EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 38, No. 4, 364-374, August 2006

Release of heat shock protein 70 (Hsp70) and the effects of extracellular Hsp70 on matric metalloproteinase-9 expression in human monocytic U937 cells

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Accepted 1 June 2006

Abbreviations: AP-1, activating protein-1; HCS, Hsp70-overexpressing cells culture supernatants; Hsp, heat shock protein; HUVEC, human umbilical vascular endothelial cells; MCS, mock transfected cells culture supernatants; MMP-9, matrix metalloproteinase-9; NF-κB, nuclear factor kappa B; SFM, serum-free medium; TLR, Toll-like receptor

Abstract

Heat shock protein 70 (Hsp70) release and its effects on pro-inflammatory cytokine production have been controversial. In this study, we investigated whether Hsp70 could be released from monocytes and activates matrix metalloproteinase-9 (MMP-9) gene expression. Hsp70 overexpression in human monocytic cell line U937 was found to increase PMA-induced MMP-9 expression and enhance cell motility. Hsp70 cDNA transfectants released Hsp70 protein into culture supernatants, and a part of released Hsp70 subsequently was bound to the surface of U937 cells. Addition of culture medium containing the extracelluar Hsp70 led to an increase not only in proMMP-9 secretion, but also the invasiveness of U937 cells through Matrigel or human umbilical vascular endothelial cells (HUVEC) *in vitro*. Immunodepletion of Hsp70 abolished its effect on MMP-9 expression. The released Hsp70 activated nuclear factor kappa B (NF-κB) and activating protein-1 (AP-1), which led to the activation of MMP-9 transcription. Taken together, these results suggest that extracellular Hsp70 induces the expression of MMP-9 gene through activation of NF-κB and AP-1.

Keywords: AP-1; gelatinase B; HSP70 heat-shock proteins; monocytes; NF-κB; transcription factor AP-1

Introduction

Most organisms express constitutive and stress-induced forms of Hsp70, which are involved in protein folding, translocation, oligomer dissociation, and prevention of protein aggregation. Since Hsp70 lacks any secretory signal sequence, it has been considered as an intracellular molecule (Gething and Sambrook, 1992; Hartl and Hayer-Hartl, 2002). However, it has been suggested that Hsp70 is released extracellularly under normal cell culture conditions (Hightower *et al.,* 1989; Broquet *et al.,* 2003; Hunter-Lavis *et al*., 2004), and is present in circulation of normal individuals, and its circulating level is increased after open heart surgery or in patients with cardiovascular diseases including atherosclerosis (Chan *et al.,* 1999; Dybdahl *et al.,* 2002). Furthermore, plasma concentrations of Hsp70 were shown to be associated significantly with postoperative infection (Kimura *et al.,* 2004). These results suggest that Hsp70 can be released into extracellular spaces and may be involved in postoperative inflammatory responses and in the pathogenesis of postoperative organ dysfunction. In fact, it has been shown to interact with the CD14 and Toll-like receptor 2 (TLR2) and TLR4 on antigen-presenting cells such

as macrophages and dendritic cells, and directly induce expression of cytokines such as interleukin-1α (IL-1α), IL-1β, IL-6, tumor necrosis factor-α (TNF-α), and granulocyte-macrophage colony-stimulating factor (GM-CSF) in macrophage cultures (Asea *et al.,* 2000). Thus, the induction of pro-inflammatory cytokines by extracellular Hsp70 may stimulate the innate immune response to promote chronic inflammation. However, some results suggest that the reported cytokine function of Hsp70 may have resulted from contamination by lipopolyscharride (LPS) (Bausinger *et al.,* 2002; Gao and Tsan, 2003a, b, c; Tsan and Gao, 2004a, b c; Wang *et al.,* 2005). For example, it was reported that LPS-free recombinant human Hsp70 could not induce the activation of dendritic cells (Bausinger *et al.,* 2002). The ability of commercially available rhHsp70 to induce TNF-α production was also shown to be entirely due to contamination by LPS as well as LPS-associated molecules (Gao and Tsan, 2003a, b). Thus, the question of whether the released Hsp70 has any cytokine effect still remains elusive.

MMP-9 is a 92-kDa type IV gelatinase that is produced by human monocytes/macrophages (Nagase *et al.,* 1992). It cleaves basement membrane collagen IV and V and gelatin, fibronectin, and elastin, (Tryggvason *et al.,* 1992) and plays a crucial role in monocyte extravasation, which is a primary event in inflammation (Watanabe *et al.,* 1993). Its expression is regulated by cytokines, chemokines, and cellular interactions through adhesion molecules (Opdenakker *et al.,* 2001; Hong *et al.,* 2005). In this study, we investigated whether extracellular Hsp70 could be released from living Hsp70 transfectants and induce MMP-9 gene expression. This study shows that Hsp70 is released actively from monocytic cells, and enhances MMP-9 expression and activity through activation of NF-κB and AP-1.

Materials and Methods

Reagents

Culture reagents were purchased from Invitrogen (Carlsbad, CA). Anti-human Hsp70 mAb was purchased from Stressgen Bioreagents (Victoria, BC, Canada). Horseradish-conjugated goat anti-mouse IgG was from Chemicon (Temecula, CA). FITC-conjugated anti-mouse IgG antibody was from Becton Dickinson (San Jose, CA). Anti-human α- and β-actin mAb and PMA were from Sigma-Aldrich Co. (St. Louis, MO). Nitrocellulose membranes and the antibody detection kit (ECL plus) were obtained from Amersham Biosciences (Piscataway, NJ). [α-P^{32}] dCTP (3,000 Ci/mmol) was supplied by Perkin Elmer (Boston, MA). TRIzol reagent was from Life Technologies (Rockville, MD). Dual-luciferase reporter assay system was from Promega (Madison, WI).

