrease was observed in the old cells treated for 4 weeks with WSP (Fig. 4). In the young cultures treated 6 weeks, however, WSP treated cells exhibited an increased cathepsin B activity (76%) (Fig. 3). A similar increase was observed in the

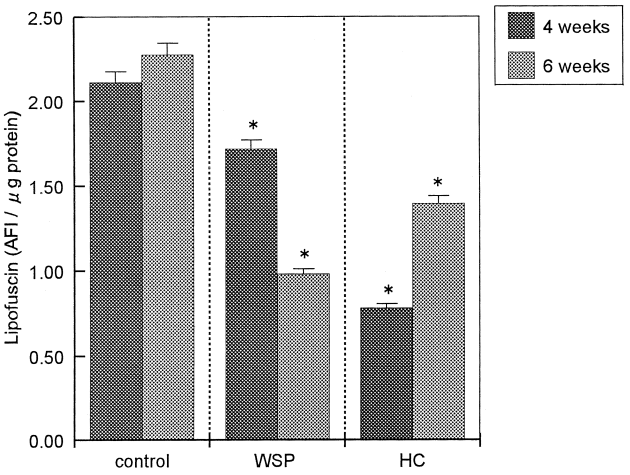

Fig. 6. Effects of WSP on lipofuscin accumulation as a function of culture age of old TIG-1 cells. Lipofuscin accumulation in old cells (PDL 50, 62.5% of the maximum life span). The cells were incubated with WSP or HC for 4 and 6 weeks. Data are expressed as the means ± standard deviation of three replicate analyses. Lipofuscin accumulation is expressed as AFI/ μg protein. *p<0.05 compared with the control.

old cells treated for 4 weeks with HC (36%) (Fig. 4). No significant changes in cathepsin B activity were detected either in the young cells treated with HC or in the WSP or HC treated old cells for 6 weeks. Our preliminary study has indicated deleterious effects of a higher concentration of WSP (25 μg/ml) in terms of cell growth, and the need for a much more dilute concentration (<1.25 μg/ml) which may fail to elicit any significant change either in cell growth or in cathepsin B activity.

Lipofuscin

Lipofuscin increased as a function of cell culture (Figs. 5 and 6). AFI/ μg protein in young cell cultures increased from 1.46±0.05 (4 weeks) to 1.55±0.04 (6 weeks). AFI/ μg protein in old cell cultures increased from 2.11±0.06 (4 weeks) to 2.27±0.05 (6 weeks). WSP treated cells exhibited a significant decrease in their AFI in response to increased WSP treatment exposure. The WSP effect was more pronounced in old cell culture treatment (Fig. 6) than in young cell culture treatment (Fig. 5). This result indicated that the extent of AFI reduction was dependent on cellular age.

On the other hand, the lipofuscin content in the young cells treated with HC for 4 weeks decreased 31%, but a statistically significant decrease in lipofuscin for 6 weeks treatments was not observed. Lipofuscin content in old cells treated with HC for 4 and 6 weeks, however, decreased by 63 and 40%, respectively.

DISCUSSION

Cathepsin B is a typical and well-characterized lysosomal cysteine protease and has been studied in many mammalian tissues (Takahashi *et al.*, 1981) and in vertebrates (Okada and Yokota, 1990). Generally, the activity of protein degradation is decreased in aged animals. The lysosomal thiol

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proteases are responsible for the majority of intracellular protein turnover (Barret, 1977). It also seems consistent with the observation that, at least in fibroblasts from elderly human subjects and from individuals with premature aging conditions such as Werner's syndrome, the activity of cathepsin B is lower than in normal adults (Gracy *et al*., 1985). In addition, Amano *et al*. (1995) suggested that the cathepsin B level in the rat tissue was significantly changed with aging (55% decrease). On the other hand, Nakanishi *et al*. (1994) reported that the activity of cathepsin B was not decreased with age. We indicated that a progressive decrease in cathepsin B activity was observed from 4 weeks to 6 weeks in both young and old cell cultures (Figs. 3 and 4). Our results are similar to the former findings.

When cathepsin B activity was studied in HC treated young cells, no differences were found between the control groups (Figs. 3 and 4). However, when cells were incubated with WSP, an 89% decrease in cathepsin B activity was found in young cells from 4 week cultures, whereas a 76% increase in activity was observed in the WSP treated cells from 6 weeks. These results may indicate either a reduction or an increase of cathepsin B activity by WSP in response to the change in protein accumulation occurred in young cells from culture age (Dice, 1982). On the other hand, cathepsin B activity was not induced in WSP treated cells from old subjects, but levels of lipofuscin accumulation in WSP treated cells were lower than in the control (Figs. 5 and 6). This result proves that the lipofuscin accumulation does not correlate with cathepsin B activity. Namely, there is growing evidence that oxidants are rapidly scavenged by WSP. In this context, we have demonstrated that BHP-induced oxidative stress increases cytosolic SOD activity. On the other hand, when the old cells incubated in WSP were subjected to a BHP challenge, the cytosolic SOD activity returned to its control value (Okada and Okada, 1999). In the present work, we found a modification of cathepsin B activity when young cells were incubated with WSP. However, this did not correlate with the decrease in lipofuscin accumulation levels. These findings suggest that when human lung cells are incubated with WSP, the decrease in lipofuscin accumulation is independent of cathepsin B activity. In a previous work, Porta *et al*. (1995) suggested that the increase in lipofuscin with age may not be due to an age-wise decline in cathepsin B activity. Our results are similar to these findings.

