Catalase Plays a Critical Role in the CSF-independent Survival of Human Macrophages via Regulation of the Expression of BCL-2 Family*

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M-colony-stimulating factor (M-CSF)-induced monocyte-derived macrophages (M-MΦ) required continuous presence of M-CSF for their survival, and depletion of M-CSF from the culture induced apoptosis, whereas human alveolar macrophages (A-MΦ) and granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced monocyte-derived macrophages (GM-MΦ) survived even in the absence of CSF. The expression of BCL-2 was higher in M-MΦ and M-CSF withdrawal down-regulated the expression. The expression of BCL-XL was higher in A-MΦ and GM-MΦ, and the expression was CSF-independent. The expression of MCL-1 and BAX were not different between M-MΦ and GM-MΦ and were CSF-independent. Down-regulation of the expression of BCL-2 and BCL-XL by RNA interference showed the important role of BCL-2 and BCL-XL in the survival of M-MΦ and GM-MΦ, respectively. Human erythrocyte catalase (HEC) and conditioned medium obtained from GM-MΦ or A-MΦ cultured in the absence of GM-CSF prevented the M-MΦ from apoptosis and restored the expression of BCL-2. The activity of the conditioned medium was abrogated by pretreatment with anti-HEC antibody. Anti-HEC antibody also induced the apoptosis of M-MΦ cultured in the presence of M-CSF and GM-MΦ and A-MΦ cultured in the presence or absence of GM-CSF and down-regulated the expression of BCL-2 and BCL-XL in these MΦs. GM-MΦ and A-MΦ, but not M-MΦ, can produce both extracellular catalase and cell-associated catalase in a CSF-independent manner. Intracellular glutathione levels were kept equivalent in these MΦs, both in the presence or absence of CSF. These results indicate a critical role of extracellular catalase in the survival of human macrophages via regulation of the expression of BCL-2 family genes.

Human tissue macrophages (MΦ) play important roles for homeostasis, and in vivo alveolar (A)-MΦ acquire a strong antioxidant phenotype that contributes to prevention of the oxidant burst in an aerobic environment and can survive for long periods (1). In a previous study, we reported that human A-MΦ and GM-CSF-induced monocyte-derived macrophages (GM-MΦ) are resistant to hydrogen peroxide (H2O2) via their high basal and inducible levels of catalase activity and that M-CSF-induced monocyte-derived macrophages (M-MΦ) are sensitive to low levels of H2O2 with low levels of catalase activity (2). GM-MΦ is phenotypically identical to A-MΦ, whereas M-MΦ closely resembles peritoneal MΦ in respect to morphology, cell surface antigen expression, and several biological functions (2–7). In vivo A-MΦ express BCL-2 family proteins such as BCL-2 and BCL-XL that prevent H2O2-induced apoptosis via inhibition of caspase-3 or -9 activation and cytochrome c release from mitochondria (8–11). These findings suggest that a high level of catalase activity enables long survival of GM-MΦ and A-MΦ with positive regulation of BCL-2 family protein. In this study, we found that M-MΦ absolutely require CSF for their survival and express high levels of BCL-2 gene and protein in the presence of M-CSF. In contrast to M-MΦ, GM-MΦ and A-MΦ can survive in the absence of CSF via high levels of BCL-XL gene and protein. We further examined the relation between catalase activity and distinct expression of BCL-2 family protein and make clear the roles of CSF in the regulation of catalase activity and BCL-2 family protein in human tissue macrophages under the influence of oxidative stress.

EXPERIMENTAL PROCEDURES

Preparation and Culture of Macrophages—Monocytes (Mo) were obtained from peripheral blood mononuclear cells of normal healthy volunteers using a magnetic cell separation system (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) with anti-CD14 monoclonal antibody-coated microbeads as described previously (7). CD14+ Mo were cultured in RPMI 1640 medium (Nissui Seiyaku Co., Ltd., Tokyo, Japan), 3 mg/ml filtered glutamine (Sigma), 100 units/ml penicillin G potassium (Banyu Seiyaku Co., Ltd., Tokyo, Japan) at 37 °C in humidified 5% CO2 for 7 days. During the culture, Mo differentiated to MΦ.

