Effects of Radical Scavenger Protein from Broad Beans on Glutathione Status in Human Lung Fibroblasts

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Abstract

Objective: Human diploid cells are more susceptible to oxidative stress at late passage than at early passage, presumably because of the decrease in cellular-reduced glutathione (GSH) concentration. Water-soluble protein (WSP) from broad beans scavenges free radicals. The effects of WSP on the glutathione system were examined in PDL 20 (early passage) and PDL 50 (late passage) human lung fibroblasts (TIG-1).

Methods: To determine cytosolic glutathione peroxidase (GSH-Px) activities, glutathione reductase (GR) activities, oxidized glutathione (GSSG) concentrations, and GSSG/reduced glutathione (GSH) ratios, WSP and hydrocortisone (HC) treatments of TIG-1 cells (PDL 20→50 and PDL 50→75) were performed for 40 days. We also investigated the GSSG concentrations and GR activities in PDL 20 cells that were continuously treated with WSP until PDL 39 and 55.

Results: GSSG concentrations decreased in WSP- and HC-treated PDL 50→75 cells. The GSSG/GSH ratios in PDL 50→75 cells became low after the treatments. Increases in GR activities were observed in treated PDL 50→75 cells. The decline in the GSSG concentration of PDL 50→75 cells correlated with the increase in GR activity. The GSSG levels in control cells were higher following cellular age, whereas the levels in treated cells were lower than those in the control. The studies on cellular age-related changes indicated that greater increases in GR activity were found in treated cells than in the control.

Conclusion: These results indicated that WSP influences the GSSG concentration that is associated with cellular aging, but the mechanism of GSSG reduction by WSP remains unknown. The enhancement of glutathione status following WSP treatment may be related to the delay in the cellular aging.

Key words: radical scavenger protein, glutathione, human lung fibroblasts, broad beans, hydrocortisone

Introduction

Yuan et al. (1) indicated that human diploid cells are more susceptible to oxidative stress at late passage than at early passage, presumably because of the decreases in cellular reduced glutathione (GSH) concentration and catalase activity. More, they suggested that GSH might serve as a primary defense against oxidative stress (1). The mechanism of intra-

cellular protection against oxidative damage may be low in late-passage cells, in which the damage is greater than that in early-passage cells. Conversely, the cellular protection capacity against oxidative damage may decrease with prolonged culture period (*in vitro* aging). GSH is the most abundant low-molecular-weight thiol in mammalian cells and, with its conversion to oxidized glutathione (GSSG), GSH is synthesized endogenously in fibroblasts using amino acid substrates that can be derived exogenously from culture medium. GSH that is present in fibroblasts appears to be required for antioxidant defense (2), in part because it protects the fibroblasts from cellular damage by free radicals and maintains the sulfhydryl/disulfide balance of proteins.

The flavoenzyme, glutathione reductase (GR), contains a redox-active disulfide that is reduced by NADPH via the flavin, constituting the reductive half-reaction (3). The resulting dithiol

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reacts with the second substrate, GSSG, yielding two molecules of GSH in the oxidative half-reaction. GR belongs to the pyridine nucleotide-disulfide oxidoreductase family that includes lipoamide dehydrogenase and thioredoxin reductase. GR differs from the other two enzymes in that two molecules of the monothiol GSH are produced, whereas in lipoamide dehydrogenase, the substrate is a dithiol and, in thioredoxin reductase, the product is a dithiol (4).

In the last few years, a wide spectrum of compounds have been screened as potential reactive oxygen species (ROS) scavengers in therapy for ROS-mediated cell damages (5–8). Among these antioxidants, water-soluble protein (WSP) from broad beans has demonstrated protection against oxidative damage in *in vitro* experiments by inhibiting or scavenging ROS (9).

In this study, we examined the effects of WSP treatment on the glutathione system in early-passage and late-passage fibroblasts. The GSSG concentration in the WSP-treated cells was measured because there is evidence that it indicates the increase in GSH concentration by WSP treatment (10). Glutathione peroxidase (GSH-Px) activity in the WSP-treated cells was also measured because there is evidence that suggests an inverse correlation between lower amounts of GSH and higher GSH-Px activity (11). On the other hand, it has been shown that hydrocortisone (HC) inhibits superoxide generation (12). In addition, HC increases the GSH concentration in human diploid fibroblasts (10). The effects of WSP were compared with those of HC in order to show the correlation between the cellular reducing potential, as indicated by the GSSG concentration.

