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A potential role for *Helicobacter pylori* heat shock protein 60 in gastric tumorigenesis

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ABSTRACT

Helicobacter pylori has been found to promote the malignant process leading to gastric cancer. Heat shock protein 60 of *H. pylori* (HpHSP60) was previously been identified as a potent immunogene. This study investigates the role of HpHSP60 in gastric cancer carcinogenesis. The effect of HpHSP60 on cell proliferation, anti-death activity, angiogenesis and cell migration were explored. The results showed that HpHSP60 enhanced migration by gastric cancer cells and promoted tube formation by umbilical vein endothelial cells (HUVECs); however, HpHSP60 did not increase cell proliferation nor was this protein able to rescue gastric cancer cells from death. Moreover, the results also indicated HpHSP60 had different effects on AGS gastric cancer cells or THP-1 monocytic cells in terms of their expression of pro-inflammatory cytokines, which are known to be important to cancer development. We propose that HpHSP60 may trigger the initiation of carcinogenesis by inducing pro-inflammatory cytokine release and by promoting angiogenesis and metastasis. Thus, this extracellular pathogen-derived HSP60 is potentially a vigorous virulence factor that can act as a carcinogen during gastric tumorigenesis.

Introduction

Helicobacter pylori is an important human pathogen and infection with this bacterium may lead to various gastric diseases [1]; particularly, it has been proposed to act as a carcinogen causing gastric tumors. *H. pylori* has been demonstrated to promote tumorigenesis. Cancer initiate processes such as preventing cells from apoptosis [2], enhancing cell proliferation [3], increasing blood vessel density [4], and augmenting cell invasion/migration ability [5] were found to be associated with *H. pylori* infection.

Persistent infection by *H. pylori* is known to trigger chronic inflammation, which is an important risk factor for malignancy [6]. Chemokine productions during inflammation have multiple effects on tumor growth, invasion, and angiogenesis [7]. Recent studies have indicated that patients with *H. pylori* infection have higher CXC chemokine expression levels of various factors, including IL-8, GRO, and epithelial neutrophil activating protein-78 (ENA-78), in the gastric mucosa, gastric epithelium cells, and macrophages [8,9].

Based on detection of auto-antibodies in patients, it has been suggested that overexpression of human heat shock protein 60 (HSP60) during the early stage of breast cancer development may be functionally correlated with tumor growth and/or progression [10]. Additionally, human HSP60 has been suggested as facilitating metastasis [11] and anti-apoptosis [12] in tumor cells. If we consider pathogen-derived HSP60s, however, there have been only a few reports suggesting an association between these proteins and tumorigenesis. HSP60 from the intracellular bacterium *Chlamydia trachomatis* has been proposed as a risk factor for ovarian cancer since it is able to inhibit apoptosis [13]; nonetheless, the effect of extracellular HSP60 from a bacterial pathogen on cancer development remains unclear. *H. pylori* HSP60 has been previously identified as an adhesion molecule that is able to interact with gastric epithelial cells and mucin [14]. It is also known to be involved in the induction of host inflammatory responses. As a potent immunogen, HpHSP60 can stimulate human monocytes/macrophages and/or human gastric epithelium cells to produce pro-inflammatory cytokines including IL-1, IL-6, IL-8, and GRO [15–18]. Recently, by

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measuring the level of anti-HpHSP60 antibodies in the sera of patients with different gastric diseases, Tanaka et al. proposed that *H. pylori* HSP60 might be associated with gastric carcinogenesis [19].

In this study, we investigate how HpHSP60 may act during tumor progression and malignancy. By screening *H. pylori* patient sera and a general examination of the cancer growth properties of HpHSP60-treated cells, we found that HpHSP60 promote inflammation, cell migration, and angiogenesis but does not increase cell proliferation or anti-death activity. These results suggest that HpHSP60 may act as a potent carcinogen during *H. pylori* infection.

