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Intracellular glutathione status regulates mouse bone marrow monocyte-derived macrophage differentiation and phagocytic activity☆

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Abstract

Although a redox shift can regulate the development of cells, including proliferation, differentiation, and survival, the role of the glutathione (GSH) redox status in macrophage differentiation remains unclear. In order to elucidate the role of a redox shift, macrophage-like cells were differentiated from the bone marrow-derived monocytes that were treated with a macrophage colony stimulating factor (M-CSF or CSF-1) for 3 days. The macrophagic cells were characterized by a time-dependent increase in three major symptoms: the number of phagocytic cells, the number of adherent cells, and the mRNA expression of c-*fms*, a M-CSF receptor that is one of the macrophage-specific markers and mediates development signals. Upon M-CSF-driven macrophage differentiation, the GSH/GSSG ratio was significantly lower on day 1 than that observed on day 0 but was constant on days 1–3. To assess the effect of the GSH-depleted and -repleted status on the differentiation and phagocytosis of the macrophages, GSH depletion by BSO, a specific inhibitor of the de novo GSH synthesis, inhibited the formation of the adherent macrophagic cells by the down-regulation of c-*fms*, but did not affect the phagocytic activity of the macrophages. To the contrary, GSH repletion by the addition of NAC, which is a GSH precursor, or reduced GSH in media had no effect on macrophage differentiation, and led to a decrease in the phagocytic activity. Furthermore, we observed that there is checkpoint that is capable of releasing from the inhibition of the formation of the adherent macrophagic cells according to GSH depletion by BSO. Summarizing, these results indicate that the intracellular GSH status plays an important role in the differentiation and phagocytosis of macrophages.

Keywords: Macrophages; Cell differentiation; Phagocytosis; GSH/GSSG ratio; Redox state

In all aerobic organisms, reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), the superoxide anion ($O_2^{\bullet -}$), and hydroxyl radicals ($OH^{\bullet}$), are produced continuously by the cellular metabolism [1,2]. ROS are toxic by-products of the cellular oxygen metabolism and can damage cells and tissues by reacting with intracellular macromolecules such as proteins, nucleic acids, and lipids. Therefore, ROS have been implicated

☆ *Abbreviations:* ROS, reactive oxygen species; GSH, glutathione; M-CSF, macrophage colony stimulating factor; BSO, L-buthionine-[*S*,*R*]-sulfoxide; NAC, *N*-acetylcysteine; DTNB, dithionitrobenzoic acid; BMMs, bone marrow-derived monocytes; DCFH-DA, 2′,3′-dichlorofluorescin diacetate; γ-GCS, γ-glutamylcysteinyl synthetase.

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in diverse cellular events such as apoptosis, cancer, aging, proliferation, and differentiation [3–6]. In order to protect against these harmful ROS, aerobic organisms have two antioxidant defense systems, enzymatic and non-enzymatic components, which contribute to the homeostasis of the intracellular redox state.

Enzymatic antioxidants include glutathione peroxidase, glutathione reductase, glutaredoxin, catalase, superoxide dismutase, thioredoxin reductase, and thioredoxin, all of which can scavenge ROS and maintain the thiol groups of the protein [7–11]. The non-enzymatic components, which can provide redox power in the cells, include vitamins A, C, and E, β-carotene, and glutathione (GSH) [12,13]. Among these components, GSH is a ubiquitous tripeptide, γ-glutamylcysteinylglycine, which plays a pivotal role in the antioxidant defense system in all aerobic organisms. GSH and its redox cycle play important roles in catabolizing H_2O_2 and other peroxides through an enzymatic coupling reaction, detoxifying electrophiles, and protecting the thiol groups of proteins from oxidation [14,15]. Several reports have indicated that modulation of the GSH redox cycle can regulate the inflammatory cytokine-induced apoptosis, cell growth, embryonic development, and chondrocyte differentiation [13,16–20]. In addition, it is known that the intracellular GSH contents are altered by various stimuli such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA), menadione, dexamethasone, hyperoxia, phorbol myristate acetate (PMA), paraquat, and the tumor necrosis factor α (TNF-α) [21–25].

In the macrophages, a growing body of evidence suggests that ROS play a role in a functioning macrophage. The phagocytic function of a macrophage is achieved as a result of an increase in the production of ROS, a so-called "respiratory burst," which is generated by the activation of the NADPH oxidase complex [26]. In addition, Boggs et al. [27] reported that the intracellular GSH levels could regulate ROS-mediated apoptosis in the macrophages.

