Induction of heat shock proteins and their possible roles in macrophages during activation by macrophage colony-stimulating factor

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(1) Treatment of resident peritoneal macrophages for 8 h with macrophage colony-stimulating factor (M-CSF) increased release of superoxide anion (O_2^-) stimulated by phorbol 12-myristate 13-acetate. Gel electrophoresis of pulse-labelled proteins with L-[^{35}S]methionine showed that a number of proteins were induced during activation by M-CSF. Immunoblot analysis with antibody against heat shock protein (HSP) 90, HSP70, or HSP60 demonstrated that M-CSF induced these stress-inducible HSPs; the timing of induction and level of each HSP correlated with the increase in O_2^- production. The activated macrophages acquired resistance to H_2O_2 -induced damage. M-CSF also stimulated the synthesis of a heat shock cognate protein (HSC70); however, the induction occurred at 1 h, when O_2^- production was not yet augmented, but at which time L-[^{35}S]methionine incorporation

into cell proteins was already enhanced. (2) Gel mobility shift assay with oligonucleotide coding for the heat shock element showed that M-CSF activated the heat shock factor within 15 min, and the activation continued for at least 8 h. Northern-blot analysis with a cDNA probe for human HSP70 or HSC70 showed that accumulations of HSP70 and HSC70 mRNAs coincided with the inductions of the respective proteins. (3) These results suggest that M-CSF may induce the transcriptional activation of heat shock genes, and that the stress-inducible HSPs as well as HSC70 may play an important role in the activation of macrophages by functioning as molecular chaperones and by protecting the macrophage against the auto-oxidative damage associated with the respiratory burst.

INTRODUCTION

The macrophage colony-stimulating factor (M-CSF), first identified as an L cell macrophage growth factor by Stanley and Heard [1], plays a crucial role in survival, proliferation, and differentiation of mononuclear phagocyte lineages [2]. In addition, M-CSF activates tissue macrophages and increases their capacity for killing microbes [3] or tumour cells [4]. In response to appropriate stimuli, macrophages release large amounts of partially reduced oxygen intermediates, including superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), through the activity of a membrane-associated NADPH oxidase [5]. These oxygen intermediates have been postulated to play a central role in microbicidal and cytotoxic activities. The capacity for O_2^- release is markedly enhanced by bacterial lipopolysaccharide (LPS) [6] and cytokines including γ -interferon [7], interleukin 3, granulocyte-macrophage CSF, and M-CSF [8,9]. During activation of macrophages, certain functional proteins have been reported to be induced [10], and some of them have been identified as a nitric oxide synthase [11], the phagocyte cytochrome *b*, an essential component of the O_2^- -generating oxidase [12], and prostaglandin endoperoxide synthase 2 [13].

In human blood monocytes, exposure to heat, glucose starvation, or oxidants and physiological oxidative stress during phagocytosis induce heat shock proteins (HSPs) [14–17]. In addition, activators of monocyte functions, such as LPS and tumour necrosis factor, and an inducer of differentiation, 1,25-

dihydroxyvitamin D_3 , have been shown to increase the synthesis of HSP70 in these cells [14,16]. These results suggest that HSPs may play a role in the activation and differentiation of monocyte macrophages as well as in autoprotection against various types of insults. On the other hand, it is not well understood how the stress response occurs during activation of tissue macrophages.

In the present experiments, we show that M-CSF induced stress-inducible HSP90, HSP70, HSP60 and a constitutively expressed HSP with a molecular mass of 73 kDa (HSC70) during activation of macrophages *in vitro* under LPS-free conditions. We also studied the mechanism of inductions of HSPs and their possible roles in macrophages.

MATERIALS AND METHODS

Reagents and media

Recombinant human C-terminal-truncated M-CSF [18] was used in the present experiments. The structure and biological activity of this recombinant M-CSF have already been described [19]. Superoxide dismutase (SOD, from horse heart), ferricytochrome *c*, phorbol 12-myristate 13-acetate (PMA) and LPS (from *Escherichia coli* K-235) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Catalase from bovine liver was purchased from Wako Pure Chemical Industries, Osaka, Japan. Dulbecco's modified Eagle's medium (DMEM) was from Flow Laboratories, McLean, VA, U.S.A. Hanks balanced salt solution without

Abbreviations used: CHX, cycloheximide; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; ECL, enhanced chemiluminescence; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSE, heat shock element; HSF, heat shock transcription factor; IEF, isoelectric focusing; HSP, heat shock protein; HSC, constitutive heat shock protein; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; M-CSF, macrophage colony-stimulating factor; PMA, phorbol 12-myristate 13-acetate; PVDF, poly(vinylidene difluoride); SOD, superoxide dismutase.