DNA constructs and transfection

The full-length cDNA coding for inducible Hsp70 was subcloned into the *Hind*III and *Xba*I sites of a stable expression vector, pcDNA3.1 (Invitrogen Corp., Carlsbad, CA). All sequences of the construct were confirmed by DNA sequencing. DNA transfection to U937 cells was performed using an electroporator (Invitrogen Corp.). pcDNA3.1 vector only was also transfected as a control. Stable transfectants were selected in RPMI1640 medium, containing 10% FBS, and 0.6 mg/ml G418 (Invitrogen Corp.), for 4 weeks. Resistant clones were selected, and their expression levels of Hsp70 were then examined by Western blot analysis.

Cell culture

Human monocytic cells (U937) were obtained from American Type Culture Collection (ATCC), and were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen Corp.). For cultures of the Hsp70- transfected U937 cells, G418 was added to the complete RPMI medium at a concentration of 0.6 mg/ml. Cell cultures were maintained and incubated in 5% CO_2 at 37°C.

Flow cytometric analysis

At an appropriate time point, U937 cells (5×10^5 cells) were centrifuged at 3,000 rpm, and the pellets were washed with serum free RPMI1640, resuspended in 200 μl of serum-free medium (SFM) including 1 μg of anti-human Hsp70 monoclonal antibody, and incubated for 30 min at 4°C. Unbound mAb was removed by washing three times with SFM. The cells were resuspended in 200 μl of SFM, including 1 μg of FITC-conjugated anti-mouse IgG antibody, and incubated for 30 min on ice. The cells were finally resuspended in 400 μl of cold 1 × PBS containing 1% formalin. Fluorescence intensity was measured with a FACS Scan Flow Cytometer (Becton Dickinson, San Jose, CA).

Gelatin zymogram assay

The amount of proMMP-9 secreted was assayed by geleatin zymography as described previously (Lee *et al.,* 2004).

Western blot analysis

Western blot analysis used for detecting Hsp70 protein expression was carried out as described previously (Lee *et al.,* 2004).

Northern blot analysis

Expression of MMP-9 mRNA was determined by Northern blot analysis as previously described (Lee *et al.,* 2004).

Immunodepletion

Concentrated culture supernatants were incubated with anti-human Hsp70 monoclonal antibody (1:100 dilution) (StressGen Bioreagents) or an equal amount of mouse IgG for 22 h and then incubated with protein A-agarose (Life Technologies) for 2 h on a roller system at 4°C. The culture supernatants and control medium were used to stimulate U937 cells in 35-mm dishes, and MMP-9 activity was analyzed by zymography.

Determination of cell viability

Cell viability was assessed by the dye exclusion test. Cells were incubated in SFM for 24 h at 37°C. For quantitative analysis of cell viability, cells were collected at the indicated time. After washing, 0.1 ml of the culture sample was stained with 0.1 ml of 0.4% trypan blue, and the number of living and dead cells was then counted using a hemocytometer.

Transmigration assay

Polycarbonate filters (8-μm pore size; Costar) were coated with Matrigel and placed in a modified Boyden chamber, filled with 600 μl of NIH3T3 cell cultured-DMEM conditioned medium, 0.005% vitamin C, and 0.1% BSA as a source of chemoattractants in the lower compartment. Then, 100 μl of U937 cell suspension (1×10^6 cells/100 μl SFM) were placed in the upper compartment of the chamber. The plates were incubated at 37°C in 5% CO_2 in air saturated with H_2O for 24 h, 48 h, and 72 h, and the cells on the lower surface of the filter were then stained with trypan-blue and counted. Each assay was carried out in triplicate. Invasion rate was determined as a percentage of control.

Endothelial leukocyte transmigration assay

HUVEC were seeded onto Matrigel-coated Transwell polycarbonate filter inserts (24-well format, pore size 8 μm) in growth medium and grown to confluence. Monolayer integrity was assessed in parallel inserts by crystal violet staining followed by light microscopy. Forty eight hours after reaching confluence, endothelial monolayers were stimulated with growth medium containing chemoattractants used in cell invasion assay. U937 cells were suspended in RPMI 1640 medium containing 10% (v/v) FBS and 1% (w/v) of glutamine, and 1×10^6 cells were added on top of the endothelial monolayers. U937 cells were allowed to migrate for 48 h at 37°C in 5% CO_2, and transmigrated cells in the lower chamber were counted under a light microscope. Assays were carried out in triplicates.

Confocal microscopy

To determine the binding of secreted Hsp70 to the surface of U937 cells, cells were harvested with 200 μl of SFM including 1 μg of anti-human Hsp70 monoclonal antibody in the presence or absence of secreted Hsp70 in fresh Eppendorf-tube and incubated for 2 h at room temperature. To eliminate unbound mAbs, they were washed with 1 ml of SFM and resuspended in 200 μl of SFM, containing 1 μg of FITC-conjugated anti-mouse IgG antibody and incubated for 2 h at room temperature. They were finally resuspended in 400 μl of cold 1 × PBS containing 1% formalin, and analyzed under a confocal microscope (Leica TCS SP; Leica, Deerfield, IL).

Transfection of promoter/luciferase constructs and luciferase assay

The structure of pGL3-M9Pwt, pGL3-M9Pmt-NF-κB, pGL3-M9Pmt-AP-1, and pRL-SV40△enh that expresses *Renilla* luciferase by an enhancerless SV40 promoter were previously described (Hah and Lee, 2003). pNFκB-TA-Luc and pAP1-TA-Luc were obtained from Clontech (Mountain View, CA) and their structures were described in the manufacturer's manual. U937 cells were transiently transfected with pGL3-M9Pwt, pGL3-M9Pmt-NF-κB, pGL3-M9Pmt-AP-1, pNFκB-TA-Luc, or pAP1-TA-Luc, using electroporation. pRL-SV40△Enh was also transfected to normalize the luciferase activity, which was measured by using the dual-luciferase reporter assay system (Promega).

Statistical analysis

The data are expressed as the average of the mean values obtained ± SD. Statistical significance was determined by the Student's *t* test with the statistical software GraphPad Prism (version 4.0). All experiments were conducted three times or more for obtaining reproducible results. The representative data are shown in the figures.