Despite the cumulative data showing that the GSH status is related to differentiation and apoptosis, and is modulated by various stimuli, the effects of the GSH status on the differentiation and phagocytic function of macrophages are still unknown. In order to understand the role of GSH in regulating differentiation and the phagocytic function in macrophages, this study examined whether the GSH/GSSG ratio altered during the macrophage colony stimulating factor (M-CSF, also called CSF-1)-induced macrophage differentiation and whether GSH depletion or repletion can modulate the differentiation and phagocytic activity in macrophages. To accomplish this, the intracellular GSH levels were changed by a treatment with L-buthionine-[*S*,*R*]-sulfoxide (BSO), an inhibitor of GSH synthesis, and *N*-acetylcysteine (NAC) or GSH, which can enhance the GSH level. This study shows that M-CSF-induced differentiation and the phagocytic activity of macrophages could be regulated by the intracellular GSH status.

Materials and methods

Chemicals and reagents. The L-buthionine-[*S*,*R*]-sulfoxide (BSO), *N*-acetylcysteine (NAC), GSH, crystal violet, dithionitrobenzoic acid (DTNB), and NADPH were purchased from Sigma (St. Louis, MO). M-CSF was obtained from the Genetics Institute (Cambridge, MA). All other reagents were of the highest commercial grade.

Differentiation of macrophage from bone marrow-derived monocytes. The preparation of the bone marrow-derived monocytes (BMMs) and the differentiation of macrophages were achieved as described previously [28]. Briefly, the BMMs were prepared from the tibia and femur of C57BL/6 female mice (6 weeks of age) and cultured under a humidified incubator with 5% CO_2 at 37 °C in an α-MEM (Gibco-BRL, Grand Island, NY) containing 10% heat inactivated fetal bovine serum and 5 ng/ml M-CSF for 12 h in 100-mm diameter culture dishes to obtain the BMMs. In order to differentiate the BMMs into macrophages, the floating cells were counted and cultured in the presence of 30 ng/ml M-CSF for 3 days.

Adhesion assay. The BMMs were differentiated into macrophages for the indicated time points. After carefully removing the floating cells, the adherent cells were determined using a crystal violet assay as described previously [29]. Briefly, after removing the medium, the adherent cells were washed with PBS and fixed with 10% formalin followed by staining with 0.5% crystal violet for 1 h. The adherent cells stained with crystal violet were dissolved with a 2% SDS solution after washing with tap water. The extent of the adherent cells was measured spectrophotometrically at 570 nm using a microplate reader (Bio-Rad, Hercules, CA).

Reverse transcription-polymerase chain reaction (RT-PCR). At various time points after exposure to M-CSF (30 ng/ml), the floating and adherent cells were harvested. The total RNA was extracted from the cell using the TRI-reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. After the RNA was denatured by a 70 °C incubation for 10 min and kept on ice for 5 min, the cDNA was prepared using reverse transcriptase (Supertranscript II, Invitrogen, Carlsbad, CA) and a random hexamer, and subjected to PCR amplification with *Taq* polymerase (Sigma–Aldrich). The PCR cycling was as follows: 30 cycles of denaturation at 94 °C for 1 min, annealing at 57 °C for 30 s, and extension at 72 °C for 1 min. The primers used for the M-CSF receptor (c-*fms*) were designed as described [30]: 5′-ACTCTCCAACCTGCATCGGCT-3′ and 5′-GCT CACAGCGTTGAGACTGAG-3′. The primers to normalize the expression level were prepared: β-actin; 5′-ATCATGTTTGAGA CCTTCAA-3′ and 5′-CATCTCTTGCTCGAAGTCCA-3′. All the primers were synthesized at Bioneer (Korea). The PCR products were resolved on a 1% agarose gel, stained with ethidium bromide, and visualized using a UV illuminator.

GSH/GSSG ratio. After the BMMs were exposed to M-CSF (30 ng/ml), the whole cells (floating and adherent cells) were harvested at 1-day intervals for 3 days and stored at −70 °C until use. The GSH/GSSG ratio was determined using a colorimetric assay kit for GSH (BIOXYTECH GSH-412, OXIS International, Portland, OR) according to the manufacturer's protocols, with GSSG used as standard for calibration.