Materials and methods

Cell culture. AGS and SNU-1 cells (BCRC, Hsinchu, Taiwan) were cultured in RPMI 1640 medium (Invitrogen, MD, USA) supplemented with 2 g/L sodium bicarbonate (BIO BASIC Inc., Canada), 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, MD, USA) and 50 μg/mL penicillin/streptomycin (Biological industries, Beithaemek, Israel). Human umbilical vein endothelial cells (HUVECs) (BCRC, Hsinchu, Taiwan) were cultured in M199 medium (Invitrogen, MD, USA) supplemented with 10% heat-inactivated FBS, 50 μg/mL penicillin/streptomycin, 30 μg/mL endothelial cell growth supplement (Sigma–Aldrich, Steinheim, Germany), and 25 U/mL heparin (Sigma–Aldrich, Steinheim, Germany). THP-1 cells (BCRC, Hsinchu, Taiwan) were cultured in RPMI 1640 medium supplemented with 0.05 mM 2-mercantoethanol (Amresco Inc., OH, USA), 2 g/L sodium bicarbonate, 50 μg/mL of penicillin/streptomycin, and 10% heat-inactivated FBS.

Measurement of serum antibody to HpHSP60. The serum samples were obtained from National Taiwan University Hospital and came from the patients identified as having *H. pylori* infection. According to the diagnostic results, the samples were divided into four groups, namely the gastric cancer group (HC), the gastritis group (HS), the duodenal ulcer group (HD), and the peptic ulcer group (HU). Serum antibodies against HpHSP60 were measured by enzyme-linked immunosorbent assay (ELISA). First, 96-well plates were coated with 1 μg HpHSP60 overnight at 4 °C. After that, the wells were blocked with 2% skim milk in 0.05% Tween 20/PBS (PBST) for 1 h, and then incubated with 100 μL of each patient's serum at a dilution of 1: 1,000,00 for 1 h at room temperature. Subsequently, 100 μL of peroxidase-labeled goat anti-human IgG, IgA, IgM antibody (Millipore Co., MA, USA) at a dilution of 1: 10,000 were added, and the plates were incubated for 1 h at room temperature. After this, the plates were washed with PBST three times and each well had 100 μL of 3,3′,5,5′-tetramethylbenzidine solution (Sigma–Aldrich, Steinheim, Germany) added; the plates were then developed for 20 min. After the reaction was finished, 100 μL HCl per well was added to stop the reaction. Finally, the optical density was measured at 450 nm on an ELISA plate reader.

MTT viability assay. Assessment of cell viability was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltertrazolium bromide (MTT) assay. Gastric tumor cells, AGS or SNU-1, were seeded (2×10^4 cells/well) into 96-well plates, which were then incubated in different concentrations of HpHSP60 for 48 h at 37 °C. The effect of HpHSP60 on cell anti-death activity in the above two cell lines was measured by pre-treating the cells with either mitomycin C (2.5 μg/mL) (KYOWA, Tokyo, Japan), or curcumin (25 or 100 μM) (Sigma–Aldrich, Steinheim, Germany) for 4 h. After washing out mitomycin C or curcumin and the cells were incubated with different concentrations of HpHSP60 for 24 h. At the end of incubation time, 0.5 mg/mL MTT solution was added to each well, and cells were cultivated for 4 h at 37°C. Conversion of MTT into purple formazan by metabolically active cells indicates the extent of cell viability. The crystals of produced formazan were dissolved with DMSO and the optical density was measured at 570 nm using a microplate reader.

Cell migration assay. AGS cells or HUVECs were incubated in medium without serum for 24 h prior to seeding into Transwell cell culture chambers (Corning, NY, USA). The cell suspensions (3×10^4 cells) with or without HpHSP60 were added to the upper side of the Transwell cambers for 8 h cultivation at 37 °C and 5% CO_2. After incubation, the wells were washed and the cells stained with 50 μg/mL propidium iodide (PI) (Sigma–Aldrich, Steinheim, Germany); finally the membranes were examined using a fluorescent microscope. The migration rate was calculated as the number of cells that moved to the lower side of the Transwell insert as a fraction of the total cells seeded.