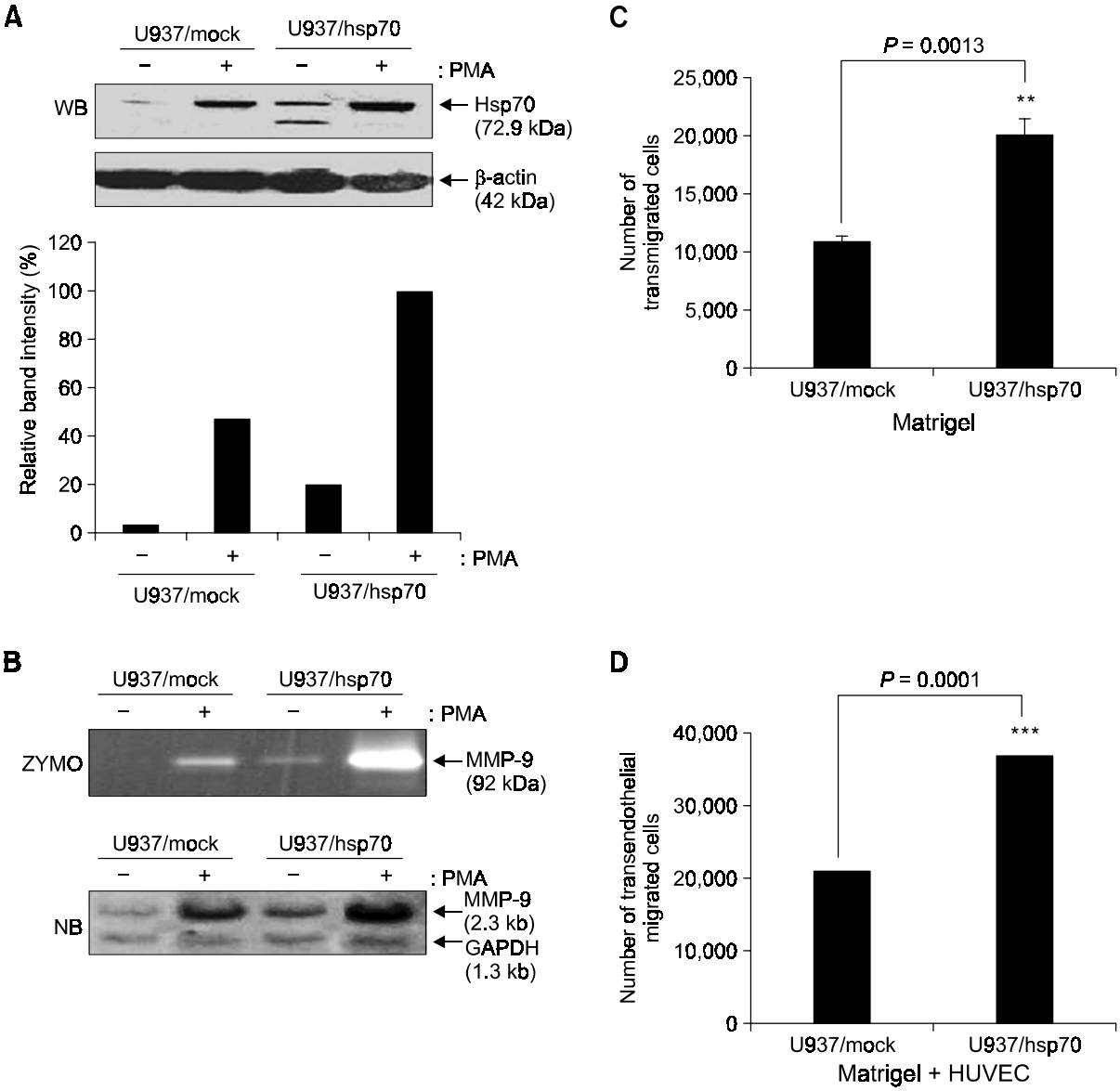

Figure 1. MMP-9 expression and secretion are upregulated in the Hsp70 overexpressing clone. (A) Expression level of Hsp70 in Hsp70 cDNA transfected clone. Mock-vector transfected U937 or Hsp70 cDNA transfected U937 cells were incubated in serum-free medium in the absence or presence of PMA (3 nM). After 24 h, the cells were lysed with 1% NP-40 lysis buffer. Expression levels of Hsp70 protein in mock-vector transfected U937 or Hsp70 transfected U937 clone were analyzed by Western blot analysis. Results were replicated in three independent experiments. A relative expression level of Hsp70 is shown as a relative intensity of bands by measuring with a densitometer. (B) U937 mock transfectants and Hsp70 cDNA transfectants were incubated for 24 h in the absence or presence of PMA (3 nM). Samples of conditioned medium were collected, and then analyzed for the proMMP-9 secretion by gelatin zymographic analysis (upper panel), or analyzed for MMP-9 mRNA expression by Northern blot analysis (lower panel). GAPDH bands were detected as loading controls. (C) Effect of Hsp70 on transmigration ability of U937 cells. Polycarbonate filters coated with Matrigel were placed in a modified Boyden chamber. Boyden chambers were filled with conditioned medium of NIH3T3 cells. Then, U937 cells or Hsp70 overexpressing U937 (1×10^6 cells) cells were placed in the upper compartment of the chamber. After 24 h, cells that had migrated to the lower wells were stained with trypan-blue and counted under the light microscope. Bar graphs were calculated from no less than three independent experiments each in duplicates. A significant difference from control is shown by asterisks as follows: $^{**}P < 0.01$; $^{***}P < 0.001$. Lines indicate additional statistical comparisons. (D) HUVECs were grown on Transwell cell culture inserts to confluence, and then transmigration assay was performed as described in (C).