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Phenol Red (HBSS) was purchased from Nissui Pharmaceutical Co., Tokyo, Japan. Methionine-free RPMI 1640 was from Gibco, Grand Island, NY, U.S.A. ^{35}S -labelled protein labelling mix containing $\geq 77\%$ L-methionine and $\geq 18\%$ L-cysteine (specific radioactivity 1000–1200 Ci/mmol) was obtained from DuPont/New England Nuclear, Boston, MA, U.S.A. Polyclonal antibodies against HSP90 [20], HSC70 [21], HSP70 [22] and HSP60 [23] were gifts from Dr. Hideaki Itoh, Akita University, Japan. Rabbit antiserum raised against heat shock transcription factor 1 (HSF1) was kindly provided by Dr. Akira Nakai and Dr. Kazuhiro Nagata, Kyoto University, Japan. Milk casein was purchased from Yukijirushi Co., Sapporo, Japan. Poly(dI-dC)·poly(dI-dC) and ampholines of pH 4.0–6.5, 5.0–8.0 and 3.5–9.5 were obtained from Pharmacia LKB Biotechnology Inc., Sweden. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (> 5000 Ci/mmol), $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ (3000 Ci/mmol), T_4 polynucleotide kinase, an enhanced chemiluminescence (ECL) Western-blotting detection system, and a random-primer labelling kit were from Amersham Japan, Tokyo, Japan. cDNA probes for human HSP70 (A.T.C.C. 57494) and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; A.T.C.C. 57091) were purchased from the American Type Culture Collection, Rockville, MD, U.S.A. The human HSC70 cDNA probe was a gift from Dr. Richard I. Morimoto, Northwestern University, Evanston, IL, U.S.A.

Isolation and cultivation of resident peritoneal macrophages

C3H/He-slc mice at 8 to 12 weeks of age were purchased from Shizuoka Laboratory Animal Center, Shizuoka, Japan. Resident peritoneal macrophages were prepared in a LPS-free system as described previously [24]. All reagents to which macrophages were exposed in culture were determined to be free from detectable LPS by the *Limulus* amoebocyte lysate assay (E-toxate, Sigma). Peritoneal cells were collected by washing the peritoneal cavity with ice-cold saline. After washing the isolated cells twice with cold saline, they were suspended in DMEM and 1×10^6 , 3×10^6 , or 1×10^7 cells were placed in each well of a 24-well culture plate (Corning Glass Works, Corning, NY, U.S.A.), a 35-, or a 60-mm-diam. plastic dish (Corning Glass Works) respectively. After incubation for 2 h at 37°C in 5% $\text{CO}_2/95\%$ air, non-adherent cells were removed by vigorously washing three times with ice-cold saline. Macrophages were further cultured overnight in DMEM without any supplement and then were left untreated or were treated with various concentrations of M-CSF for the indicated times. Differential counts of the adherent cells used for experiments were determined microscopically after Giemsa and non-specific esterase staining [24]. Resident preparations were $> 95\%$ macrophages.

Assay of O_2^- release

O_2^- release from macrophages was assayed by measuring the SOD-inhibitable reduction of ferricytochrome *c* as described previously [24]. The amount of O_2^- release was spectrophotometrically determined at 550 nm and expressed in terms of nmol/mg of protein per h. Cell protein was determined by the Lowry method using BSA as a standard.

Metabolic labelling with ^{35}S methionine and gel electrophoresis

Macrophages were treated with $1 \mu\text{g}/\text{ml}$ M-CSF for the indicated times. After washing with normal saline prewarmed at 37°C , they were further cultured for 2 h in methionine-free RPMI 1640 containing $50 \mu\text{M}$ L- ^{35}S methionine ($80 \mu\text{Ci}/\text{ml}$), $1 \mu\text{g}/\text{ml}$ M-CSF and 0.1% mouse autologous serum. Control cells were labelled in the same manner without M-CSF. The labelling was