Human A-MΦ were obtained from healthy volunteers (non-smokers without diseases) by bronchial alveolar lavage method (2, 7). All volunteers gave informed consent to the use of their A-MΦ in this study.

Antisense Oligonucleotide (AS) Treatment—2′-O-methyl-modified oligoribonucleotide phosphorothioate 18-mer two CpG motifs targeted to the BCL-2 initiation codon (G3139 (BCL AS): 5′-TCTCCCCAGCGT−O−methyl-modified oligoribonucleotide phosphorothioate 18-mer to the 5′-splice site of BCL-XL; MS, missense; anti-Cat ab, anti-HEC antibody.
GCGCCAT-3'), G3139 variant with single base mismatch at each CpG motif (G4126 (BCL missense (MS)): 5'-TCTCCAGCAGTGCCAT-3') (12) and 18-mer to the 5'-splice site of BCL-XL (5'-BCL-X AS, ACCCAAGCCCGCUUCCUCG) (13) and MCL-1 AS (ISIS 20 408, TTG-GCTTTGTCTCTTGGCCG) (14) were synthesized by Prologo France SAS (Paris, France). Oligonucleotides with random sequences were used as negative controls. Cells were treated with oligonucleotides complexed with Lipofectin (5 mg/ml; Invitrogen) cationic lipid delivery agent according to the manufacturer's directions.

Assessment of Cell Number and Cell Viability—The number of adherent Mo and MΦ was determined by counting the liberated intact nuclei from lysed cells stained with 1% (w/v) cetyltrimethyl ammonium bromide (Cetavlonave; Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 0.1 M citric acid with 0.05% (w/v) naphthol blue black (Sigma). Cell viability was assessed by the trypan blue dye exclusion test.

Assessment of Apoptosis—DNA fragmentation was detected by immunohistochemical staining using the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling method (Apo-tag kit; Oncor Co.) or visualized as DNA ladder formation (5). Cells were preincubated with 1% H2O2 in phosphate-buffered saline for inactivation of endogenous peroxidase for 5 min at room temperature. Incorporation of digoxigenin-conjugated dUTP to the terminal 3'-OH of fragmented DNA by exogenous terminal deoxynucleotidyl transferase was carried out at 37 °C for 1 h. The reaction products were incubated with horseradish peroxidase-linked anti-digoxigenin antibody at 37 °C for 30 min and visualized with the substrate 3–3-diaminobenzidine plus 0.6% H2O2.

Cells were lysed with hypotonic lysis buffer (10 mM Tris-Cl (pH 7.4), 10 mM EDTA (pH 8.0), 0.5% Triton-X), and crude DNA was extracted from the lysed cells by incubation with 40 μg/ml RNase and 40 μg/ml proteinase K for 1 h at 37 °C. The DNA was precipitated with final 50% propanol at −20 °C overnight and washed with 70% (w/v) ethanol. Electrophoresis was performed in 2% agarose at 50 V, and the migrated DNA was visualized by ethidium bromide staining.

Transmission Electron Microscopy—Cells were fixed by immersion in a 2.5% glutaraldehyde-2% paraformaldehyde mixture (10), followed by 1% glutaraldehyde-0.5% tannic acid diluted in 0.1 M cacodylate buffer (Wako Pure Chemical Industries, Ltd.) at 4 °C for 2 h. Samples were post-fixed with 1% OsO4 (osmium tetroxide; Wako) at 4 °C for 2 h and then embedded in epoxy resin (Epok 812; Okenshoji. Co., Ltd., Tokyo). Thin sections were cut using a LKB-8800 ultratome (LKB, Uppsala, Sweden) and observed using a transmission electron microscope (Hitachi H-7000) after staining with uranyl acetate (Serva Electrophoresis GmbH)-0.2% lead citrate buffer (Wako).