Tube formation assay. HUVECs (1.5×10^4 cells) were cultured in a 96-well plate coated with Matrigel (BD Biosciences, Bedford, MA, USA) with or without HpHSP60 (10 μg/mL) for 6 h. Tube formation was quantified by measuring the number of tube branch points using a phase contrast microscope (100×).

ELISA assays for cytokine expression. THP-1 and AGS cells were seeded in 24-well culture plates with 1 mL of cell suspension (10^5 cells/mL) and incubated at 37 °C in a 5% CO_2 atmosphere for 2 h. After incubation, 10 μg of HpHSP60 was added to cells for the remainder of the 16 h incubation. Supernatants were harvested after the 16 h incubation period and the IL-8, IL-6, and TNF-α levels were measured by various ELISA assays (R&D systems, Minneapolis, MN) according to the manufacturer's specifications.

Statistical analysis. All results were expressed as means ± SEM. Student's *t* test was applied to compare the titers of anti-HpHSP60 antibodies, cell proliferation assays, migration assays, pro-inflammatory cytokine profiles, and tube formation assays.

Results

Analysis of the anti-HpHSP60 antibody titers in H. pylori-positive patients with gastric cancer or other gastro-duodenal diseases

To determine whether HpHSP60 was associated with gastric diseases, the levels of anti-HpHSP60 antibodies in the sera of *H. pylori*-infected patients were measured as an indicator. According the

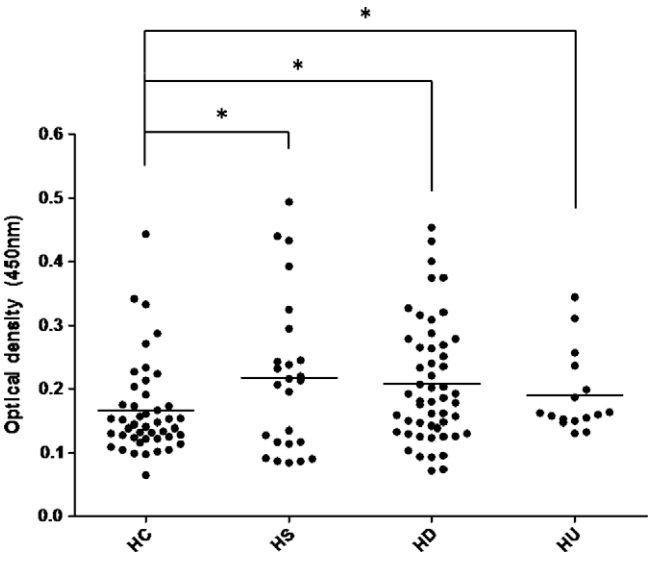

Fig. 1. Anti-HpHSP60 titers in the sera from *H. pylori*-infected patients. Sera collected from patients with *H. pylori* infection and various gastro-duodenal diseases were analyzed to measure their anti-HpHSP60 antibody titers. HC: gastric cancer ($n = 45$); HS: gastritis ($n = 26$); HD: duodenal ulcer ($n = 76$); HU: gastric ulcer ($n = 16$). $^{**}p < 0.01$.

clinical symptoms, samples were collected from patients suffering from gastric cancer (HC, $n = 45$), gastritis (HS, $n = 26$), duodenal ulcer (HD, $n = 76$), and gastric ulcer (HU, $n = 16$). The titers of anti-HpHSP60 antibody in sera of patients with gastric cancer were significant lower than in the sera of the other gastro-duodenal disease groups (Fig. 1, $p < 0.05$). The lower titers of anti-HpHSP60 antibody in the cancer patients might indicate that HpHSP60 exposure was associated with progression to gastric cancer; however, more evidence is needed to prove this correlation.