No significant changes in cathepsin B activity were detected either in the young cells treated with HC or in the HC treated old cells for 6 weeks (Figs. 3 and 4). In the old cultures treated 4 weeks, however, HC treated cells exhibited an increased cathepsin B activity (36%) (Fig. 4). However, the lipofuscin content in the young cells treated with HC for 4 weeks decreased 31%, but a statistically significant decrease in lipofuscin for 6 week treatments was not observed. On the other hand, lipofuscin content in old cells treated with HC for 4 and 6 weeks, decreased by 63 and 40%, respectively (Fig. 6). Similarly, these findings suggest that when human lung cells are incubated with HC, the decrease in lipofuscin accumulation is independent of cathepsin B activity.

The results of this study provide evidence for the age-related elevation in lipofuscin content of human lung fibroblasts. Lipofuscin accumulation in the cytoplasm of culture cells serves as an important biomarker of ageing (Dreamer, 1973; Jongkind *et al*., 1982; Terman and Brunk, 1998). The increase in lipofuscin with culture age coincided with a decrease in a growth rate of both single cells and groups of cells.

An interesting finding of the present work was a significant reduction in the lipofuscin accumulation in the cells treated with WSP or HC. In young cells, WSP treatment for 6 weeks or HC treatment for 4 weeks showed a decrease in lipofuscin content compared to the control level, and old cells treated with WSP or HC exhibited a decrease in lipofuscin content (Figs. 5 and 6).

Because free radicals are potentially toxic, there are some free radical deactivating enzymes (preventing antioxidants) such as superoxide dismutase (SOD), glutathione peroxidase and catalase in biological systems. In addition to free radical deactivating enzymes, biological systems are using free radical scavengers (chain-breaking antioxidants) which detoxify radicals by cap-turning an extra electron and forming fewer toxic radicals. WSP is an important free radical scavenger. Similarly, HC inhibited the superoxide generation (Korchak and Weissman, 1978). Furthermore, the effects of WSP treatment have been observed in cell culture conditions (Okada and Okada, 1999; Okada and Okada, 2000). Moreover, WSP showed scavenging activity in an oxidative stress model system. Therefore, WSP or HC may come in contact with polyunsaturated fatty acids of lipids to stabilize the membrane while inhibiting the free radical reactions. It was revealed by quantitative fluorescence photometer that WSP or HC supplementation may be able to distinctly reduce lipofuscin accumulation related to cellular aging.

Thaw *et al*. (1984) and Sohal *et al*. (1989) reported that the rate of lipofuscin accumulation was retarded by antioxidants such as vitamin E. However, it is unlikely that antioxidants contained in the serum in the culture medium, if any, were enough to suppress lipofuscin accumulation, since it is reported that stored serum lacks antioxidative activity (Baker *et al*., 1988). Our results demonstrated that longer WSP treatment periods resulted in greater reductions in AFI in cultured lung fibroblasts. Devi *et al*. (1990) have also reported a similar trend for the action of vitamin E in rat heart cells. Their study shows that lipofuscin accumulation is reduced by antioxidants.

The notable outcome in WSP or HC-treated cultures in the present study was not related to the cathepsin B activity of treated cells. The fact that cathepsin B activity in the WSP-treated cultures was lower than in the controls was not related to suppressed lipofuscin accumulation. Studies on human lung fibroblasts have indicated that antioxidant (such as vitamin E) deficiency can lead to high vulnerability of the membranes, especially the mitochondrial membrane, to peroxidative damage. This peroxidative damage could possibly spread over the cytoplasm with simultaneous damage to the macromolecules such as proteins and interfere with en-

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zyme activities (Packer and Smith, 1974). Perhaps such a condition was reversed in WSP-treated cells which exhibited a decrease in lipofuscin accumulation as compared to the cultures grown in the normal medium.

The present investigation shows that the cellular growth rate in old TIG-1 cells is not associated with lipofuscin accumulation occurring during their proliferation in culture. On the other hand, a much higher dose of α-tocopherol (5×10^{-2} IU/ml) interfered with cell growth, and this could be due to the alterations induced in the structure and function of serum lipoproteins (Sakagami and Yamada, 1977). This may be the reason WSP decreased the cellular growth of TIG-1 cells. A balance probably exists between the occurrence of environmentally induced oxidative damage to cells in culture and their ability to neutralize it. It is possible that by decreasing the occurrence of this damage, lipofuscin accumulation may be prevented.

WSP treatment clearly had a significant effect on lipofuscin accumulation with culture age in young and old cells. Similarly, lipofuscin content in the old cells was decreased by the HC treatment. Nevertheless, it was also revealed that HC treatment for 6 weeks in young cells did not have a decisive effect on lipofuscin accumulation. Although these results may be attributable to age-dependent alterations in free-radical production and compensatory systems of antioxidant mechanisms as described above, there may also be other unknown factors which could have caused these results. For further details, age-dependent alterations in the activities of the free-radical deactivating enzymes as well as in free-radical production of WSP or HC supplemented cells should be measured in the young and old cells throughout the culture age.

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