Neutralization of Conditioned Medium—Conditioned medium was obtained from MΦ cultured in medium alone for 48 h at 37 °C and incubated for 60 min at 37 °C with 10 μg/ml rabbit anti-human erythrocyte catalase (HEC) IgG (lot PTC 9301; Athens Research and Technology, Inc.), mouse anti-BCL-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-BCL-xL/S antibody (Santa Cruz Biotechnology), rabbit anti-BCL-X antibody (Transduction Laboratories, Lexington, KY), mouse anti-MCL-1 antibody (Santa Cruz Biotechnology), rabbit anti-BAX antibody (Santa Cruz Biotechnology), or normal rabbit or mouse IgG and then at room temperature for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG or anti-mouse IgG (Santa Cruz Biotechnology). The blots were visualized with Amersham ECL reagent on Hyper ECL film (Amersham Biosciences).

Reverse Transcription and Polymerase Chain Reaction—Total RNAs (1 mg) were prepared by use of RNA Zol B (Cinna/Biotecx Laboratories, Friendswood, TX) and reverse transcribed by incubation in 50 μl of 10 mM Tris-HCl (pH 8.3), 6.5 mM MgCl2, 50 mM KCl, 10 mM dithiothreitol, each dNTP at 1 mM, 2 μM random primer, and 2.4 units/ml Moloney murine leukemia virus reverse transcriptase for 1 h at 42 °C (Takara Shyzo, Otsu, Japan). The conditions for PCR were as follows: in a 50-μl reaction, 0.15 mM each primer, each dNTP at 2.5 mM, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, and 1.25 units of Taq polymerase (Takara Shyzo). Primers used were as follows: glyceraldehyde-3-phosphate dehydrogenase: sense, 5'-CTCTCATTGACCTCAACTACT-3' and antisense, 5'-AGTGAAGCTGAGCGAGTGTC-3'; catalase: sense, 5'-CATTCTACCAGCTCAAGAATTG-3' and antisense, 5'-AGCAAGATTGTAGATTTCCAT-3'; BCL-XL: sense, 5'-CTTTCCACGTCACAGAACATTT-3' and antisense, 5'-GACACGATTGTAGATTTCCAT-3'; MCL-1: sense, 5'-TTGGAACATGCTGAGTTCTA-3' and antisense, 5'-GATAGGTGAGGGTCTCATG-3'; BAX: sense, 5'-AGAAGCTGAGGCAAGTGC-3' and antisense, 5'-GG-CCCGCAGTTGAGTTGC-3'. Reactions were incubated in a PerkinElmer DNA thermal cycler for 25 cycles (with each cycle consisting of denaturation for 30 s at 95 °C, annealing for 30 s at 60 °C, and extension for 60 s at 72 °C) (5).

Statistical Analyses—Statistical analyses of the data were performed using Student's t-test. p values <0.01 were considered significant. The results shown are representative of three to seven independent experiments.
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FIGURE 1. Susceptibility of CSF-induced monocyte-derived MΦ and A-MΦ to CSF withdrawal. M-MΦ and GM-MΦ (2.5 × 10\textsuperscript{5}/ml/well) were cultured in medium with or without M-CSF or GM-CSF, and A-MΦ were cultured in medium without GM-CSF. A, cell number and viability of MΦs were assessed using Cetavlon and trypan blue dye as described under “Experimental Procedures.” Values are expressed as the means of triplicates (standard deviation). B, CM was collected from M-MΦ, GM-MΦ, and A-MΦ cultured in the presence of M-CSF or GM-CSF and floated in serum-free medium supplemented with a low level of extracellular catalase (21). Therefore, we examined whether extracellular catalase prevents apoptosis but both GM-MΦ and A-MΦ can survive in the absence of CSF. C, transcriptional levels of the BCL-2 and BCL-X \textsubscript{L} genes in M-MΦ, GM-MΦ, and A-MΦ were assessed using RT-PCR. MCL-1 and BAX proteins were not affected by CSF deprivation (Fig. 2A). In contrast to M-MΦ and GM-MΦ, A-MΦ expressed strongly the BCL-X \textsubscript{L} gene but weakly the BCL-2 gene, and GM-CSF withdrawal had no significant effect on the expression of these two genes (Fig. 2A). In contrast to BCL-2 and BCL-X \textsubscript{L}, the transcriptional levels of the MCL-1 and BAX genes were not significantly different between M-MΦ and GM-MΦ and were not affected by CSF deprivation (Fig. 2A). In accordance with the gene expression, the expression of the BCL-2 protein was higher in M-MΦ compared with GM-MΦ and M-CSF withdrawal decreased the expression of BCL-2 protein in M-MΦ (Fig. 2B). Similarly, the expression of BCL-X \textsubscript{L} protein was higher in GM-MΦ than in M-MΦ, but GM-CSF withdrawal had no significant effect on the expression of BCL-X \textsubscript{L} protein in GM-MΦ (Fig. 2B).