terminated by aspirating the medium and washing three times with ice-cold PBS. A $50 \mu\text{l}$ aliquot of lysis buffer consisting of 50 mM Tris/HCl (pH 7.4), 6 M urea, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF and 0.33 mM leupeptin was promptly added. The macrophages were scraped with a 'rubber policeman' and collected in microcentrifuge tubes. Cell extracts were then passed through a 22-gauge needle 20 times, and soluble proteins, obtained by centrifugation at $11300 g$ for 20 min at 4°C , were subjected to SDS/PAGE or two-dimensional PAGE. For SDS/PAGE, samples were mixed with the same volume of double-strength Laemmli's buffer and boiled for 5 min. The sample containing 5×10^4 c.p.m. ^{35}S was resolved in SDS/10%-PAGE. The gel was stained with Coomassie Brilliant Blue R-250 and dried for autoradiography. For two-dimensional PAGE, soluble proteins were dissolved in $40 \mu\text{l}$ of lysis buffer consisting of 0.4% pH 4.0–6.5 ampholine, 0.8% pH 5.0–8.0 ampholine, 0.8% pH 3.5–9.5 ampholine, 5% (v/v) glycerol, 0.5% Nonidet P-40, 2% (v/v) 2-mercaptoethanol and 0.001% Bromophenol Blue. The mixture containing 1×10^6 c.p.m. ^{35}S was subjected to isoelectric focusing (IEF) in disc gels of 4% polyacrylamide containing the above lysis buffer lacking 2-mercaptoethanol and Bromophenol Blue. After IEF, the gels were incubated with Laemmli's SDS buffer for two 15-min periods. The second electrophoresis was carried out in SDS/10%-PAGE. The gels were treated with DMSO/2,5-diphenyloxazole for fluorography, dried and exposed to Fuji RX-R film (Fuji Photo Film, Tokyo, Japan). The radioactivity of labelled proteins precipitated with 5% (v/v) trichloroacetic acid was counted using a liquid scintillation counter, and the results were expressed as c.p.m./mg of protein.

Western-blot analysis

Cell extracts ($40 \mu\text{g}$ of protein) were subjected to SDS/10%-PAGE and transferred to a poly(vinylidene difluoride) (PVDF) filter. After blocking with 4% (w/v) milk casein for 30 min, the filter was incubated for 1 h at room temperature with a 1:1000 dilution of polyclonal antibody against HSP90, HSP70, HSC70, or HSP60. Bound antibodies were detected using an ECL system.

Assay of H_2O_2 -induced cell damage

Cell viability after exposure to 1 mM H_2O_2 for 1 h was assessed by measuring the percentage release of lactate dehydrogenase (LDH) as described previously [25]. Macrophages cultured in 24-well culture plates (1×10^6 cells/well) were left untreated or treated with $1 \mu\text{g}/\text{ml}$ M-CSF for 8 h, washed three times with HBSS, then exposed to 1 mM H_2O_2 in HBSS for 1 h.

Northern-blot analysis

Total RNA was isolated with an acid guanidinium thiocyanate-phenol-chloroform mixture [26]. A sample ($15 \mu\text{g}$) of total RNA was subjected to electrophoresis in a 1% agarose gel containing 0.6 M formaldehyde and transferred to Hybond N-plus filters (Amersham). The filters were prehybridized in hybridization solution containing 50% formamide, $5 \times \text{SSC}$ ($1 \times \text{SSC}$; 0.15 M NaCl, 0.015 M sodium citrate), $5 \times$ Denhardt's solution and 0.1% SDS for 4 h at 42°C . The filters were hybridized for 16 h at 42°C by incubating with the cDNA probe specific for human HSP70, human HSC70, or human GAPDH in the presence of $100 \mu\text{g}/\text{ml}$ heat-denatured salmon sperm DNA and 10% (w/v) dextran sulphate. These probes were labelled with $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ to a specific radioactivity of $> 1 \times 10^8$ c.p.m. using a random-primer kit. The filters were washed twice with $2 \times \text{SSC}$ containing 0.1% SDS at 50°C and then three times with $0.2 \times \text{SSC}$

containing 0.1% SDS at 65 °C. Bound probes were detected using a Fujix Bio-Analyzer BAS-2000 (Fuji Photo Film) and autoradiographed by exposure of the filters to Fuji RX-R films for 2 or 3 days at -80 °C.