Flow cytometric analysis of c-fms expression. In order to avoid non-specific binding the cells were incubated in PBS with 1% bovine serum albumin (BSA) and rabbit anti-mouse IgG (Santa Cruz Biotechnology, CA) for 15 min at 4 °C. After washing twice with PBS containing 1% BSA, the cells were incubated with rabbit anti-mouse c-*fms* antibodies (Upstate Biotechnology, Lake placid, NY) for 45 min, washed twice, and stained with FITC-labeled goat anti-rabbit IgG (Santa Cruz Biotechnology) for 45 min. After washing three times, the cells were analyzed using a flow cytometer [31].

Assays of cell proliferation and viability. The cells were cultured in the presence or absence of BSO, NAC, or GSH for the indicated times. Cell proliferation and viability were determined by assessing the level of trypan blue exclusion [32] and by a colorimetric MTT assay [33], respectively.

Measurement of intracellular peroxide. After the cells were exposed to BSO, NAC, or GSH for 15 h, the intracellular peroxide concentration was measured by a flow cytometer after loading the cells with the oxidant sensitive dye, 2′,3′-dichlorofluorescin diacetate (20 μM DCFH-DA, Sigma), for 30 min, as described previously [32].

Determination of free thiol groups. After exposing the cells to 300 μM BSO for 15 h, the whole cells, including the floating and adherent cells, were harvested using a scrapper and centrifugation, washed with ice-cold PBS, centrifuged, suspended in a lysis buffer (80 mM sodium phosphate, 2 mM EDTA), and homogenized by passing the cells through a 25-gauge syringe. The total free thiol groups in the cytosolic fractions of the cell lysates were measured by exposing the cells to DTNB as described previously [34].

Measurement of the phagocytic activity of macrophage using a flow cytometer. The BMMs (1×10^6 cells) were seeded on 60-mm culture dishes, cultured for 3 days in the presence of M-CSF (30 ng/ml), and then further incubated in the absence or presence of BSO, NAC or GSH for 15 h. A final 20 μg/ml of Fluorescein-conjugated zymosan A Bio Particles (Molecular Probes, Eugene, OR) was added to the macrophages in the culture plates, incubated for 1 h, and washed with PBS three times in order to remove the non-incorporated particles. The cells were scrapped into PBS and resuspended by pipetting. The FITC-intensity of the cells was measured using an excitation and emission set of 488 and 530 nm, respectively, using a flow cytometer with a FACS-Calibur instrument (Becton–Dickinson Immunocytometry Systems).

Results

Macrophage was differentiated from BMMs

The BMMs were isolated from the tibia and femur of the mice and differentiated into macrophages by exposing them to M-CSF. The macrophages display a broad range of phenotypes in function, morphology, cell adhesion, and cell surface molecule expression. In order to assess the differentiation of BMMs into macrophage, this study first measured the phagocytic capacity, in which the cells incorporate the FITC-conjugated particles. As expected, the percentage of phagocytic cells to total cells showed 14% in day 1, 25% in day 2, and 77% in day 3 after differentiation (Fig. 1A). It was also observed that the BMMs cultured in the M-CSF showed morphological changes from a spindle shape on day 2 to a round shape on day 3. Consistent with these results, the extent of the adherent macrophagic cells was increased significantly in a time-dependent manner by