Different Expression of BCL-2 and BCL-X \textsubscript{L} Genes in M-MΦ, GM-MΦ, and A-MΦ and the Opposite Effect of CSF on Expression——BCL-2 family genes play important roles in the apoptosis of many types of cells (10, 16—20). We therefore examined the expression of BCL-2 family genes and the effect of CSF on their expression in M-MΦ and GM-MΦ by RT-PCR. In M-MΦ, the transcript of the BCL-2 gene was stronger than that of the BCL-X \textsubscript{L} gene, and M-CSF withdrawal decreased the expression of the BCL-2 gene but induced a slight decrease in the expression of the BCL-X \textsubscript{L} gene (Fig. 2A). In contrast to M-MΦ, GM-MΦ expressed strongly the BCL-X \textsubscript{L} gene but weakly the BCL-2 gene, and GM-CSF withdrawal had no significant effect on the expression of these two genes (Fig. 2A). In contrast to BCL-2 and BCL-X \textsubscript{L}, the transcriptional levels of the MCL-1 and BAX genes were not significantly different between M-MΦ and GM-MΦ and were not affected by CSF deprivation (Fig. 2A). In accordance with the gene expression, the expression of the BCL-2 protein was higher in M-MΦ compared with GM-MΦ and M-CSF withdrawal decreased the expression of BCL-2 protein in M-MΦ (Fig. 2B). Similarly, the expression of the BCL-X \textsubscript{L} protein was higher in GM-MΦ than in M-MΦ, but GM-CSF withdrawal had no significant effect on the expression of BCL-X \textsubscript{L} protein in GM-MΦ (Fig. 2B).

The expression of BCL-2 family proteins in A-MΦ resembles that of GM-MΦ, and the expression was independent of CSF (Fig. 2B). As shown in Figs. 6 and 7, the expression of MCL-1 and BAX proteins in both MΦs was not changed in the presence or absence of CSF, in accordance with the expression patterns of the MCL-1 and BAX genes.

HEC Stimulates the Survival and the Expression of BCL-2 in CSF-withdrawn M-MΦ, and Anti-HEC Antibody Abolishes the Survival-stimulating Activity of CM Obtained from CSF-withdrawn GM-MΦ and A-MΦ——The above findings suggest that GM-MΦ and A-MΦ, but not M-MΦ, can produce factor(s) that maintain their survival in the absence of CSF. To investigate this possibility, the conditioned medium (CM) obtained from these MΦ cultured for 48 h without CSF was used to examine its effect on the survival of CSF-withdrawn M-MΦ. CM of GM-MΦ or A-MΦ, but not of M-MΦ, prevented cell death of M-CSF-depleted M-MΦ (Fig. 3A).

A previous study showed that human CEM T cells can survive in serum-free medium supplemented with a low level of extracellular catalase (21). Therefore, we examined whether extracellular catalase prevents apoptosis in M-CSF-withdrawn M-MΦ and stimulates the expression of both BCL-2 gene and protein. Addition of HEC prevented M-CSF-withdrawn M-MΦ from undergoing cell death in a dose-dependent manner, and 10 units/ml of HEC completely rescued the cells.
from apoptosis. Addition of 10 units/ml HEC to GM-CSF-withdrawn GM-MΦ had no such effect (Fig. 3A).