Results

Hsp70 overexpression enhances MMP-9 activity

In order to confirm the previously reported cytokine function of Hsp70 under the condition of excluding a possibility of bacterial lipopolysacharide contamination (Bausinger *et al.,* 2002; Gao and Tsan, 2003a, b, 2004; Tsan and Gao, 2004a, b), Hsp70 was overexpressed in the human monocytic U937 cells, and its effect on MMP-9 expression was then

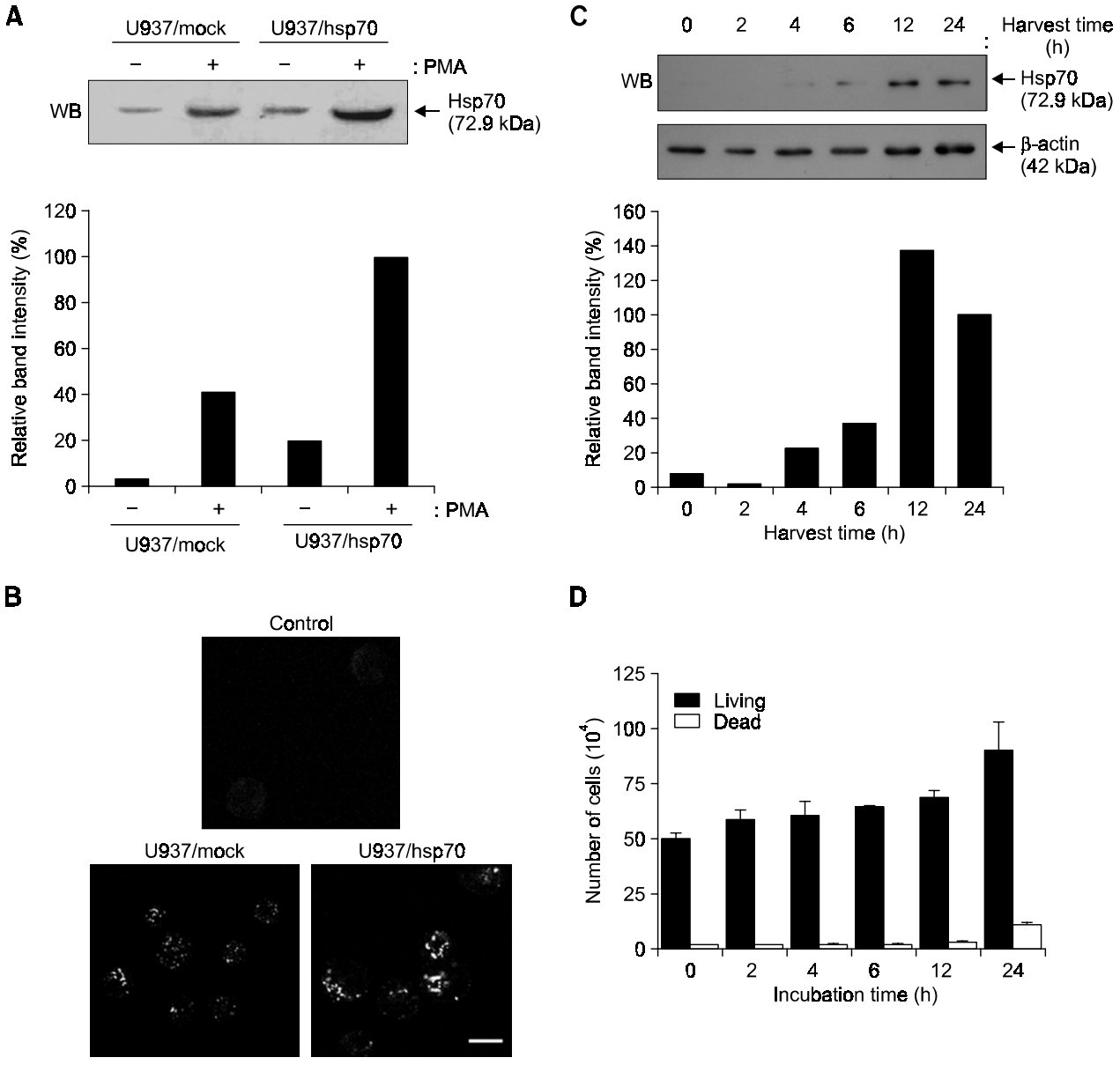

Figure 2. Hsp70 is secreted from the U937 cells. (A) U937 mock transfectants and Hsp70 cDNA transfectants were incubated for 24 h in the absence or presence of PMA (3 nM). After 24 h, culture supernatants were collected, and analyzed for Hsp70 expression by Western blot analysis. The graph represents relative expression level of Hsp70, shown as relative band intensity by measuring with a densitometer. (B) The secreted Hsp70 binds onto the surface of U937 cells. To confirm the binding of secreted-Hsp70 to the cell surface, U937 mock transfectants and Hsp70 cDNA transfectants were incubated for 24 h in the presence of PMA (3 nM). After 24 h, culture supernatants were collected, and then added to the freshly cultured U937 cells, respectively. After 1 h, the cells were stained with anti-Hsp70 mAb and FITC-conjugated anti-mouse IgG, and analyzed by confocal microscopy. Scale bar, 200 μm. (C) Kinetics of Hsp70 secretion from Hsp70 cDNA transfectants. Hsp70 cDNA transfectants were incubated for 24 h in the absence of PMA. The equal amounts of culture supernatants were collected at the indicated time, and then analyzed for Hsp70 and actin expression by Western blot analysis. The graph represents relative expression levels of Hsp70 and actin, shown as a relative intensity of bands by measuring with a densitometer. (D) Cell viability was assessed by the dye exclusion test as depicted in Materials and Methods.

investigated. As shown in Figure 1A, Hsp70 was detected in both Hsp70- and mock-transfected cells. However, increased level of Hsp70 protein expression was observed only in the Hsp70-transfected cells. There was about a five-fold increase in the Hsp70 expression level after stimulating the Hsp70 transfected cells with PMA (3 nM). Hsp70 and mock transfectants were cultured in the presence or absence of PMA in serum free medium for 24 h, and then supernatants were collected to measure MMP-9 expression by gelatin zymography (Figure 1B upper panel). Quiescent mock transfectants secreted proMMP-9 at barely detectable levels. In contrast, the overexpression of Hsp70 induced detectable levels of MMP-9 expression. Stimulation of mock and Hsp70 transfectants with PMA markedly augmented proMMP-9 expression levels in the supernatants. We next investigated by Northern blot analysis whether the stimulatory effect of Hsp70 on proMMP-9 secretion resulted from increased MMP-9 mRNA expression. As shown in Figure 1B lower panel, the result indicates that U937 cells expressed the 2.3 kb mRNA species for MMP-9, and Hsp70 transfectants induced more MMP-9 mRNA expression than mock transfectants. These results demonstrate that overexpression of Hsp70 increases MMP-9 mRNA expression in monocytes/macrophages.