The effects of HpHSP60 on the viability/growth of gastric cancer cells

Tumorigenesis consists of several main aspects that create the microenvironment necessary for cancer development. Enhancing cell proliferation and the anti-death ability of the cancer cells are two of major procedures that allow tumors to grow. HpHSP60 was used to determine whether the presence of the protein affects the viability or the cell growth of gastric cancer cell lines. Both AGS and SNU-1 cells were subject to serial dosages of HpHSP60 for 48-h incubation; the results showed that these two cell types did not increase their cell proliferation rate (Fig. 2A and B). Subsequently, two chemicals that induce apoptosis through different cell signal pathways were used to evaluate if HpHSP60 was able to reverse the cell death fate. Treatment with mitomycin C (2.5 μg/mL), which cross-links DNA bases to promote apoptosis, causes ~55% cell death with AGS or SNU-1 cells. Incubation for 24-h with HpHSP60 was not able to rescue either cell type from death (Fig. 2C and D). Similar results were found for the curcumin-treated cells; 25 μM or 100 μM curcumin gave rise to ~60% cell death when AGS or SNU-1 cells were treated; however, treatment with HpHSP60 also failed to reduce the apoptosis rate (Fig. 2E and F).

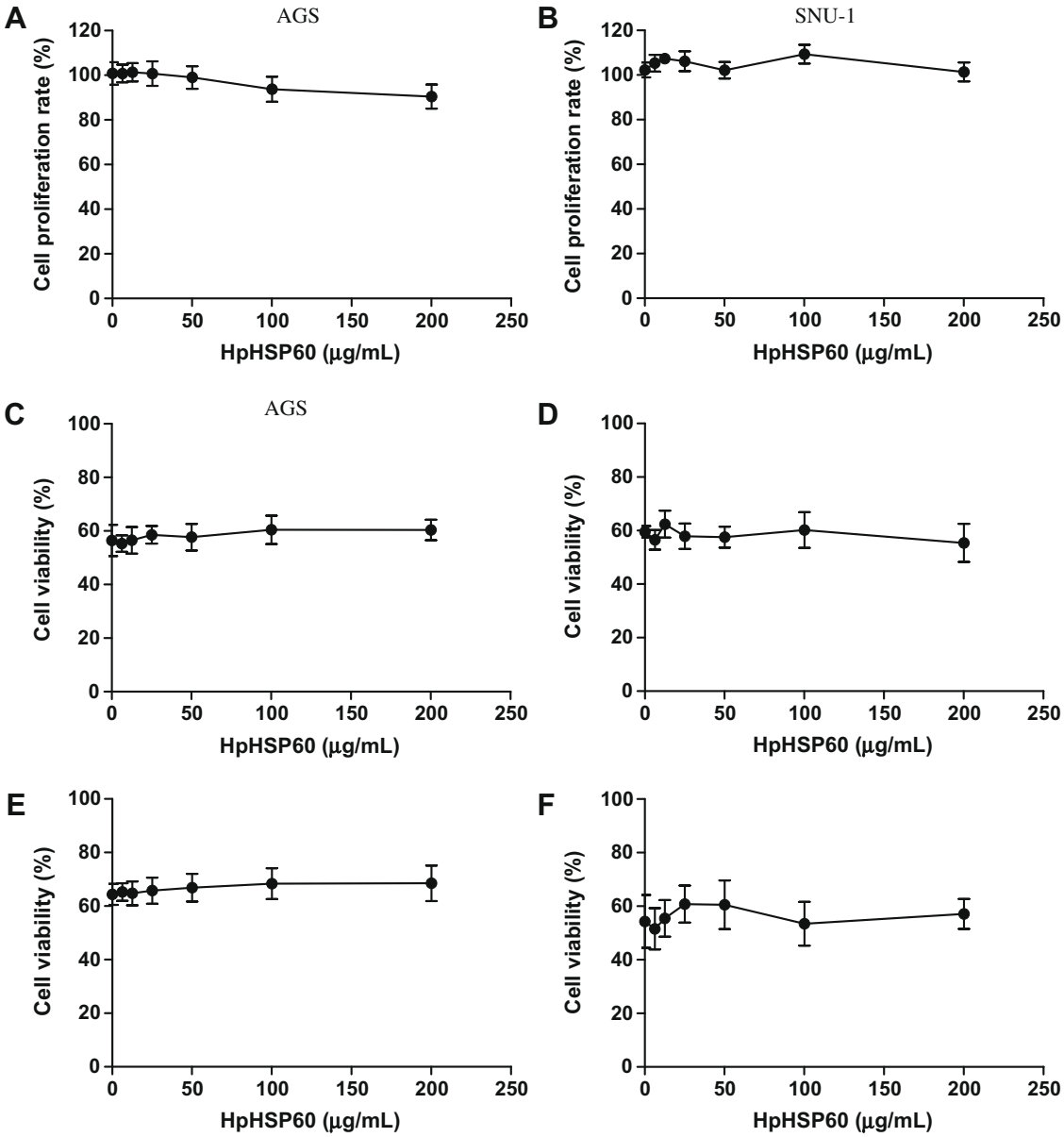

Fig. 2. The effect of HpHSP60 on cell proliferation and anti-apoptosis ability. Serial dosages of HpHSP60 (0, 6.25, 12.5, 25, 50, 100, and 200 μg/mL) were incubated with AGS (A) or SNU-1 (b) cells for 48 h at 37 °C and the cell proliferation rate measured. For the anti-apoptotic evaluation, mitomycin c (2.5 μg/mL) was used to treat AGS (C) or SNU-1 (D) cells for 4 h prior to HpHSP60 incubation in order to achieve cell death rate of about 50%. A 24-h cultivation with HpHSP60s was then performed to assess the effect of this protein in terms of any anti-death effect. Similarly, curcumin was used to pre-treat AGS (25 μM) (E) or SNU-1 (100 μM) (F) before HpHSP60 treatment. Data are expressed as means ± SEM for three replicates ($n = 9$).