Materials and Methods

Materials

HC, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and Fungizone were obtained from Sigma Ltd. (St. Louis, MO). TIG-1-20 cells (JCRB0501) and TIG-1-50 cells (JCRB0504) were obtained from the Tokyo Metropolitan Institute of Gerontology (Tokyo, Japan). Fetal bovine serum (FBS) was purchased from Flow Laboratories (North Ryde, N. S. W., Australia), and trypsin from Difco Laboratories (Detroit, MI., U.S.A.). Eagle's minimum essential medium (MEM) was from Nissui Pharmaceutical (Tokyo, Japan). All other chemicals were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

WSP preparation

WSP was prepared as follows (8). Briefly, the purification procedures included ammonium sulfate precipitation followed by sequential chromatography on Sephadex G-75. The final gel filtration step yielded two peaks that indicate scavenging activity, each having *M_r* values of 70 kDa and 28 kDa. The 28 kDa protein 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 diploid fibroblasts (TIG-1 cells) were cultured by methods previously described (13). Briefly, the cells

were cultured in an atmosphere of air containing 5% CO₂ at 37°C in MEM supplemented with 10% FBS, 28 mM HEPES and 1.5 µg/ml Fungizone (referred to as MFHF medium). TIG-1 cells were seeded onto 60 mm culture dishes at 1×10⁵ cells/dish in MFHF medium with or without 2.5 µg/ml WSP. This medium was replaced every 3 or 4 days with fresh medium, with or without WSP. The cell number was counted at regular intervals. Similarly, HC treatment was carried out by the addition of 5 µg/ml HC to the growth medium. Subculture at 1×10⁵ cells/dish was performed at approximately 1 week intervals for population doubling level (PDL) 20 cells (TIG-1-20, 25% of the maximum life span), or at approximately 10 day intervals for PDL 50 cells (TIG-1-50, 62.5% of the maximum life span). The PDL number indicates the subculture number. Treatment with WSP or HC was carried out continuously for 40 days (PDL 20→50) in PDL 20 cells, and for 40 days (PDL 50→75) in PDL 50 cells by the addition of WSP or HC to the medium. To determine the cellular age-related changes, the treatment was carried out continuously for 60 days (PDL 20→55) in PDL 20 cells.

Effect of WSP on cytosolic antioxidant enzymes

The effect of WSP on cytosolic antioxidant enzymes was examined using TIG-1 cells. The cells were cultured in MFHF medium supplemented with WSP or HC. 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 antioxidant enzymes.

Biochemical analysis

Total glutathione concentration was measured in PDL 20→50 and PDL 50→75 fibroblasts by the spectrophotometric recycling assay (14) in the presence of 5,5'-dithiobis-(2-nitrobenzoic acid), NADPH and GR at 412 nm. GSSG was assayed by the same method after derivatization of GSH with 2-vinylpyridine (15). GSH values were obtained after subtracting GSSG from total glutathione. GSH and GSSG values were corrected for spontaneous reaction in the absence of a sample. The assay was performed at 25°C. The GSSG/GSH ratio was calculated as an estimate of the oxidative stress of the cells.

The cytosolic GR activity was assayed in PDL 20→50 and PDL 50→75 cells by following the NADPH oxidation at 340 nm in the presence of GSSG (16). The GR activity was corrected for spontaneous reaction in the absence of a biological sample (without an enzyme). The assay was performed at 25°C. The cytosolic GSH-Px activity was assayed according to the procedure of Tappel (11). The protein content was measured by the method of Bradford (17) using bovine serum albumin as the standard.

Statistical analysis

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

Antioxidative capacity of control cells

Cell cultures were started at PDL 20, and increased to PDL 50 after 40 days. Also, cell cultures were started at PDL 50, and increased to PDL 75 after 40 days. Such increases in PDL per culture period were similarly observed in subsequent experiments.

Tables 1-1 and 1-2 show variations in the GSSG levels and GSSG/GSH ratios of PDL 20→50 and PDL 50→75 cells as a function of culture time. The GSSG level in PDL 20 cells cultured for 40 days (PDL 20→50) was about 3 times that of PDL 50 cells for 40 days (PDL 50→75). Similarly, the GSSG/GSH ratio tripled in PDL 50 cells cultured for 40 days (PDL 50→75) relative to that of PDL 20 cells (PDL 20→50), as shown in Table 1-2.