MMP-9 has been known to play a crucial role in leukocyte migration from the circulation to inflamed tissues (Hah and Lee, 2003). Having found the stimulatory effect of overexpression of Hsp70 on PMA-induced MMP-9 mRNA expression, we were interested to determine whether Hsp70-induced proMMP-9 expression could increase the ability of monocytes to infiltrate through extracellular matrix. Therefore, transmigration ability of Hsp70-transfected U937 cells was compared with that of mock-transfectants by a Matrigel invasion assay. The number of transmigrated Hsp70-transfected U937 cells was an about two-fold more than that of mock-transfectants (Figure 1C). In addition, we examined whether Hsp70 had an effect on leukocyte transendothelial migration. HUVECs were grown on Transwell cell culture inserts to confluence and subjected to a transmigration assay by adding a defined number of mock transfectants or Hsp70-transfected U937 cells to the upper wells. As shown in Figure 1D, the overexpression of Hsp70 led to a marked increase of U937 transendothelial migration (migrated cell number; 35,000), as compared with control (20,000). This finding suggests that Hsp70 may play a role in the human microvasculature as a potential proinflammatory mediator.

Hsp70 is secreted from the cells to culture supernatants, and binds to the cell surface

There are a number of reports on the secretion of Hsp70 independent of necrotic cell death (Hightower *et al.,* 1989; Broquet *et al.,* 2003; Esparza *et al*., 2004; Hunter-Lavis *et al*., 2004; Adewoye *et al*., 2005; Dybdahl *et al*., 2005; Svensson *et al*., 2005). To explore whether Hsp70 could be released from Hsp70 transfectants to the culture medium, Hsp70 and mock transfectants were cultured in the absence or presence of PMA, and the amount of Hsp70 released in the culture medium was then measured by Western blot analysis. As shown in Figure 2A, there was about two-fold more Hsp70 in the culture medium of Hsp70-transfected cells than that of mock-transfectants. Next, we investigated whether the released Hsp70 was bound to the surface membrane of U937 cells. The result showed that the released Hsp70 was bound to the surface membranes of approximately 20% of the cells (Figure 2B). Consistent with Western blot analysis, the staining intensity was higher in the Hsp70 transfectants than in mock transfectants. Mock transfectants treated with only FITC-conjugated anti-mouse IgG antibody showed barely detectable staining pattern. These results were confirmed by flow cytometric analysis by using an indirect immunofluorescence technique with anti-Hsp70 mAb and FITC-conjugated anti-mouse IgG antibody. Mean fluorescence intensity was 48.80 arbitrary units, compared with mock-transfected cells of 13.72 arbitrary units. To determine whether extracellular Hsp70 was released from living Hsp70 transfectants, we measured the protein abundance of Hsp70 released into medium and compared it with that of an intracellular protein, β-actin (Figure 2C). Hsp70 transfectants were cultured in serum-free medium in the absence of PMA for 24 h, and then culture supernatants were collected at the indicated time. Culture media at 0 h and 2 h showed very low levels of Hsp70, but relatively high levels of β-actin (lane 2 and 3). From 4 h-culture medium, however, the secretion of Hsp70 started to increase. After culture for 12-24 h, there was an about 10-14 fold increase in Hsp70 present in serum-free medium (Figure 2C, lane 6), but an about two-fold increase in β-actin. To verify that the Hsp70 release was not due to cell death, cell viability was determined every 2 h for 24 h by trypan blue staining. There was no significant decrease of cell viability when cells were cultured for 24 h (Figure 2D). In addition, there was no noticeable change of morphology in Hsp70 transfectatns for 24 h (data not shown). Taken together, these results suggest that regulated secretion of Hsp70 occurred for 24 h of culture.