Preparation of whole-cell extracts and the gel mobility shift assay

Preparation of whole-cell extracts and gel mobility shift assays were performed by the method of Mosser et al. [27]. Cells (1×10^7) in 60-mm-diam. culture dishes were left untreated or treated with 1 $\mu\text{g}/\text{ml}$ M-CSF for the indicated times. Cells were harvested and collected in microcentrifuge tubes. After washing with PBS by centrifugation at 1200 g for 5 s in a microcentrifuge, pelleted cells were rapidly frozen in liquid nitrogen. The frozen pellets were suspended in lysis buffer containing 20 mM Hepes (pH 7.9), 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM MgCl_2 , 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM leupeptin and 0.5 mM dithiothreitol (DTT). The mixture was gently pipetted using a Pasteur pipette and allowed to stand on ice for 15 min. Whole-cell extracts, obtained by ultracentrifugation at 100000 g for 5 min at 4 °C, were dialysed against binding reaction buffer consisting of 10 mM Tris/HCl (pH 7.8), 50 mM NaCl, 1 mM EDTA, 5% (v/v) glycerol, 0.5 mM DTT and 0.5 mM PMSF, and stored at -80 °C.

The heat shock elements (HSEs) in the mouse and human *hsp70* promoters are composed of five inverted pentamers (5'-nGAAn-3'), and the first, third and fourth repeats are the same consensus motif [28]. Therefore, a synthetic double-stranded HSE oligonucleotide encoding nucleotides -73 to -107 of the HSE (5'-GGAGGCGAAACCCCTGGAATATTCCTGACCTGGCA-3') located in the human *hsp70* promoter [29] was used to detect the activation of the heat shock transcription factor (HSF). The HSE oligonucleotide was radiolabelled with [γ - ^{32}P]ATP by 5'-end labelling one strand with T_4 polynucleotide kinase. The radiolabelled oligonucleotide was annealed to the complementary strand. Whole-cell extract (5 μg of protein) was mixed with 0.1 ng of ^{32}P -labelled oligonucleotide and 1 μg of poly(dI-dC)·poly(dI-dC) in the binding reaction buffer. Binding reaction was carried out at 4 °C for 20 min. The HSE-containing complexes were separated in 4% non-denaturing polyacrylamide gels, and electrophoresis was done with 0.5 $\times$ Tris-borate-EDTA at 150 V for 3.5–4 h at 4 °C. Antibody perturbation experiments were performed as described by Sarge et al. [30]; 1 μl of 100-fold diluted rabbit antiserum specific for HSF1 was preincubated with whole-cell extracts at 4 °C for 20 min prior to the binding reaction.

RESULTS

Effect of M-CSF on PMA-stimulated O_2^- release by macrophages

Resident macrophages, isolated and cultured overnight under LPS-free conditions, released 56 ± 8 nmol of O_2^-/mg of protein per h (mean $\pm$ S.D., $n = 10$) with 0.5 $\mu\text{g}/\text{ml}$ PMA and 6.6 ± 0.8 ($n = 6$) without any stimulant. The amounts of PMA-stimulated O_2^- release after treatment overnight with different concentrations of M-CSF are shown in Figure 1(A). M-CSF at 1 $\mu\text{g}/\text{ml}$ or higher increased the release of the untreated control cells about 3-fold. Macrophages cultured overnight were treated with 1 $\mu\text{g}/\text{ml}$ M-CSF for the indicated times, and the amounts of O_2^- release without or with 0.5 $\mu\text{g}/\text{ml}$ PMA were measured (Figure 1B). M-CSF did not change the spontaneous production of O_2^- , but PMA-stimulated release began to increase 6 h after addition of 1 $\mu\text{g}/\text{ml}$ M-CSF and reached a maximum at 8 h. The time required for activation by M-CSF was similar to that by LPS [6],

Figure 1 Effect of M-CSF on O_2^- release from macrophages

Resident macrophages were incubated overnight with M-CSF at different concentrations (A). Macrophages cultured overnight were treated with 1 $\mu\text{g}/\text{ml}$ M-CSF for the indicated times (B). The amounts of O_2^- production in the absence (●) or presence (■) of 0.5 $\mu\text{g}/\text{ml}$ PMA for 1 h were measured as described in the Materials and methods section. Values are mean $\pm$ S.D., $n = 10$ for PMA-stimulated O_2^- release and $n = 6$ for spontaneous release.

which has been shown to induce several functional proteins during activation of macrophages [10].

Figure 2 shows the effect of the protein synthesis inhibitor, cycloheximide (CHX), on PMA-stimulated O_2^- release. In macrophages, a half-maximum inhibition of protein synthesis, estimated by [^{35}S]methionine incorporation into protein precipitated

Figure 2 Effect of CHX on M-CSF-induced increase in O_2^- production

Cells were treated with various concentrations of CHX and 1 $\mu\text{g}/\text{ml}$ M-CSF for 8 h. Amounts of O_2^- released with PMA were compared with those of untreated or M-CSF-treated cells. Values are mean $\pm$ S.D., $n = 10$. Statistical significance: * $P < 0.01$ by Student's t -test (M-CSF versus M-CSF + CHX). # $P < 0.01$ by Student's t -test, compared with untreated or CHX-treated cells.