Table 2-1 shows variations in the cytosolic GR activities of PDL 20→50 and PDL 50→75 cells as a function of culture time. The GR activity of PDL 50→75 cells decreased by about 50% as compared with that of PDL 20→50 cells. On the other hand, the GR activity in PDL 20 cells increased throughout the culture period (from PDL 20→39 to PDL 20→55) (Table 3-1). Although the GR activity of PDL 20 cells increased with increasing culture period (from PDL 20→39 to PDL 20→55), the GR activity of PDL 50→75 cells decreased as compared with that of PDL 20→50 cells (Table 2-1). In other words, their activities appeared changeable during the cellular aging from PDL 20 to PDL 75.

Table 2-2 shows variations in the cytosolic GSH-Px activities of PDL 20→50 and PDL 50→75 cells as a function of culture time. The cytosolic GSH-Px activities of PDL 50→75 cells were half those of PDL 20→50 cells.

Table 3-1 shows variations in the cytosolic GSSG levels of PDL 20→39 and PDL 20→55 cells as a function of culture time. The GSSG level of PDL 20 cells cultured for 60 days (PDL 20→55) was about 6 times that of cells cultured for 30 days (PDL 20→39).

Antioxidative capacity of WSP- or HC-treated cells

The GSSG concentrations in PDL 20→50 and PDL 50→75 cells are shown in Table 1-1. WSP and HC treatments resulted in a significant increase in the GSSG concentration in PDL 20→50 cells as compared with the control, but the GSSG concentration in PDL 50→75 cells decreased after treatment with WSP or HC. The GSSG/GSH ratios in PDL 20→50 and PDL 50→75 cells are summarized in Table 1-2. The GSSG/GSH ratios in PDL 20→50 cells were significantly higher in HC groups than in the control group, whereas the ratios in PDL 50→75 cells even after the treatment were lower than those in the control. Also, the ratio in PDL 20→50 cells treated with WSP was similar to that obtained in the control. However, the ratios in PDL 50→75 cells after WSP treatment were lower than those in the control. Changes in these ratios were greater in

Table 1-1 GSSG concentrations in PDL 20→50 and PDL 50→75 human lung fibroblasts

	Control	WSP	HC
PDL 20→50	0.853±0.282	1.751±0.368*	3.234±0.565**
PDL 50→75	2.696±0.279	0.737±0.065**	0.296±0.036**

Table 1-2 GSSG/GSH ratios in PDL 20→50 and PDL 50→75 human lung fibroblasts

	Control	WSP	HC
PDL 20→50	0.0174	0.0201	0.0539**
PDL 50→75	0.0528	0.0121**	0.00591**

Treatment of PDL 20 and PDL 50 cells with WSP or HC was performed continuously for 40 days.

GSSG concentrations are expressed in nmol GSH equivalents per mg protein.

Data are expressed as means±standard deviation (n=4).

* *p*<0.05, ** *p*<0.01 compared with the control.

Table 2-1 Glutathione reductase (GR) activities in PDL 20→50 and PDL 50→75 human lung fibroblasts

	Control	WSP	HC
PDL 20→50	16.6±2.62	11.9±1.03*	20.6±2.93
PDL 50→75	8.50±1.02	32.0±3.56**	47.0±3.62**

Table 2-2 Glutathione peroxidase (GSH-Px) activities in PDL 20→50 and PDL 50→75 human lung fibroblasts

	Control	WSP	HC
PDL 20→50	103.0±9.05	53.4±6.83*	91.3±9.13
PDL 50→75	62.7±7.69	72.3±7.98	68.6±7.62

Treatment of PDL 20 and PDL 50 cells with WSP or HC was performed continuously for 40 days.

GR activity is expressed in nmol NADPH per min per mg protein.

GSH-Px activity is expressed as units per mg protein. One unit of GSH-Px is defined as the oxidation of 1 nmol NADPH per min.

Data are expressed as means±standard deviation (n=4).

* *p*<0.05, ** *p*<0.01 compared with the control.

Table 3-1 Cellular age-related changes in GSSG levels in human lung fibroblasts

PDL	20→39	20→55
Control	0.661±0.168	4.098±0.369
WSP	0.247±0.055**	0.093±0.029**

Table 3-2 Cellular age-related changes in glutathione reductase (GR) activities in human lung fibroblasts

PDL	20→39	20→55
Control	8.80±1.29	43.3±1.89
WSP	18.1±1.68**	50.6±1.91**

GSSG concentrations are expressed in nmol GSH equivalents per mg protein.