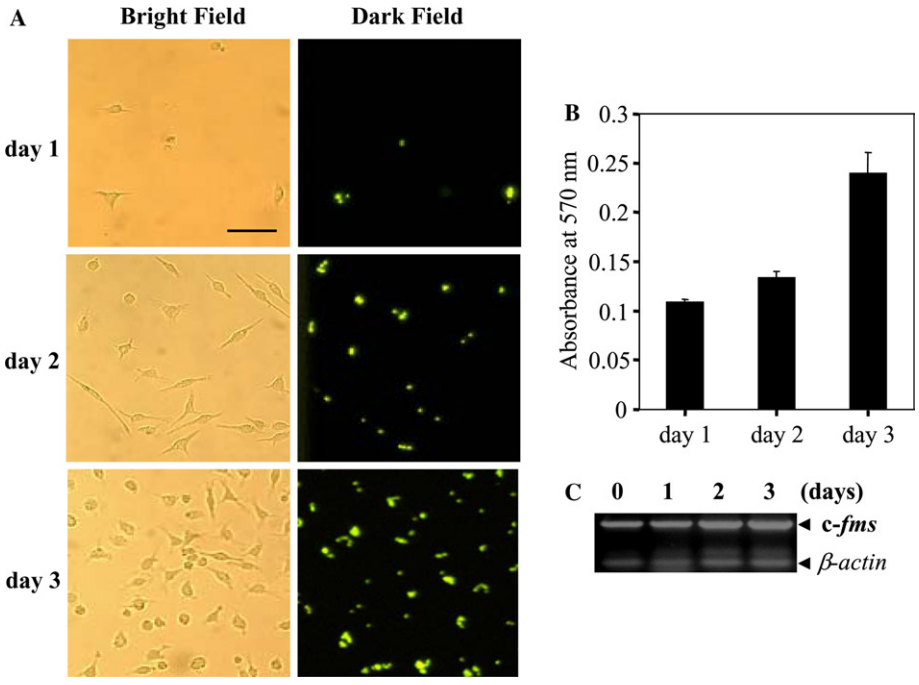

Fig. 1. The characteristics of the macrophages derived from the BMMs. (A) Phagocytic activity. The BMMs were obtained from the tibia and femur of the C57BL6 mice. After incubating the cells in the medium containing 5 ng/ml M-CSF for 12 h, the floating cells (1×10^5 cells/well) were seeded on a 24-well plate and cultured in the presence of 30 ng/ml M-CSF for the indicated time points. The cells were incubated with FITC-conjugated zymosan particles (20 μg/ml) for 1 h, washed three times with PBS, added to PBS, and photographed using a fluorescence microscope (Olympus BX51 microscope) under visible and UV illumination. Bar, 50 μm. (B) M-CSF-induced formation of the adherent macrophages. The BMMs (1×10^4 cells/well) were seeded on a 96-well plate and cultured in the presence of 30 ng/ml M-CSF. The adherent extent of the cultured cells was analyzed using crystal violet as described in Materials and methods. Data are expressed as means ± SD of the results from quintuplicate values of at least three independent experiments. (C) RT-PCR for M-CSF receptor, c-*fms*. At 1-day intervals after differentiation, the total RNA was prepared from the cells and reverse-transcribed to produce the cDNA. This cDNA allowed for PCR amplification of c-*fms* and β-actin (a housekeeping gene). The PCR products (expected fragment sizes: c-*fms*, 756 bp; β-actin, 320 bp) were analyzed on a 1% agarose gel by electrophoresis.

M-CSF (Fig. 1B). This study next tested whether the expression level of the macrophage-specific marker was increased in differentiation. The mRNA level of c-*fms*, which is known to promote the differentiation of BMMs into macrophage-like cells, which is one of the macrophage-specific markers [30], was analyzed using RT-PCR. The results showed that the mRNA level of c-*fms* gradually increased in a time-dependent manner by M-CSF (Fig. 1C). The c-*fms* expression level was 1.9-fold higher in the cells incubated with M-CSF for 3 days than in the control. Taken together, these results indicate that the differentiation of BMMs into macrophage-like cells was achieved in our model system.

GSH/GSSG ratio was decreased upon macrophage differentiation

Cells have a variety of antioxidants to protect themselves from oxidative damage. One of these antioxidants is GSH, which is an intracellular ROS scavenger in the cells. The GSH status was evaluated at 1-day intervals for 3 days after macrophage differentiation by M-CSF. Upon M-CSF-driven macrophage differentiation, the GSH/GSSG ratio was significantly decreased on day 1 compared with the day 0 control. In contrast, the GSH/GSSG ratio was maintained on day 1–3 after exposing the cells to M-CSF (Fig. 2). This ratio could be caused by changes of total GSH (GSH + GSSG) or GSSG contents. In this study, the decrease of this ratio was tightly dependent on total GSH contents. These findings indicate that the GSH status was connected to macrophage differentiation.

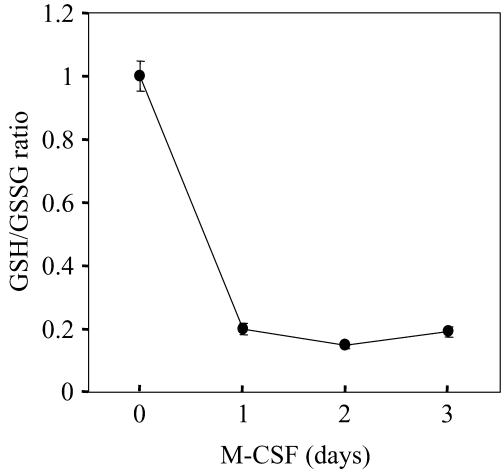

Fig. 2. Changes of GSH/GSSG ratio during differentiation. The total cytosolic extracts were prepared from the cells at the indicated time points, deproteinized, and the GSH level was assayed with a kit from OXIS International. After the concentration of the total GSH and GSSG is converted as nmol/mg of protein, the final data are expressed as the relative ratio for the day 0 control. Each value indicates the mean ± SD of triplicate results of one of three independent experiments.