Figure 3 Effect of M-CSF on protein synthesis in macrophages

After incubation with 1 μ g/ml M-CSF for 2 (lane 2) or 6 h (lane 3), cells were labelled with 50 μ M L-[35 S]methionine and 1 μ g/ml M-CSF for 2 h. Untreated cells (lane 1) were also labelled for 2 h without M-CSF. Cell proteins containing 5×10^4 c.p.m. 35 S were separated in SDS/10%-PAGE. The gel was stained with Coomassie Brilliant Blue R-250, dried and exposed to Fuji RX-R film. Molecular masses indicated by arrows were calculated from the positions of the molecular-mass standards (Bio-Rad) shown on the left. Similar results were obtained in four separate experiments.

with trichloroacetic acid, was achieved at 200 ng/ml CHX. CHX at concentrations over 5 μ g/ml inhibited 94% of total protein synthesis. As shown in Figure 2, 100 ng/ml CHX completely inhibited the M-CSF-induced increase in PMA-stimulated O_2^- release, suggesting that M-CSF induces certain functional proteins responsible for the enhanced capacity for O_2^- release.

Effect of M-CSF on protein synthesis

Resident peritoneal macrophages were incubated with 1 μ g/ml M-CSF for 2 or 6 h and then metabolically labelled with L-[35 S]methionine for 2 h in the presence of 1 μ g/ml M-CSF. Whole-cell proteins were separated by SDS/PAGE or two-dimensional PAGE, and labelled proteins were analysed by autoradiography (Figures 3 and 4). Several proteins including 73, 67, 57, 56, 54, 43, 38 and 30 kDa proteins were induced within 2 to 4 h of addition of M-CSF (Figure 3, lane 2), at which time macrophages did not yet increase the capacity for O_2^- release (Figure 1B). Macrophages acquired the maximum activity of O_2^- release 8 h after treatment with M-CSF, at which time several other proteins including 90, 72, 60, 52, 48 and 40 kDa proteins were additionally induced.

Autoradiograms of two-dimensional PAGE show the marked changes in protein synthesis during activation by M-CSF (Figure 4). Inductions of low-molecular-mass proteins of less than 30 kDa were more clearly detected by two-dimensional PAGE than SDS/PAGE. At the same time, the autoradiograms revealed that M-CSF decreased the syntheses of certain proteins indicated by open arrows in Figure 4(A).

Detection of HSPs by immunoblotting

The molecular masses and pI values of several proteins, indicated by arrows in Figure 4(B), were compatible with those of known HSPs. Therefore, Western-blot analyses with anti-HSP antibodies were carried out to confirm this, and the results are shown

Figure 4 Detection of synthesized proteins by two-dimensional PAGE

Macrophages were labelled for 2 h with [35 S]methionine before (A) or 6 h after (B) addition of 1 μ g/ml M-CSF as described in the legend to Figure 3. Cell proteins containing 1×10^6 c.p.m. 35 S were separated by two-dimensional PAGE as described in the Materials and methods section. The gels were treated with DMSO/2,5-diphenyloxazole for fluorography, dried and exposed to Fuji RX-R film. Molecular masses indicated by arrows were calculated from molecular-mass markers shown on the left. The pH gradient was determined from the position of IEF markers (Bio-Rad). These results are representative of those obtained in four experiments.

Figure 5 Detection of HSPs by immunoblotting

Whole-cell proteins were extracted from cells freshly isolated and cultured for 2 h (lane 1, 0_1) or overnight (lane 2, 0_2) as described in the Materials and methods section. Macrophages were then treated with 1 μ g/ml M-CSF for the indicated times (h) (lanes 3–7). Macrophages were also cultured for 8 h with no supplement (lane 8), 1 μ g/ml M-CSF (lane 9), or 1 μ g/ml M-CSF plus 300 units/ml SOD and 3500 I.U./ml catalase (lane 10). An equal amount of extracted cell proteins (40 μ g of protein per lane) was subjected to 10% SDS/PAGE and transferred to a PVDF membrane. Immunoblot analysis with antibody against HSP90, HSP70, HSP60, or HSC70 was carried out as described in the Materials and methods section. Similar results were obtained in three separate experiments.