GR activity is expressed in nmol NADPH per min per mg protein.

Data are expressed as means±standard deviation (n=4).

** *p*<0.01 compared with the control.

HC-treated cells than in WSP-treated cells. The GR activities in PDL 20→50 and PDL 50→75 cells are shown in Table 2-1. Compared with the control cells, greater increases in GR activity were found in PDL 50→75 cells after WSP and HC treatments except for the treated PDL 20→50 cells. The GR activity in PDL 20→50 cells treated with WSP was lower than that in the control. Table 2-2 shows the GSH-Px activities in PDL 20→50 and PDL 50→75 cells treated with WSP and HC. The GSH-Px activities in treated cells were nearly equal to those in control cells, but the activity in PDL 20→50 cells was lower in the WSP-treated group than in the control group. The GSSG levels in treated cells were relatively unaffected by the GSH-Px activities. Cellular age-related changes in GSSG levels in TIG-1 cells are shown in Table 3-1. The GSSG levels in control cells were significantly higher following *in vitro* aging (from PDL 20→39 to PDL 20→55), whereas the levels in WSP-treated cells were lower than those in the control. Table 3-2 shows the cellular age-related changes that were observed in terms of GR activities in human lung fibroblasts. The GR activity was markedly increased (105% in PDL 20→39 and 17% in PDL 20→55) by WSP treatment. Compared with the control samples, greater increases in GR activity were found in WSP-treated cells.

Discussion

It has been reported that during the *in vitro* aging of cultured cells, the GR activity tends to decrease and the GSH-Px activity increases (1). As shown in Table 2-2, the GSH-Px activity in PDL 50→75 cells decreased as compared with that in PDL 20→50 cells, while the GR activities in TIG-1 cells increased with prolonged culture period from PDL 20→39 to PDL 20→55 (Table 3-2). However, the GR activity in PDL 50→75 cells decreased as compared with that in PDL 20→50 cells with increasing culture period (*in vitro* aging) as shown in Table 2-1. Namely, the GSH-Px activity decreased with increasing culture period (from PDL 20→50 to PDL 50→75), and the GR activity increased in the case of PDL 20→39 to PDL 20→55, but decreased in the case of PDL 20→50 to PDL 50→75. The mechanism of intracellular protection against oxidative damage may be low in late-passage cells, in which the damage was greater than that in early-passage cells. Conversely, the cellular protection capacity against oxidative damage may decrease with prolonged culture period (*in vitro* aging). Our observations on control cells support this inference. As shown in Table 1-1, the GSSG concentrations increased with increasing culture period (from PDL 20→50 to PDL 50→75). The GSH system is considered to be one of the major cellular redox buffering systems in mammals. Hydrogen peroxide (H_2O_2) formed by the dismutation of superoxide is catabolized at the expense of reducing equivalents from GSH by GSH-Px to form GSSG or mixed protein disulfides. It is considered that GSH depletion occurs with aging (18). Namely, GSH metabolism appears to be closely linked to antioxidant enzymes responsible for removing superoxide and H_2O_2 from the cell, demonstrating the synergistic balance between these enzymes (GR and GSH-Px) and GSH in maintaining cellular redox homeostasis. The significant change in antioxidant enzyme

activity levels after the progress of cellular aging indicates that the antioxidant enzymes' status is an important factor in maintaining GSH balance and overall redox homeostasis. However, it appears that this balance is precarious.

It is now clear that the levels of endogenous antioxidants such as GSH decrease with age (18, 19). We investigated the potential of the dietary antioxidant, WSP, to reverse the age-related influences of GSH depletion using the *in vitro* human lung fibroblast (TIG-1) model.

In this study, we have examined the changes caused by the radical scavenger, WSP in the fibroblast glutathione system. The effects of WSP were compared with those of the superoxide inhibitor HC (as a reference compound). Also, we previously showed that the GSH concentration in HC-treated fibroblasts is higher than that in the control (10). We have examined the effects of WSP and HC on the glutathione system and have found that WSP treatment has a significant effect *in vitro* as indicated by the decrease in the GSSG level in the PDL 50→75 human fibroblasts.