BSO blocked macrophage differentiation by the down-regulation of c-fms, a M-CSF receptor gene

The effect of GSH depletion on differentiation was examined in order to determine if the alteration of M-CSF-induced GSH contents was related to macrophage

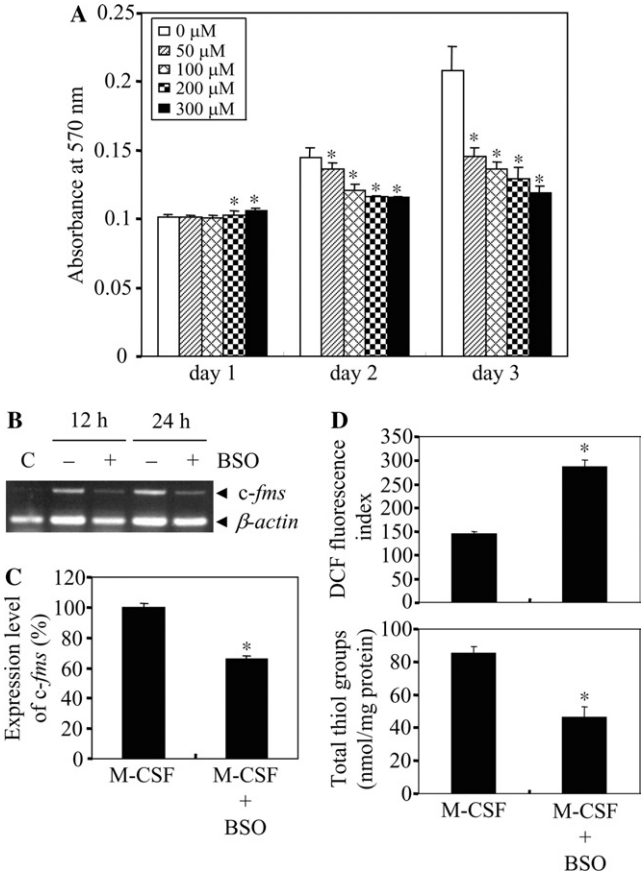

Fig. 3. Inhibitory effects of BSO on differentiation of macrophage and physiological changes as a result of GSH depletion. (A) Inhibition of the dose-dependent M-CSF-induced adherent cell formation by BSO. The BMMs (1×10^4 cells/well) were seeded on a 96-well plate and exposed to the media containing 30 ng/ml M-CSF and various concentrations of BSO. The extent of the adherent cells was assayed with crystal violet at 1-day intervals for 3 days. Data are represented as means ± SD of the values from quintuplicate experiments. $^*P < 0.01$ vs BSO-untreated control (Student's *t* test). (B) RT-PCR for the M-CSF receptor, c-*fms*. The total RNA was obtained from the cell and reverse-transcribed to make the cDNA. PCR amplification was executed by using the specific primer for c-*fms* and β-actin. (C) Flow cytometric analysis of cell surface expression of c-*fms*. The BMMs were treated with M-CSF for 24 h in the presence or absence of 300 μM BSO, harvested by scrapping, incubated with a specific antibody to the M-CSF receptor, stained with FITC-labeled IgG, and analyzed with a flow cytometer. The c-*fms* expression level as a result of BSO was represented as a percentage of M-CSF alone. (D) Determination of the redox status. After the BMMs were treated with M-CSF alone or the combined M-CSF and BSO for 15 h, the intracellular peroxide concentrations and free thiol contents in the cytosolic fraction were measured by the DCF fluorescence and DTNB, as described in Materials and methods, respectively. Data in the C and D are shown as means ± SD of the results from three independent experiments. $^*P < 0.01$ vs M-CSF alone.

differentiation. In order to deplete the endogenous GSH level, BSO, which is a specific inhibitor of GSH synthesis, was used. The BMMs treated with increasing concentrations (0–300 μM) of BSO for 3 days resulted in a BSO dose-dependent decrease in the adherent macrophagic cells (Fig. 3A). Most of the BMMs treated with 300 μM BSO remained as non-adherent cells and had a smaller size than the BSO-untreated BMMs (data not shown).