in Figure 5. Lane 1 (0_1) shows the amounts of HSPs in macrophages freshly isolated and attached to plastic plates after culture for 2 h, and lane 2 (0_2) shows those in cells cultured overnight without M-CSF. Small amounts of HSP90, HSP70 and HSC70 were detected in freshly isolated macrophages (Figure 5, lane 1). Overnight culture slightly increased the contents of these proteins (lane 2). These inductions may reflect environmental stress during isolation and cultivation. In addition, the macrophages are known to release small amounts of O_2^- during attachment to plastic plates [31], which may cause oxidative stress and induce the HSPs. In fact, freshly isolated macrophages released 10–20 nmol of O_2^- /mg of protein per h during the initial 4 to 5 h of culture, then the release gradually decreased to the basal level (4–8 nmol of O_2^- /mg of protein per h). When macrophages cultured overnight were treated with M-CSF,

Figure 6 Time-dependent changes in the amounts of HSPs, PMA-stimulated O_2^- release and [35 S]methionine incorporation into cell proteins

The level of each HSP was quantified by densitometry and standardized by the level of respective protein in lane 0_1 (A). Before or after treatment of cells with 1 μ g/ml M-CSF for 0, 2, 4, or 6 h, cells were incubated for 2 h with 20 μ M L-[35 S]methionine and 1 μ g/ml M-CSF. Time-dependent changes in the incorporation of 35 S into cell proteins were determined as described in the Materials and methods section and compared with the time course of the PMA-stimulated O_2^- release (B). Values are mean $\pm$ S.D., $n = 10$.

HSC70 began to increase 1 h after addition of M-CSF, and the levels continued to increase until 8 h. In contrast, M-CSF increased the levels of HSP90 and HSP70 at 6 h, and HSP60 was first detected at this time. M-CSF did not increase spontaneous O_2^- release from macrophages (Figure 1B), but it still up-regulated HSP synthesis in the presence of SOD and catalase (Figure 5, lane 10), suggesting that spontaneously produced oxidants, which were scavenged by SOD and catalase, did not participate in the up-regulation of HSP synthesis by M-CSF.

The levels of the HSPs were quantified by densitometry (Figure 6A), and their changes were compared with both time-dependent changes in [35 S]methionine incorporation into cell proteins and PMA-stimulated O_2^- release (Figure 6B). The increase of the capacity for O_2^- release coincided with the inductions of the stress-inducible HSPs. On the other hand, HSC70 protein was more rapidly induced than the stress-inducible HSPs, and its time-dependent accumulation preferentially correlated with the M-CSF-induced increase in protein synthesis (Figure 6B).

Macrophages treated with M-CSF for 8 h were exposed to 1 mM H_2O_2 for 1 h, and cell viability was estimated by measuring the percentage LDH release. M-CSF-untreated cells released

Figure 7 Northern-blot analyses of HSP70, HSC70 and GAPDH mRNAs in macrophages

After culture of isolated macrophages overnight, cells were incubated without M-CSF for 8 h (lane C) or with 1 μ g/ml M-CSF for the indicated times (h). Total RNA (15 μ g), extracted as described in the Materials and methods section, was subjected to 1% agarose/0.6 M formaldehyde gel electrophoresis and transferred to a nylon filter. The filters were hybridized with the 32 P-labelled cDNA probe for human HSP70, human HSC70, or human GAPDH (A). (B) The level of each mRNA was quantified by densitometry and standardized by the level of 18 S ribosomal RNA shown in the bottom of (A). These results are representative of those obtained in four separate experiments.

$21.1 \pm 1.3\%$ of the total LDH activity (mean $\pm$ S.D., $n = 6$), while the percentage release of M-CSF-treated cells decreased to $8.0 \pm 0.7\%$ (mean $\pm$ S.D., $n = 6$). Thus, the activated macrophages significantly acquired resistance to H_2O_2 -induced damage ($P < 0.001$ by Student's t -test). The acquisition of the resistance with M-CSF was completely blocked by a protein synthesis inhibitor (CHX); macrophages cultured for 8 h with 100 ng/ml CHX released $24.6 \pm 4.0\%$ ($n = 6$) of the total LDH activity upon exposure to 1 mM H_2O_2 for 1 h, and M-CSF did not significantly suppress the release ($20.0 \pm 3.5\%$, $n = 6$).