The cellular GSH level is maintained by two pathways: *de novo* synthesis and the NADPH-dependent reduction of GSSG by GR. As can be seen from Table 1-1, there were significant decreases in GSSG concentration after WSP and HC treatments in PDL 50→75 cells as compared with the control. The GSSG concentration in the WSP-treated PDL 50→75 cells was about one-quarter that in the control cells. The decrease in the GSSG concentration observed in PDL 50→75 cells following WSP and HC treatments could be explained by the increase in GR activity, because the decrease in the GSSG concentration correlates with an increase in the GR activity. In fact, the induction of GR activity has been demonstrated with other antioxidants such as α -tocopherol, butylated hydroxyanisole, and propyl gallate (20). The induction of GR activity suggests that the critical factor was the maintenance of the low GSSG level (4).

Although the mechanisms underlying the increase in GSH and/or decrease in GSSG are as yet unknown, it is plausible that in late-passage cells, as has been reported for other antioxidants (21), large quantities of stable free radicals of WSP produced from hydrogen abstraction could have induced GR activity which in turn resulted in increases in GSH and decreases in GSSG concentrations. The reducing potential of the cell in terms of the additional bioconversion of GSSG to GSH may protect the cells from free-radical-mediated damage and account for the increase in the cellular growth as has been observed in old human fibroblasts treated with antioxidants (9). This hypothesis is consistent with previous studies that indicate that cellular aging is associated with a general deficiency of glutathione (1, 22).

Table 1-1 shows that WSP and HC treatments resulted in a significant increase in GSSG concentration in PDL 20→50 cells. Ochi reported that the incubation of cultured Chinese hamster V79 cells with menadione (2-methyl-1,4-naphthoquinone), a generator of superoxide anion radicals, caused a rapid increase in the GSSG level and a decrease in the GSH level, following a 1.5- to 2-fold increase in the GSH level during the posttreatment incubation (23). Menadione also caused a concentration- and time-dependent increase in the

activity of gamma-glutamylcysteine synthetase (gamma-GCS), a rate-limiting enzyme in the synthesis of GSH. These results suggested that the increase in the GSH level after treatment with menadione was due to the increase in the gamma-GCS activity. Thus, our findings indicate that the modification of reactive oxygen radicals by WSP and HC may be responsible for the decreased gamma-GCS activity, resulting in the increase in GSSG concentration in PDL 20→50 cells. The GSSG/GSH ratios in HC-treated PDL 20→50 cells were significantly higher than those in the control group (Table 1-2). It may be the reason behind the observed changes in the glutathione system due to HC, consisting of glutathione depletion and lowered GR activities in alveolar epithelial-type II cells, in contrast with unchanged activities in a fibroblast-like lung cell line (24).

Our comparison (Tables 1 & 2) of the amounts of GSSG and related enzymes (GR and GSH-Px) in PDL 20→50 and PDL 50→75 cells is highly informative given the importance of these enzymes in the delay of cellular aging. With regards to WSP-treated PDL 50→75 cells, the GR activity increases while the GSSG concentration decreases just before the cells start to degenerate. Table 3-1 shows that there were significantly high decreases in GSSG concentration in WSP-treated PDL 20→55 cells as compared with that in the control. Although the reasons for this are unknown, we demonstrated previously that WSP (2.5 µg/ml) has a small amount of sulfhydryl groups (1.8 µM). The capability of WSP-treated cells to synthesize glutathione may be observed in the present experiments. Because of their functions, these changes might provide an explanation for the considerable decrease in GSSG concentration.

GSH is a nonprotein, soluble thiol, present at millimolar

levels in the cells, which plays an important role in the maintenance of cellular thiols (25). Because *N*-acetylcysteine (NAC) is known to be a precursor of GSH synthesis in some cells (26), it is often used to maintain GSH levels after various cell treatments. WSP has sulfhydryl groups similar to NAC. The results of the GSSG reduction in the WSP-treated PDL 50→75 or PDL 20→55 cells presented here may be consistent with this mechanism of NAC. It might be hypothesized that WSP is the substance that enhances GSH synthesis, e.g., NAC.

WSP may slightly modify GSH-Px activity in PDL 50→75 human fibroblasts. This behavior may be a defense mechanism against cellular aging. Reactive behavior is well documented for other components (10) and is consistent with our previous findings on cellular aging modification (9). One interpretation of WSP-instigated reactive behavior is based on a composite view of all the modifications and degenerative processes associated with cellular aging. We can therefore confidently assert our belief that this reaction stops the degenerative process. Because lower levels of GSSG were observed in the WSP-treated PDL 50→75 or 20→55 cells than in the control cells (Tables 1-1 and 3-1), we hypothesize that the antioxidant molecule WSP protects cells against cellular aging. In future experiments, we will explore the mechanism of these responses in more detail.

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