It is known that M-CSF is a key regulator of macrophage development. This event is initiated by an interaction between M-CSF and M-CSF receptor that is encoded by the c-*fms* gene and is expressed on the surface of the cells during development [35,36]. In order to identify the cellular mechanisms that lead to the inhibition of macrophage differentiation by GSH depletion, the pattern of c-*fms* expression in the presence or absence of BSO was first analyzed using RT-PCR (Fig. 3B) and flow cytometric analysis (Fig. 3C). The expression of the c-*fms* gene was increased in M-CSF alone, but was markedly repressed when combined with M-CSF and BSO. It was also observed that the c-*fms* expression level was lower in the presence of BSO, as determined by Northern blot and immunoblot analyses, and all experimental conditions to analyze c-*fms* expression did not affect the cell viability, as measured by the trypan blue exclusion and a colorimetric MTT assay (data not shown). In order to access why the BSO-treated BMMs caused the inhibition of cell differentiation, the redox status was determined by measuring the intracellular peroxide (H_2O_2) and the cytosolic free thiol content. BSO-treated BMMs triggered a higher level of H_2O_2 production (Fig. 3D, upper panel) and lower thiol contents than the BSO-untreated BMMs (Fig. 3D, lower panel). This suggests that the inhibitory effects of BSO on macrophage differentiation were caused due to the low expression of c-*fms*, which resulted from excessive ROS production by GSH depletion.

Antioxidants had no effects on macrophage differentiation

As shown in Figs. 2 and 3A, it was observed that the GSH/GSSG ratio altered upon macrophage differentiation, and the GSH depletion by BSO resulted in the inhibition of differentiation. Since multiple steps are involved in the process of macrophage differentiation, which is implicated in cell adhesion, proliferation, and motility, it was hypothesized that there is a checkpoint in macrophage differentiation, at which the differentiation is released as a result of GSH depletion by the BSO. In order to determine this checkpoint, the BMMs were treated with BSO at the indicated times after exposing them to M-CSF. On 2 day after treating the cells with M-CSF, the cells were unable to alter their fate caused by GSH depletion (Fig. 4A). This suggests that the cells have a commitment step regulated by the GSH status during macrophage differentiation. Since GSH depletion by BSO is correlated to the inhibition of macrophage differentiation (Fig. 3), the effects of the antioxidants that are capable of enhancing the GSH content in the cell on macrophage differentiation were next tested. It was found that NAC, a GSH precursor, and the reduced GSH did not affect the differentiation capacities of the BMMs (Fig. 4B).

Antioxidants, NAC and GSH, but not BSO, inhibited phagocytic activity of macrophage

It is well known that the level of ROS is increased in the course of macrophage phagocytosis [26]. In order to determine if the changes in the intracellular redox state can affect M-CSF-induced macrophagic phagocytosis,

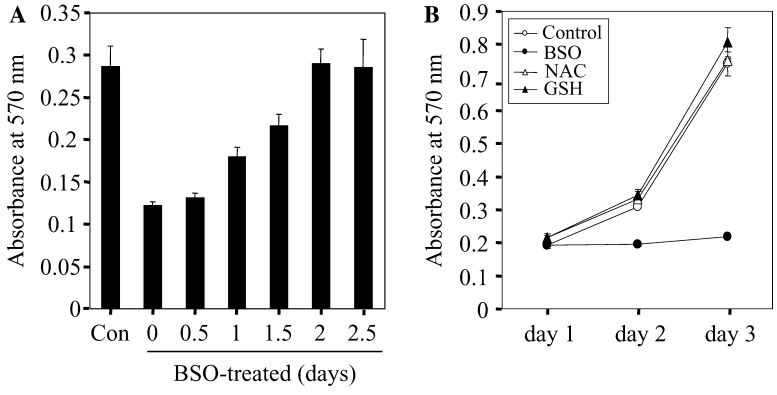

Fig. 4. Effects of BSO, NAC, and GSH on M-CSF-induced macrophage differentiation. (A) Checkpoint of differentiation as a result of GSH depletion. The BMMs were prepared, seeded on a 96-well culture plate, and cultured as shown in Materials and methods. The BSO was then added to cells at 12 h-intervals after exposing them to M-CSF. On day 3, the extent of the adherent cells was assayed using crystal violet. (B) The effects of the alteration of the intracellular redox state on macrophage differentiation. The BMMs were seeded on a 24-well culture plate (1×10^5 cells/well) and cultured in media containing 30 ng/ml M-CSF in the presence or absence of BSO (300 μM), NAC (2 mM), and GSH (2 mM). The extent of the adherent cells was assayed with crystal violet at 1-day intervals for 3 days. Data are represented as means ± SD of the results from quintuplicate experiments.