Detection of HSP70, HSC70, or GAPDH mRNA

Northern-blot analysis was performed to detect the accumulations of HSP70, HSC70 and GAPDH mRNAs during activation of macrophages by M-CSF (Figure 7A), and the levels of these mRNAs were standardized with the amount of the 18 S ribosomal RNA (Figure 7B). Isolated and cultured resident macrophages constitutively expressed HSP70 and HSC70 mRNAs (Figure 7A) and these levels remained constant in the absence of M-CSF during the experimental period (results not shown). The level of HSP70 mRNA began to increase 6 h after treatment with M-CSF and the GAPDH mRNA level also increased at this point. However, the accumulation of HSC70 mRNA started within 1 h and continued until 8 h.

Activation of HSF by M-CSF

The stress response mainly occurs via transcriptional induction of heat shock genes mediated by activation of HSF. HSF binds to the HSE, which consists of contiguous arrays of the alternately oriented pentanucleotide unit 5'-nGAAn-3' found in the promoter of heat shock genes [32].

Figure 8 Detection of HSF-binding activity in M-CSF-treated macrophages

After incubation of cells without M-CSF for 8 h (lane 1), or with 1 μ g/ml M-CSF for the indicated times (lane 2–11), whole-cell proteins were extracted, and the gel mobility shift assay was carried out as described in the Materials and methods section. Lane 10 marked 'anti-HSF1' contained rabbit antiserum specific for HSF1. Lane 11 marked 'self' contained a 30-fold molar excess of the non-labelled HSE oligonucleotide. A HSF–HSE complex is indicated by 'HSF'. Non-specific DNA–protein complexes are denoted by 'NS'. Similar results were obtained in five separate experiments.

To examine whether M-CSF treatment caused the activation of HSF in macrophages, the gel mobility shift assay was performed with the synthetic HSE oligonucleotide located in the human *hsp70* promoter (Figure 8). Untreated macrophages contained an HSE-binding activity indicated by 'NS', which was not competed for by a 30-fold molar excess of unlabelled HSE oligonucleotide (Figure 8, lane 11). M-CSF treatment produced another protein–DNA complex within 15 min, which migrated slowly as indicated by 'HSF'. This HSE-binding activity was considered to represent the HSF–HSE complex because a 30-fold molar excess of unlabelled oligonucleotide eliminated this band (Figure 8, lane 11), and an antibody against HSF1 supershifted this band and produced a more slowly migrating band as indicated by an open arrow (Figure 8, lane 10). This HSE-binding activity was continuously detected during the experimental period.

DISCUSSION

The present experiments indicate that the stimulation of resident peritoneal macrophages by M-CSF induced a discrete set of proteins, which were required to enhance their capacity for O_2^- release. It has already been shown that M-CSF induced several functional proteins of macrophages including cytosolic phospholipase A_2 [33], urokinase-type plasminogen activator [34], and the phagocyte cytochrome *b* heavy- and light-chain proteins [35]. To the best of our knowledge, the present experiments provide the first evidence that M-CSF increased the expression of the major stress-inducible HSPs (HSP90, HSP70 and HSP60) and a constitutive HSC70.

A significant amount of work has clearly shown that HSPs are essential for the survival of the cell confronted with various types of insults (for a review see ref. [36]). In addition, HSPs play an essential role for facilitating protein maturation events, being known as molecular chaperones, in a number of important biological processes including proliferation and differentiation as well as functional activation of the cells [36]. Macrophages might

provide a unique opportunity to study the induction of HSPs, since activation requires inductions of many functional proteins and, at the same time, macrophages must properly function even under enhanced oxidative stress due to oxygen radicals released by themselves. However, the induction of HSPs in tissue macrophages has not been well documented, since it is difficult to keep macrophages quiescent; trace amounts of contaminated LPS are enough to activate macrophages and adherence to plastic plates can stimulate O_2^- release that may induce HSPs, as shown in blood monocytes [14].