Then we examined the effect of anti-HEC antibody (anti-Cat Ab) on the activity of the CM of GM-MΦ. Pretreatment of the CM with anti-Cat Ab, but not with control IgG, completely abrogated the ability to rescue the cell death of M-CSF-withdrawn M-MΦ (Fig. 3B). Similar results were obtained in experiments using the CM of A-MΦ (data not shown).

Next, we examined the role of catalase in the expression of BCL-2 family genes in M-CSF-withdrawn M-MΦ. Addition of catalase to M-CSF-withdrawn M-MΦ restored the expression of BCL-2 but did not affect the gene expression of BCL-XL, MCL-1, or BAX (Fig. 2A). Addition of catalase did not significantly change the gene and protein expression of BCL-2 family genes in GM-CSF-withdrawn GM-MΦ (Fig. 2A). These results suggest that the active molecule in the CM of GM-MΦ and A-MΦ that can rescue the survival of M-CSF-withdrawn M-MΦ via restoration of BCL-2 expression is catalase.

Anti-catalase Antibody Abolished the Expression of BCL-2 in M-MΦ Cultured with M-CSF and BCL-XL in GM-MΦ or A-MΦ Cultured with or without GM-CSF and Induced Apoptosis of These MΦs—The above results indicate that catalase can stimulate the expression of BCL-2 and rescue the survival of M-CSF-withdrawn M-MΦ. Therefore we next examined whether extracellular catalase also plays a critical role in the CSF-dependent survival of M-MΦ and in the survival of GM-MΦ and A-MΦ. Addition of anti-Cat Ab suppressed the expression of BCL-2 protein in M-MΦ cultured in the presence of M-CSF and of BCL-XL.
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In accordance with these results, addition of anti-Cat Ab induced cell death of M-MΦ cultured with M-CSF and GM-MΦ cultured with or without GM-CSF, and addition of control IgG had no effect on the cell viability of these MΦs (Fig. 3C). The cell death of these MΦs induced by anti-Cat Ab was due to apoptosis, as indicated by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling staining (data not shown).

GM-MΦ and A-MΦ, but Not M-MΦ, Can Produce Enough Levels of Extracellular Catalase in the Absence of CSF—The above results suggest that GM-MΦ and A-MΦ, but not M-MΦ, can produce extracellular catalase in a CSF-independent manner and that the catalase supports the survival of those MΦs via maintenance of the expression of BCL-2 family genes. To examine this possibility, we measured catalase enzyme activity and catalase protein levels in the CMs of M-MΦ, GM-MΦ, and A-MΦ cultured for 48 h in the presence or absence of CSF. The extracellular catalase activities in the CMs obtained from GM-MΦ and A-MΦ cultured both in the presence or absence of GM-CSF were not significantly different, and the levels were ~4-fold higher (~240 milliunits/ml/well) than that of M-CSF-treated M-MΦ (~60 milliunits/ml/well) (Fig. 4A). In contrast to GM-MΦ or A-MΦ, the extracellular catalase activity in CM of M-MΦ was dependent on CSF and that in CM of M-CSF-withdrawn M-MΦ was significantly lower than that in CM of M-CSF-treated M-MΦ (~20 milliunits/ml/well (Fig. 4A)). Western blot analysis of the extracellular catalase of CMs using anti-HEC antibody showed similar results to the data of the enzyme activity (Fig. 4A).

Next, we examined the cell-associated levels of catalase activity in these MΦs (Fig. 4B). The levels of catalase activity in GM-MΦ and A-MΦ lysates was ~5 units/mg protein both in the presence or absence of CSF, whereas that in M-MΦ in the presence of M-CSF was ~1 unit/mg. The catalase activity in M-CSF-withdrawn M-MΦ lysates (~250 milliunits/mg protein) was ~4-fold lower than that in M-CSF-treated M-MΦ lysates. In agreement with the measurements of enzyme activity, similar results were observed in Western blot analyses (Fig. 4B).