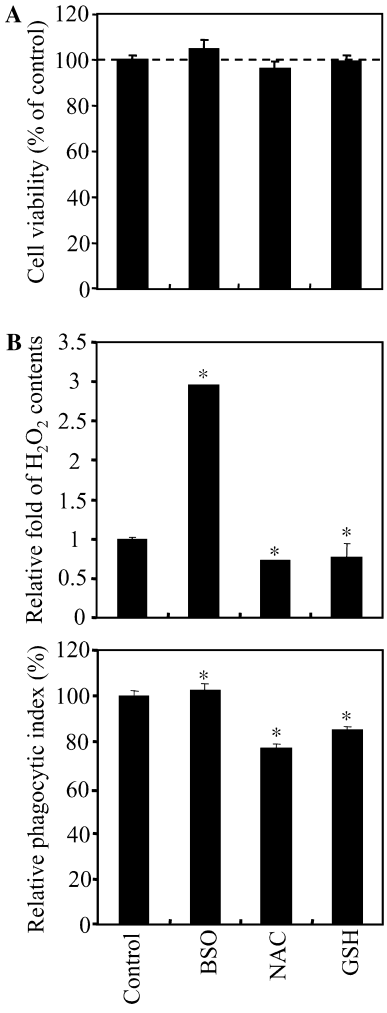

Fig. 5. Inhibitory effects of antioxidants on phagocytosis. (A) Cell viability. The M-CSF-treated BMMs (1×10^4 cells/well) were seeded on a 96-well culture plate in quintuplicate, differentiated for 3 days, and then treated with 300 μM BSO, 2 mM NAC, or 2 mM GSH for 15 h. The cell viability for the macrophage was monitored using a colorimetric MTT assay. (B) Flow cytometric analysis for the intracellular H_2O_2 level (upper panel) and phagocytosis (lower panel). On day 3 after differentiation, the cells were further incubated with 300 μM BSO, 2 mM NAC, or 2 mM GSH for 15 h at 37 °C. In order to determine the intracellular peroxide concentration, the cells were loaded with DCFH-DA for 30 min, washed with PBS, harvested in PBS using a scrapper, and then subjected to flow cytometry analysis. The intracellular peroxide contents were expressed as the relative fold to the control. In order to measure the phagocytic capacities, the cells were treated with FITC-conjugated zymosan particles for 1 h, washed with PBS to remove the particles that were not incorporated by the macrophage, harvested in PBS using a scrapper, and subjected to flow cytometry. At least 20,000 cells were then sorted. Histograms were analyzed by the CELLQuest programs (Becton–Dickinson). The phagocytic indices are expressed as a percentage of the control values. Data are represented as means ± SD of triplicate values from one of three independent experiments. $^*P < 0.01$ vs control (Student's t test).

the macrophage-like cells were induced by exposing them to M-CSF for 3 days, pretreated them with BSO, NAC, and GSH for 15 h, and then the phagocytic activity was measured using FITC-conjugated zymogen particles incorporated in the cells. Under these experimental conditions, BSO, NAC, and GSH, in addition to all the reagents used, did not cause any cytotoxicity to the cells, as measured by a colorimetric MTT assay (Fig. 5A). Since the phagocytic function of macrophages correlates with the level of intracellular ROS production, the intracellular H_2O_2 content was measured under the perturbation of the redox status. As expected, GSH depletion by BSO resulted in an increase in the H_2O_2 content compared with the control, but did not affect the phagocytic function of the macrophages. However, NAC and GSH, which can enhance the redox potential in the cells, induced a lower H_2O_2 content (Fig. 5B, upper panel) and phagocytic activity (Fig. 5B, lower panel) than control. These findings suggest that the redox shift by GSH can regulate the phagocytic capacities of the macrophages.

Discussion

It has been reported that redox shifts can modulate cell development. GSH, which is the most abundant redox equivalent, is one of determinants of the intracellular redox status. However, the role of GSH in regulating macrophage differentiation in addition to its role in the phagocytic function is unclear. This study demonstrated that (i) the GSH/GSSG ratio decreased during the differentiation of the BMMs into macrophage-like cells; (ii) GSH depletion inhibited macrophage differentiation; (iii) the inhibitory effect of GSH depletion on differentiation was elicited by the down-regulation of c-*fms* gene expression; (iv) there was checkpoint that the GSH status was capable of committing the differentiation of macrophage; and (v) the GSH status can regulate the phagocytic activity of macrophage.