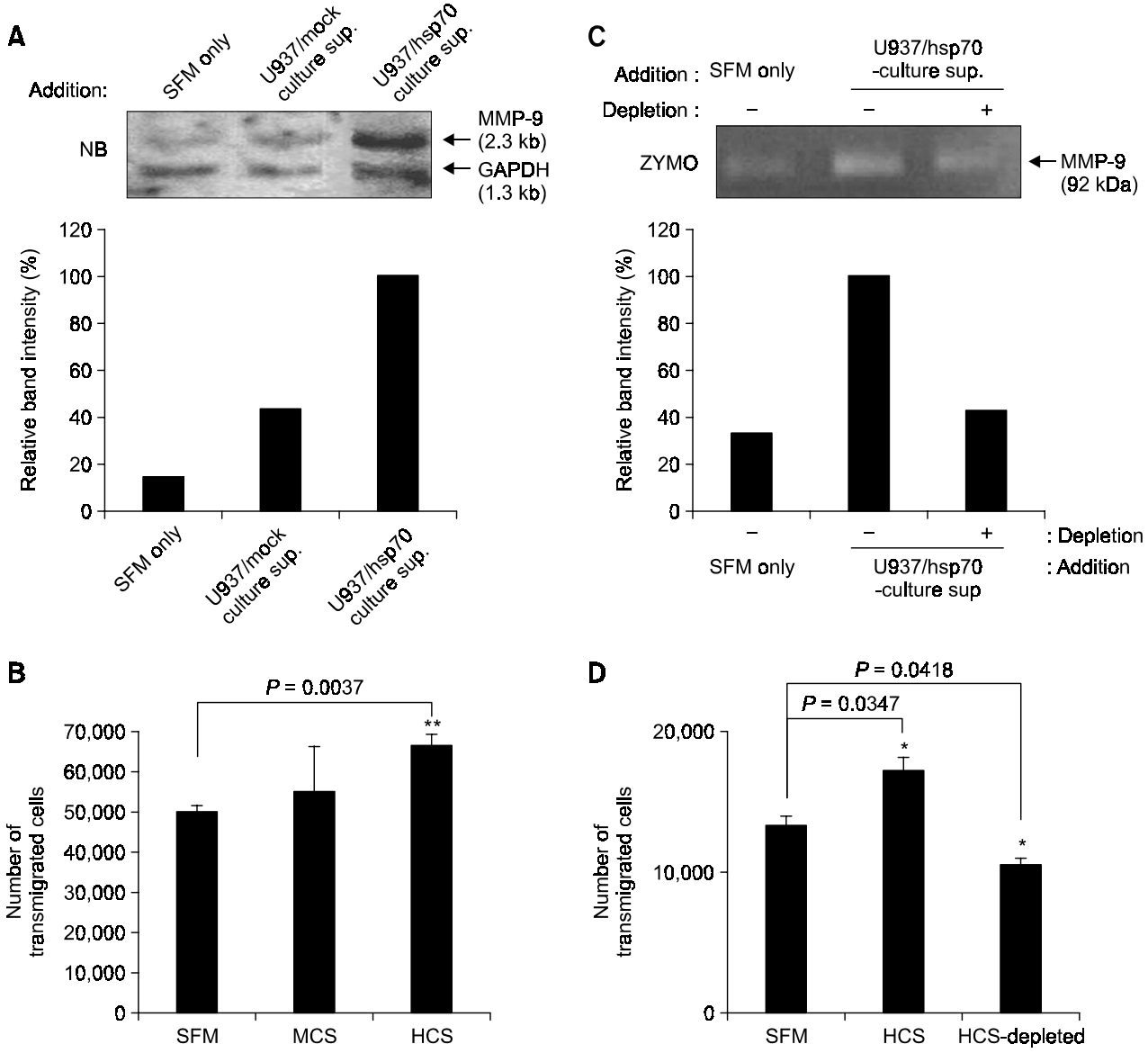

Figure 3. Effects of secreted Hsp70 on MMP-9 expression, secretion, and migration ability of U937 cells. (A) Hsp70-overexpressing U937 cells and mock transfectants were cultured in the absence of PMA. After 24 h, culture supernatants were collected, and added to U937 cell cultures. After another 24 h incubation, the cultured cells were harvested, and total RNA was isolated from them. MMP-9 mRNA expression was analyzed by Northern blotting analysis. (B) Hsp70-overexpressing U937 cells and mock transfectants were incubated for 24 h in serum-free medium. After that, culture supernatants was collected, and mixed with serum-free medium in the ratio of 1:1 (v/v). Then, U937 cells suspended in mixed culture medium were placed in the upper compartment coated with Matrigel of a modified Boyden chamber. The lower compartment of Boyden chambers was filled with conditioned medium of NIH3T3 cells. The cells migrated to the lower chamber were counted every 24 h for 72 h. Each assay was carried out in triplicates. Asterisks show a significant difference from control as follows: $^{**}P < 0.01$. Lines indicate additional statistical comparisons. SFM, serum free media; MCS, mock transfected cells culture supernatant; HCS, Hsp70-overexpressing cells culture supernatant. (C) Effect of depletion of secreted Hsp70 on the proMMP-9 secretion. Hsp70-overexpressing U937 cells were incubated for 24 h in serum-free medium. After that, culture supernatants were collected and divided into two groups. One group was incubated overnight with mouse IgG and protein A-conjugated beads. The other group was incubated overnight with anti-Hsp70 polyclonal antibody and protein A-conjugated beads. After centrifugation, culture supernatants were added to U937 cells for 24 h. Culture medium was taken, and analyzed for MMP-9 activity by the zymographic analysis. The bottom panel depicts densitometric analysis of upper panel result. (D) Hsp70-overexpressing U937 cells were incubated for 24 h in serum-free medium, and then culture supernatants were collected and divided into two groups. Immunodepletion of Hsp70 protein was performed as described in (C). Then, transmigration assay was carried out as described in (B). Asterisks show a significant difference from control as follows: $^{*}P < 0.05$. Additional statistical comparisons are indicated by lines.

The secreted Hsp70 increases MMP-9 expression and migrating ability of cells

Since Hsp70 could be released from cells as described above, we next examined whether the released Hsp70 could induce MMP-9 mRNA expression. After U937 cells were incubated in the culture supernatants containing the released Hsp70, MMP-9 mRNA expression levels were determined by Northern blot analysis. Figure 3A shows that culture supernatants from Hsp70 transfectants induced the MMP-9 mRNA expression about a 2.5-fold more than those from mock transfectants. Addition of serum- free medium alone had little effect on MMP-9 mRNA expression. These findings indicate that extracellular Hsp70 activates MMP-9 gene expression. To further evaluate the relationship between the released Hsp70 and MMP-9 gene expression in U937 monocytes, we treated U937 cells for 24 h with serum-free medium plus culture supernatants taken from mock or Hsp70 transfectants, and then determined the effect of culture supernatants on invasiveness using the modified Boyden chamber assay. The invasion rate was the highest in U937 cells incubated with culture supernatants from Hsp70 overexpressing cells, reflecting the amount of Hsp70 released (Figure 3B). To determine the extent to which secreted Hsp70 increases MMP-9 expression and cell invasion, Hsp70 in culture medium was immunodepleted with anti-Hsp70 polyclonal antibody or rabbit IgG. As shown in Figure 3C, immunodepletion with anti-Hsp70 polyclonal antibody resulted in a more than 60% decrease in MMP-9 secretion, as compared with contol. Figure 3D shows that depletion of Hsp70 led to a marked decrease in invasiveness (migrated cell number, 10,000), as compared with control (17,000). These results indicate that the secreted Hsp70 can induce MMP-9 mRNA expression and proMMP-9 secretion, consequently increasing invasive ability of the cells.