When mouse peritoneal macrophages are isolated and cultured in LPS-free conditions, the amount of O_2^- stimulated by PMA is reported to be less than 100 nmol/mg of protein per h [6]. Resident peritoneal macrophages, prepared and cultured overnight in our LPS-free system, released 56 ± 8 nmol of O_2^- /mg of protein per h (mean $\pm$ S.D., $n = 10$) with PMA. Treatment of cells with M-CSF enhanced this capacity about 3-fold. Treatment for at least 8 h was necessary to induce the maximum capacity for O_2^- production. Using this system, we examined in detail the relationship between induction of the major HSPs and activity of the respiratory burst. Western-blot analyses showed that resident macrophages, freshly isolated and cultured overnight under LPS-free conditions, contained small amounts of HSP90 and HSP70 as well as HSC70. Resident macrophages expressed both HSP70 and HSC70 mRNAs. These expressions might be due to the environmental stress during isolation and cultivation. On the other hand, it was reported that human peripheral blood myelomonocytic cells, but not lymphocytes, constitutively expressed a heat-inducible *hsp70* gene, and that activation of monocytes by LPS, bacteria, phorbol ester and tumour necrosis factor augmented the expression of HSP70 mRNA [14]. In addition, an inducer of monocyte differentiation, 1,25-dehydroxyvitamin D_3 , is known to up-regulate the expression of HSP70 [16]. These findings suggest a possible role for the *hsp70* gene in leucocyte differentiation. However, Fincato et al. showed that granulocyte-macrophage CSF and M-CSF did not increase HSP70 mRNA levels in monocytes [14].

When resident macrophages were treated with M-CSF, it apparently induced the stress-inducible HSP90, HSP70 and HSP60, and a cognate HSC70. After cultivation overnight, macrophages spontaneously released 6.6 ± 0.8 (mean $\pm$ S.D., $n = 6$) nmol of O_2^- /mg of protein per h without any stimulant. M-CSF did not change this spontaneous release and it also up-regulated the synthesis of HSPs even in the presence of SOD and catalase, suggesting that oxidative stress due to oxidants spontaneously released by macrophages may not participate in the induction of HSPs by M-CSF. The timing of induction of the stress-inducible HSPs coincided with that of increase in the capacity for O_2^- release, and the extents of their inductions were paralleled to the capacity for O_2^- production. Northern-blot analysis with the cDNA probe for human HSP70 mRNA showed that the HSP70 mRNA level also began to increase at 6 h. The macrophages activated by M-CSF acquired resistance to H_2O_2 -induced cell damage, estimated by measuring the percentage LDH release, supporting the idea that the stress response may play an autoprotective function in macrophages.

M-CSF also stimulated the synthesis of constitutively expressed cognate HSC70, but in this case the level of HSC70 protein started to increase within 1 h, when PMA-stimulated O_2^- release was not yet enhanced. Northern-blot analysis showed that the accumulation of HSC70 mRNA started within 1 h after addition of M-CSF, suggesting that HSC70 may have a functional role during the pre-activation period. After addition of M-CSF, the rate of total protein synthesis measured by incorporation of L-[35 S]methionine into cell proteins started to increase within 2 h,

and autoradiograms of gel electrophoreses demonstrated that inductions of several specific proteins were already detected within 4 h. It has been suggested that the constitutive HSC70 and stress-inducible HSPs exhibit functional similarities and interact with newly synthesized protein. However, nascent peptides have been shown to preferentially interact with pre-existing HSC70 [37]. The mechanism of induction of HSC70 remains unsolved; however, our results suggest that HSC70 may be induced to interact with newly synthesized proteins during the pre-activation period.

The stress response in mammalian cells is suggested to occur mainly via transcriptional induction of heat shock genes mediated by activation of a pre-existing pool of HSF that binds to HSE [32]. Multiple, distinct HSF genes have been cloned; two from human and mice, three from chickens [38–40]. The differential activation of distinct HSF family members has been suggested to be attributed to the complexity of transcriptional regulation of heat shock genes [41]. It has been suggested that HSF1 is the mediator of stress-induced heat shock genes [41], whereas HSF2 DNA-binding activity is induced during haemin treatment of human K562 cells [42]. Gel mobility shift assays showed that treatment with M-CSF rapidly induced the HSE-binding activity within 15 min, and the activity persisted during the experimental period. This protein–DNA complex was supershifted with anti-serum raised against HSF1, suggesting that M-CSF may activate HSF1. HSEs in both human *hsp70* and *hsc70* promoters contained three perfect inverted nGAAn repeats [29,43]. Therefore, the rapid activation of HSF may reflect the transcriptional induction of the *hsc70* gene.

The results described here show that M-CSF induced both the constitutive and stress-inducible HSPs, at least in part, at the transcriptional level during activation of macrophages *in vitro*, and suggest that these HSPs as well as HSC70 may play an essential role in the priming of macrophages for enhanced capacity for O_2^- production by functioning as molecular chaperones and by protecting the macrophage against the auto-oxidative damage associated with the respiratory burst.

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