The redox status in the cells plays an important role in the cellular development, such as proliferation, differentiation, and apoptosis [20,35,36]. Many studies have been reported that most developing cells represent redox shifts to more oxidizing environments with a low reducing state during development [18,20,37]. In general, the redox state decreased according to the following rank order: proliferation > differentiation > apoptosis. Under the cellular development, the balance between the reducing and oxidizing equivalents determines the intracellular redox state. Therefore, the redox state in the cells could modulate the ratio of the reversible oxidized and reduced biological redox couples, such as [NADH/NAD^+], [NADPH/$NADP^+$], [GSH/GSSG], [thioredoxin-SH2/thioredoxin-SS], and [protein-SH/protein-SS] [20]. Among these redox couples, GSH is a major reducing agent that can catabolize H_2O_2 and other peroxides by enzymatic coupling reactions and protect the protein thiol groups from oxidation. Since GSH contributes to approximately 90% of all the cellular reducing

equivalents in the cell [19,38,39], many reports estimate the value of the redox status of the cell by taking the ratio, GSH/GSSG. These results demonstrate that GSH has multiple functions ranging from antioxidant defense to the modulation of cell development. In this study, it was observed that the GSH/GSSG ratio altered in the cell during M-CSF-driven macrophage differentiation. This ratio was steeply decreased on day 1 compared with the day 0 control. Otherwise, this ratio was observed to maintain over the time course (day 1–3) of differentiation. From these results, it was hypothesized that the GSH status may be connected to macrophagic development. In order to assess the influence of the GSH status on differentiation, an attempt was made to either deplete GSH using BSO, a specific inhibitor of GCS that is a key enzyme in de novo GSH synthesis [40], or to replete it by treating the cells with NAC, a GSH precursor [41], or reduced GSH to the media. While antioxidants such as NAC and GSH, which can enhance the GSH content in the cell, had no effect on M-CSF-driven macrophage differentiation, the GSH depletion as a result of BSO inhibited the formation of the adherent macrophagic cells. It was also observed that there is checkpoint that is capable of determining their fates by the GSH status upon macrophage differentiation. This suggests that the intracellular GSH status can regulate the M-CSF-driven macrophage differentiation.

The next stage of these experiments was aimed at determining how GSH depletion inhibits macrophage differentiation. Until recently, a growing number of studies have shown that the coordination between M-CSF and M-CSF receptor mediates a serial cascade reaction for the cellular signaling pathway of macrophage development [35,36,42,43]. M-CSF, which is one of the growth factors, is essential for the development of BMMs. It acts via the M-CSF receptor, which is a cell surface tyrosine kinase receptor. The binding of M-CSF to its receptor induces the autophosphorylation of tyrosine kinase, which then propagates the cellular signaling for proliferation, differentiation, and survival. It was also reported that M-CSF stimulates c-*fms* expression via the binding of several transcription factors to the c-*fms* promoter [44–47]. In this model, GSH depletion down-regulated the expression of c-*fms*, which resulted in a decrease in M-CSF-dependent differentiation. The main question to be answered is 'What is the underlying cause of the inhibition of macrophagic differentiation?'. It was postulated that one of the possible candidates in this inhibition was ROS caused by a reduction in the redox state upon M-CSF-driven differentiation, and an additional decrease in the redox state due to artificial GSH depletion. In this study, GSH depletion caused by the combined effect of M-CSF and BSO induced a higher ROS level than M-CSF alone, elevated the redox shifts to a more oxidizing environment, and finally led to the inhibition of macrophagic differentiation.

It is well known that the phagocytic function of the macrophage is elicited by an increase in the production of ROS, which is termed a "respiratory burst" [26]. Consistent with these results, GSH repletion to promote the reducing redox status in the cell induced a decrease in the phagocytic function of the macrophages, while GSH depletion caused a low reducing redox status in the cell and failed to regulate its phagocytic function. This study showed that GSH depletion induced a block of M-CSF/M-CSF receptor-mediated signaling pathway by the down-regulation of c-*fms* gene expression, which resulted in a decrease in the differentiation of BMMs into macrophages. Overall, these findings suggest that GSH has several vital functions. These include protecting the cells from cytotoxic effects of ROS, regulating the differentiation of macrophages, and modulating its phagocytic activity.

Acknowledgments

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