To further confirm the distinction between the regulation of extracellular and cell-associated catalase activity by CSF in M-MΦ versus GM-MΦ, we examined the levels of transcription of the catalase gene in these MΦs cultured with or without CSF (Fig. 4C). The level of the transcript of the catalase gene in M-CSF-withdrawn M-MΦ was ~3-fold lower than that in M-CSF-treated M-MΦ. In contrast, the level of the catalase transcript in GM-CSF-withdrawn GM-MΦ and A-MΦ was similar to that in GM-CSF-treated GM-MΦ and A-MΦ, and the level was 5-fold higher than that in M-CSF-treated M-MΦ.

Thus, the difference of total extracellular plus intracellular catalase activity and the difference in the levels of catalase gene expression between CSF-withdrawn M-MΦ and GM-MΦ reached ~15–20- and ~15-fold, respectively. The results indicate that extracellular catalase activity is regulated at the transcription levels by CSF-dependent M-MΦ but CSF-independent GM-MΦ and A-MΦ.

Thiol Derivatives Act as Additive Effectors That Rescue the Cell Death of MΦ—The above data suggest that extracellular catalase plays a major role in MΦ survival. However, several reports have demonstrated that thiol proteins and thiol compounds such as GSH, adult T cell leukemia-derived factor, and l-cysteine play important roles in the survival of protein in both GM-MΦ and A-MΦ cultured without GM-CSF (Fig. 2B).
lymphocytes or neuronal cells in the absence of growth factors (22, 23, 24). Thus, thiol derivatives may help Mφ/H9021 survival. The level of intracellular GSH, however, was almost the same in M-Mφ/H9021 and GM-Mφ/H9021 cultured with or without CSF or cultured with catalase (Fig. 5A). Diamide, which can bind the SH groups of reduced thiols and oxidize them (22), induced cell death of M-Mφ/H9021 and GM-Mφ/H9021, but the levels of cell death were not very high (~20%) and no significant difference was observed between the effects of diamide on these two Mφs (Fig. 5B). 40 μM diamide and 10 μg/ml anti-Cat Ab showed synergistic effects on the cell viability of M-Mφ cultured with M-CSF and GM-Mφ cultured without CSF, causing reduction of their viability to <10% at 48 h (Fig. 5B). These results suggest that, in contrast to that of catalase, lymphocytes or neuronal cells in the absence of growth factors (22, 23, 24). Thus, thiol derivatives may help Mφ survival. The level of intracellular GSH, however, was almost the same in M-Mφ and GM-Mφ cultured with or without CSF or cultured with catalase (Fig. 5A), and this level (~300 pmol/10⁶ cells) was similar to that in A-Mφ (data not shown). Diamide, which can bind the SH groups of reduced thiols and oxidize them (22), induced cell death of M-Mφ and GM-Mφ, but the levels of cell death were not very high (~20%) and no significant difference was observed between the effects of diamide on these two Mφs (Fig. 5B). 40 μM diamide and 10 μg/ml anti-Cat Ab showed synergistic effects on the cell viability of M-Mφ cultured with M-CSF and GM-Mφ cultured without CSF, causing reduction of their viability to <10% at 48 h (Fig. 5B). These results suggest that, in contrast to that of catalase,
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the levels of thiol derivatives were constant in these MΦs with CSF withdrawal-induced oxidative stress and that thiol derivatives have only a minor effect and cannot support the full survival of MΦ.

BCL-2 AS and BCL-X₇ AS Dominantly Induce Cell Death of M-MΦ and GM-MΦ, Respectively—M-MΦ and GM-MΦ were treated with BCL-2 AS targeted to BCL-2 initiation codon (G3139) and 5'-BCL-X AS targeted to the downstream alternative 5'-splice site of exon 2 of the BCL-X gene (12, 13). As controls, these MΦs were treated with BCL-2 MS (G4126, variant G3139), control oligonucleotides with random sequence (oligonucleotide MS). Cell number and viability of MΦ were assessed as described in Fig. 1. Values are expressed as the means of triplicate cultures ± S.D. Western blot analysis of BCL-2, BCL-X₇, MCL-1, and β-actin proteins in cell lysates of MΦ probed using BCL-2, BCL-X (antibody from Santa Cruz Biotechnology for XL/S band, from Sinal transduction Labo for MCL-1, and β-actin antibody was performed as described in Fig. 4. The relative amounts of these proteins in cells were measured using NIH image software, and the expression levels were shown as photo-stimulating luminescence (PSL).