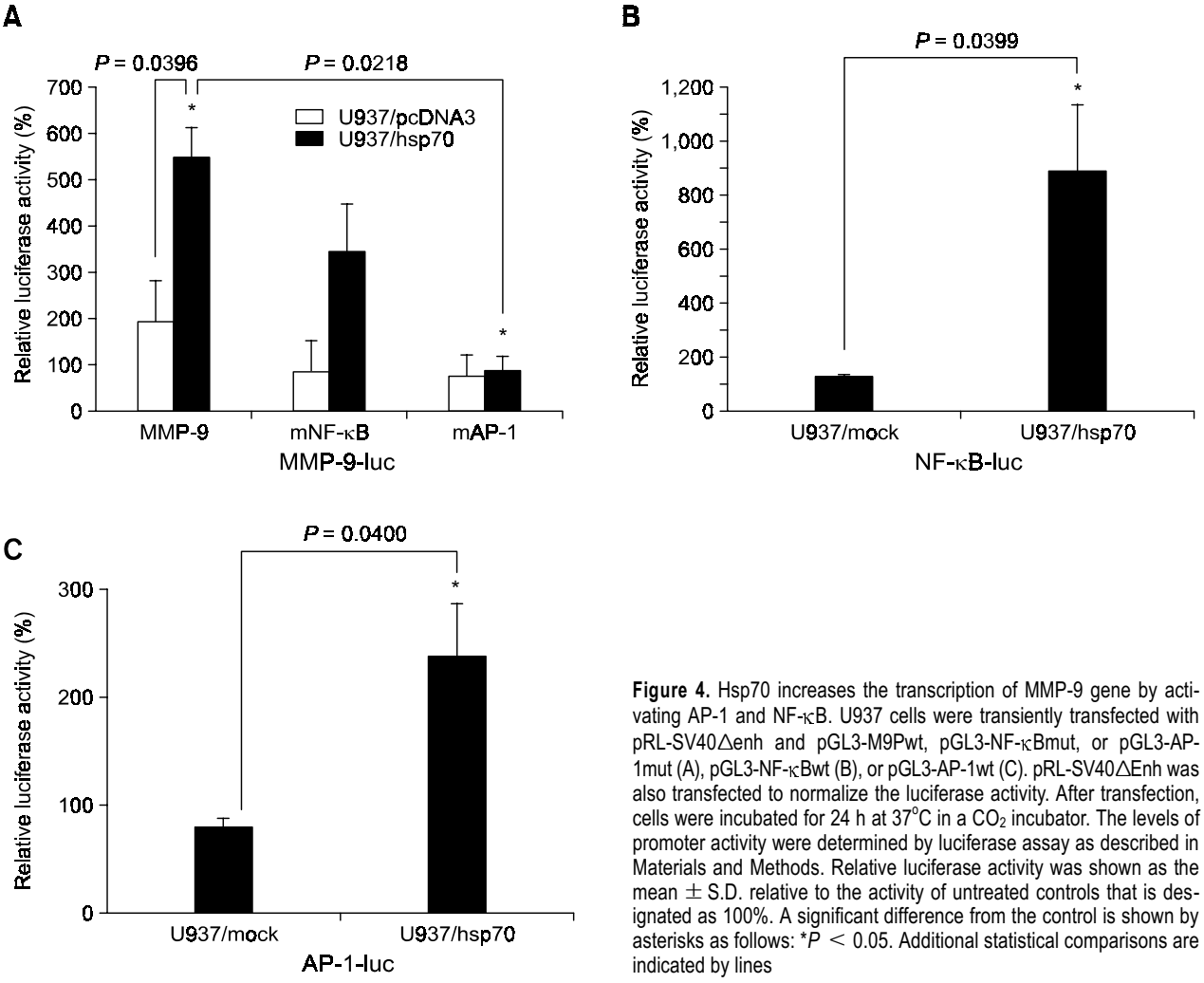

Figure 4. Hsp70 increases the transcription of MMP-9 gene by activating AP-1 and NF-κB. U937 cells were transiently transfected with pRL-SV40Δenh and pGL3-M9Pwt, pGL3-NF-κBmut, or pGL3-AP-1mut (A), pGL3-NF-κBwt (B), or pGL3-AP-1wt (C). pRL-SV40ΔEnh was also transfected to normalize the luciferase activity. After transfection, cells were incubated for 24 h at 37°C in a CO_2 incubator. The levels of promoter activity were determined by luciferase assay as described in Materials and Methods. Relative luciferase activity was shown as the mean ± S.D. relative to the activity of untreated controls that is designated as 100%. A significant difference from the control is shown by asterisks as follows: $^*P < 0.05$. Additional statistical comparisons are indicated by lines

The released Hsp70 activates NF-κB and AP-1

To investigate whether extracellular Hsp70 activates MMP-9 expression at the transcriptional level, the effect of Hsp70 on MMP-9 gene expression was assessed using the luciferase reporter gene assay. Hsp70 overexpression induced about a three-fold increase in the MMP-9 promoter-mediated luciferase activity in U937 cells transiently transfected with the MMP-9 promoter construct (Figure 4A). These results are consistent with Northern blot analysis described above. In addition, a mutation of the NF-κB binding site (pGL3-M9Pmt-NF-κB) decreased the activation of the MMP-9 promoter by Hsp70 and mutations of the two AP-1 binding sites (pGL3-M9Pmt-AP-1) drastically decreased the induction (Figure 4A). These results show that both NF-κB and AP-1 binding sites in the MMP-9 promoter are necessary for Hsp70-induced MMP-9 activation. Next, using NF-κB and AP-1 promoter luciferase constructs, we determined whether Hsp70 activated NF-κB and AP-1 activities, which have been shown to play a key role in regulating MMP-9 gene expression (Sato *et al*., 1993; Hah and Lee, 2003; Lee *et al*., 2004). NF-κB activity was approximately a seven-fold higher in the Hsp70 transfectants than in control (Figure 4B). This result is consistent with the previous result showing that exogenous Hsp70 activates the transcription activity of NF-κB (Asea *et al*., 2002). Similarly, AP-1 activity was nearly a three-fold higher in the Hsp70 transfectants than in control (Figure 4C). Taken together, these results indicate that extracellular Hsp70 induces MMP-9 transcription by activating NF-κB and AP-1.