In contrast, treatment of GM-MΦ with 5'-BCL-X AS down-regulated the expression of BCL-X₇ protein to 20% of that of control cells at 7 days after the oligonucleotide treatment and induced cell death in a dose-dependent manner. The cell viability markedly decreased to ~15% of that of control cells at 7 days of cultivation. In GM-MΦ, however, treatment with G3139 even in the high dose such as 10 μM induced only ~10% cell death, in agreement with the low expression of BCL-2 protein in this MΦ (Fig. 7).

In contrast, treatment of GM-MΦ with 5'-BCL-X AS down-regulated the expression of BCL-X₇ protein to 20% of that of control cells treated with oligonucleotide MS at 2 days after the oligonucleotide treatment and induced the cell death in a dose-dependent manner (Fig. 7). The cell viability markedly decreased to ~25% of that of control cells at 7 days of cultivation. As shown in Fig. 6, however, ~90% of the cells are viable in M-MΦ treated with 5 mM 5'-BCL-X AS in agreement with the low expression of BCL-X₇ protein.

MCL-1, a main molecule of BCL-2 family protein (14), is expressed in both M-MΦ and GM-MΦ, but down-regulation of this protein by treatment with MCL-1 AS did not stimulate the cell death of either MΦ or affect the expression levels of BCL-2 in M-MΦ and BCL-X₇ in GM-MΦ. These findings suggest that BCL-2 and BCL-X₇ expression supported by catalase prevents cell death of M-MΦ and GM-MΦ, respectively, in agreement with the dominant expression levels of gene and protein of BCL-2 in M-MΦ and BCL-X₇ in GM-MΦ.

DISCUSSION

The present study showed that extracellular catalases has a novel role in the prevention of apoptosis in human MΦ through the dominant expression of BCL-2 in M-MΦ and BCL-X₇ in GM-MΦ and that the regulation of catalase production is CSF-dependent in M-MΦ but CSF-independent in GM-MΦ and A-MΦ. Recently, H₂O₂ has been shown to enhance oxidative damage and apoptosis in C2-ceramide-pretreated HL-60 cells via a mechanism in which C2-ceramide inhibits catalase activity by increasing caspase-3-dependent proteolysis of catalase and down-regulation of catalase mRNA (25). Overexpression of catalase inhibits oxidation-mediated apoptosis through phosphorylated BCL-2, a reduced form of BCL-2 and BAX interaction (20). Thus, constant high activity of extracellular catalases plays an important role in the prevention of ceramide- and caspase-3-induced apoptosis in CSF independent of GM-MΦ and A-MΦ and CSF dependent of M-MΦ (11, 26).

GM-CSF and M-CSF stimulate catalase induction during the differentiation of Mo into MΦ, but GM-CSF alone establishes a CSF-independent autoregulatory system of catalase production in MΦ. We previously showed that human Mo-derived GM-MΦ resembles human A-MΦ in several respects (2, 5–7). In this study, we showed that GM-MΦ and A-MΦ have a similar phenotype of the resistance to apoptosis via CSF-independent expression of catalase and BCL-X₇. Con-

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A

B

Day 7

Day 7

FIGURE 7. BCL-X<sub>L</sub> AS dominantly induced cell death of GM-MΦ. A, cell number and viability of MΦ at 7 days after 5 μm AS treatment (upper panel) and immunoblot analysis at 2 days (lower panel). B, cell number and viability of MΦ at 7 days after the indicated concentrations of AS treatment (upper panel) and immunoblot analysis at 2 days (lower panel). The experimental procedure was performed as described in Fig. 6.

consistent with our present study, A-MΦ from human smokers express higher levels of p21<sup>CDP1/WAF1</sup> and BCL-X<sub>L</sub>, but not BCL-2, and the former two molecules may reduce apoptosis (9). The autoregulatory mechanism of catalase induction in GM-MΦ and A-MΦ is not yet understood but may have a strong correlation with endogenously generated low levels of H<sub>2</sub>O<sub>2</sub> (8), because we previously showed that GM-MΦ and A-MΦ, but not M-MΦ, can increase catalase expression at both the protein and mRNA levels when stimulated with H<sub>2</sub>O<sub>2</sub> (2). Adequate low levels of H<sub>2</sub>O<sub>2</sub> may keep catalase activity constant to prevent CSF deprivation-induced apoptosis in GM-MΦ and A-MΦ.

Thiol derivatives such as GSH, adult T cell leukemia-derived factor, and i-cysteine play an important role in the survival of lymphocytes or neuronal cells in the absence of growth factors (22–24). In our study, however, the activity of thiol derivatives was not significantly different between M-MΦ and GM-MΦ before and after CSF deprivation, and they had only a partial effect on MΦ survival. The reason for the differences between our study and previous studies might have been the differences in the experimental conditions (CSF-withdrawal versus serum depletion) or the cell type examined (MΦ versus lymphocytes/neuronal cells). Compared with MΦ, lymphocytes or neuronal cells may produce lower levels of catalase so that the thiol derivatives play a dominant role in their survival (21–24).

We demonstrated that catalase regulates apoptosis through the expression of BCL-2 and BCL-X<sub>L</sub> in human MΦ used in the present study. In fact, down-regulation of these proteins by RNA interference treatment induced the cell death of the MΦs. Proapoptotic BCL-2 family protein, BAX, can induce apoptosis with permeabilization of mitochondrial membranes and cytochrome c release (10, 18, 27). In a recent study, induction of apoptosis of TF-1 cells by GM-CSF withdrawal is shown to be related to down-regulation of the MCL-1 gene, and overexpression of MCL-1 is shown to delay apoptosis (16). In our study, however, BAX and MCL-1 were not associated with the regulation of apoptosis of MΦ, because both mRNA and protein levels of these genes were not significantly changed in M-MΦ and GM-MΦ after CSF deprivation. Moreover, we showed that down-regulation of the expression of MCL-1 protein by MCL-1 AS treatment did not affect the viability of the MΦs.

Our interesting finding is that BCL-2 and BCL-X<sub>L</sub> are differently expressed in MΦ during the Mo differentiation into MΦ in the presence of M-CSF and GM-CSF; the expression of BCL-2 is dominant in M-MΦ and that of BCL-X<sub>L</sub> is dominant in GM-MΦ or A-MΦ. In accordance with such different expression patterns, we found by RNA interference experiments that BCL-2 and BCL-X<sub>L</sub> play a critical role for the survival of M-MΦ and GM-MΦ, respectively. Similar differential expression of BCL-2 and BCL-X<sub>L</sub> is also observed during the selection and maturation of mouse thymocytes toward splenic T cells (17, 19). At present, we do not know the mechanisms that control the different induction of BCL-2 and BCL-X<sub>L</sub> in M-MΦ and GM-MΦ or A-MΦ, respectively. The distal promoter region of the BCL-X<sub>L</sub> gene responds to very low levels of H<sub>2</sub>O<sub>2</sub> at exon 1B-1D in rodent cardiac myocytes, and the expression of BCL-X<sub>L</sub> protein is increased by H<sub>2</sub>O<sub>2</sub> treatment (28). Thus, GM-MΦ and A-MΦ, which possess high catalase activity even in the absence of CSF, limit the levels of endogenously generated H<sub>2</sub>O<sub>2</sub> to low levels that are suitable for BCL-X<sub>L</sub> expression.

In conclusion, the present study is the first to reveal that CSF is a critical regulator of extracellular catalase activity that maintains selective expression of BCL-2 family genes and prevents tissue-specific MΦ from apoptosis. These distinct patterns of CSF-induced regulation of catalase activity may greatly contribute to the oxidant stress-induced selection of tissue MΦs suitable for their respective microenvironments.
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