Discussion

This study demonstrates that overexpression of Hsp70 in human monocytic cell line U937 induces the expression of MMP-9 gene through the activation of NF-κB and AP-1. Hsp70 cDNA transfectants released Hsp70 protein into culture supernatants, and the protein subsequently bound to the surface of U937 cells. Addition of culture medium, containing the released Hsp70, to U937 cells led to an increase in proMMP-9 secretion and invasiveness *in vitro*. Immunodepletion of the extracellular Hsp70 abolished its effect on induction of MMP-9 expression. The released Hsp70 activated NF-κB and AP-1, leading to induction of MMP-9 transcription. These results support previous observations that extracellular Hsp70 induces the expression of pro-inflammatory cytokines in macrophages (Asea *et al*., 2000; Dybdahl *et al*., 2002).

Hsp70 overexpression led to an increase in the amount of Hsp70 in culture medium. Hsp70 has been known to act not only as an intracellular chaperon, but also as a secretory protein (Chan *et al*., 1999; Asea *et al*., 2000; Guzhova *et al*., 2001; Somersan *et al*., 2001; Dybdahl *et al*., 2002; Njemini *et al*., 2003; Hunter-Lavis *et al*., 2004; Kimura *et al*., 2004; Njemini *et al*., 2004). Secretion of Hsp70 from a variety of cells in response to cellular stress has been widely reported. For example, it has been shown that Hsp70 is present in the sera of normal individuals and its circulating levels are related to the levels of inflammation markers (Chan *et al*., 1999; Dybdahl *et al*., 2002; Njemini *et al*., 2003, 2004). Treatment of macrophages with oxidized low density lipoprotein significantly increased Hsp70 concentration in supernatants, compared with untreated control (Svensson *et al*., 2005). Analysis of supernatants of transformed cell lines and primary human tumor tissue lysates showed that they contained considerably higher levels of Hsp70 than their normal autologous tissue counterparts (Somersan *et al*., 2001). The glioma cells were shown to release 5 to 15 pg of Hsp70 per 10^6 cells per day into the culture medium under normal conditions, but the amount increased following heat shock (Guzhova *et al*., 2001). These results indicate that Hsp70 can be released into the cultured medium or extracellular space from a variety of cell types in certain circumstances.

Hsp70 in the culture medium or serum may result from cell death-mediated release or its active secretion from cells. Our kinetic analysis of extracellular Hsp70 showed that during 24 h culture, there was about a 14-fold increase in Hsp70 present in the culture medium, but about a two-fold detectable increase in β-actin, an intracellular protein control. This result is consistent with previous data, suggesting that a major fraction of Hsp70 is actively released into the cell culture medium (Broquet *et al*., 2003; Hunter-Lavis *et al*., 2004; Lancaster and Febbraio, 2005). Two recent studies have provided evidences that Hsp70 can be actively released into the culture medium via specialized membrane microdomains, termed lipid rafts (Broquet *et al*., 2003; Hunter-Lavis *et al*., 2004). In addition, exonemes have been shown to play a role in the active secretion of Hsp70 from human peripheral blood mononuclear cells in both basal and heat shock-induced states (Lancaster and Febbraio, 2005).

Purified Hsp70 has been shown to act as a novel cytokine, which causes monocytes to express proinflammatory cytokines such as IL-1, IL-6, and TNF-α (Asea *et al*., 2000; Esparza *et al*., 2004). However, other reports suggested that Hsp70-mediated induction of proinflammatory cytokines might be due to contamination by LPS as well as LPS-associated molecules (Gao and Tsan, 2003a, b). In the present study, we treated cells with LPS-free Hsp70 proteins

expressed in the U937 cells rather than Hsp70 produced in bacterial cells, and then investigated its effect on MMP-9 activity. Addition of culture supernatants containing the secreted Hsp70 increased MMP-9 activity and subsequently migrating ability of cells. Furthermore, immunodepletion of the secreted Hsp70 inhibited the induction of MMP-9 expression, thereby clearly showing that extracellular Hsp70 itself rather than endotoxin contamination activated inflammatory-related genes.

Our present study showed that Hsp70 overexpression induced MMP-9 gene expression by activating NF-κB and AP-1. Hsp70 may activate the innate immune system in a manner similar to that of LPS and bacterial lipoproteins. Binding of LPS to TLRs induces activation of the transcription factor NF-κB in host cells upon infection (Kirschning *et al*., 1998). In this study, we showed that the secreted Hsp70 was bound to cell surfaces, although we did not determine whether it was bound to TLRs. Extracellular Hsp70 might potentiate NF-κB activation, inducing a pro-inflammatory response typical of LPS. Indeed, soluble Hsp70 appears to induce production of proinflammatory cytokines by stimulating the CD14, TLR2 and TLR4 complex signaling pathways (Asea *et al*., 2002; Vabulas *et al*., 2002). Interestingly, a recent study demonstrated that heat shock induced the expression of TLR2 and TLR4 prior to that of Hsp70 in monocytes (Zhou *et al*., 2005). Induction of heat shock response may activate proinflammatory gene expression by up-regulating not only the secretion of extracellular Hsp70 proteins, but also their binding to TLRs.

In summary, we demonstrated that overexpression of Hsp70 led to the release of Hsp70, which was bound to the cell surface of monocytes, and induced the expression of MMP-9 gene through activation of NF-κB and AP-1. These data not only support the previous reports suggesting that the extracellular Hsp70 may activate pro-inflammatory cytokine genes in human monocytes, but also suggest that Hsp70-induced MMP-9 activation may play a role in the transmigration of leucocytes into the inflamed tissues. Further studies are required to investigate the functional role of extracellular Hsp70 that can be induced under pathological conditions.

Acknowledgment

This work was supported by Vascular System Research Center grant of the Korea Science and Engineering Foundation. We thank Yoon-Kyoung Kim and Si-Eun Kim for their technical help.

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