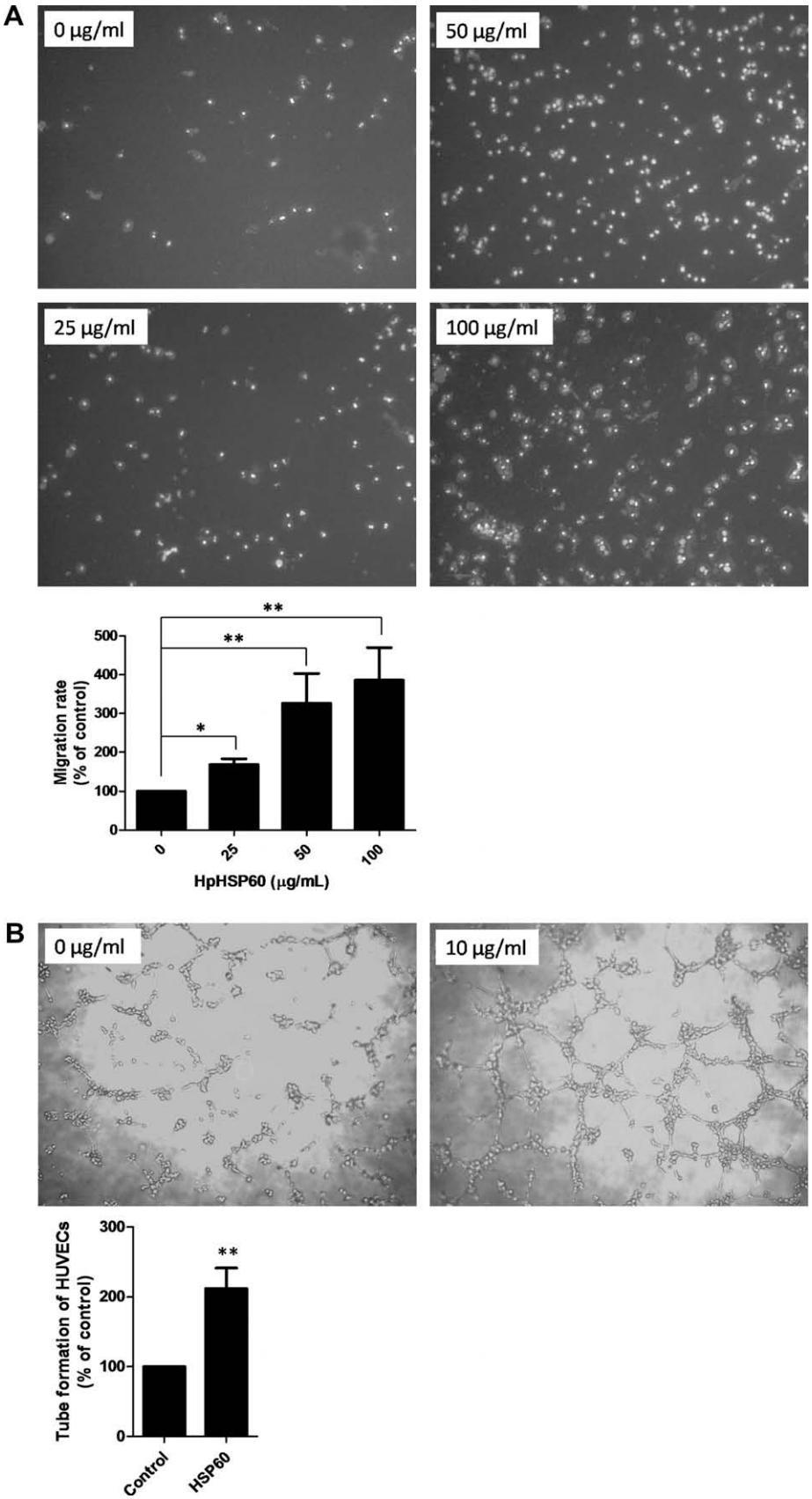

Fig. 3. The effects of HpHSP60 on cell migration and angiogenesis. (A) 25, 50, and 100 μg/mL HpHSP60 was co-cultured with AGS cells in the upper side of a Transwell for 8 h. The cells moving to the lower side of the Transwell membrane were stained with propidium iodide (PI) and analyzed by fluorescent microscopy ($n = 9$, $^{*}p < 0.05$). (B) To evaluate angiogenesis, HpHSP60 (10 μg/mL) was incubated with HUVECs for 6 h and the number of tube branch points counted ($n = 9$, $^{**}p < 0.01$). Data are expressed as means ± SEM for three replicates.

The effect of HpHSP60 on cell migration and angiogenesis

Migration and angiogenesis activities are also important factors that promote cancer formation, so we next examined whether HpHSP60 has any effect on those areas. The effect on migration of HpHSP60 when gastric cancer cells were treated was monitored by the Transwell assay. The results revealed that the migration behavior of AGS cells was significantly enhanced by HpHSP60 in a dose-dependent manner; specifically, 25–100 μg/mL of HpHSP60 gradually increased the number of migratory cells found on the lower side of the Transwell (Fig. 3A, $p < 0.05$). When the correlation between HpHSP60 and angiogenesis was evaluated using the *in vitro* capillary-like tube formation assay, it was found that, after HSP60 treatment of HUVECs for 6 h, the branch point number, which is used as a measure of capillary-like tube formation, was significantly increased compared to the untreated group (Fig. 3B, $p < 0.01$).

Induction of pro-inflammatory cytokines by HpHSP60 in gastric tumor and monocytic cells

Persistent and chronic inflammation is thought to be a risk factor for tumor progression [20]. Previous studies have shown HpHSP60 can act as a potent immunogen because it induces pro-inflammatory cytokines such as IL-6 or IL-8 that augment inflammation [16,17,21]. To elucidate the effects on the pro-inflammatory cytokine profile of induction by HpHSP60, AGS, and monocytic THP-1 cells were treated with HpHSP60 and the effect of the expression of various cytokines measured. The reason monocytes were also investigated is because of their role as the first line of innate defense against extracellular bacteria and because they are the main source of inflammatory cytokine secretion. The results showed HpHSP60 was able to induce higher expressions of IL-6, IL-8, and TNF-α in THP-1 cells (Fig. 4A–C, $p < 0.01$). However, in contrast, there was only a small increase in the expression of IL-6 and IL-8 in AGS cells and no effect on TNF-α expression.

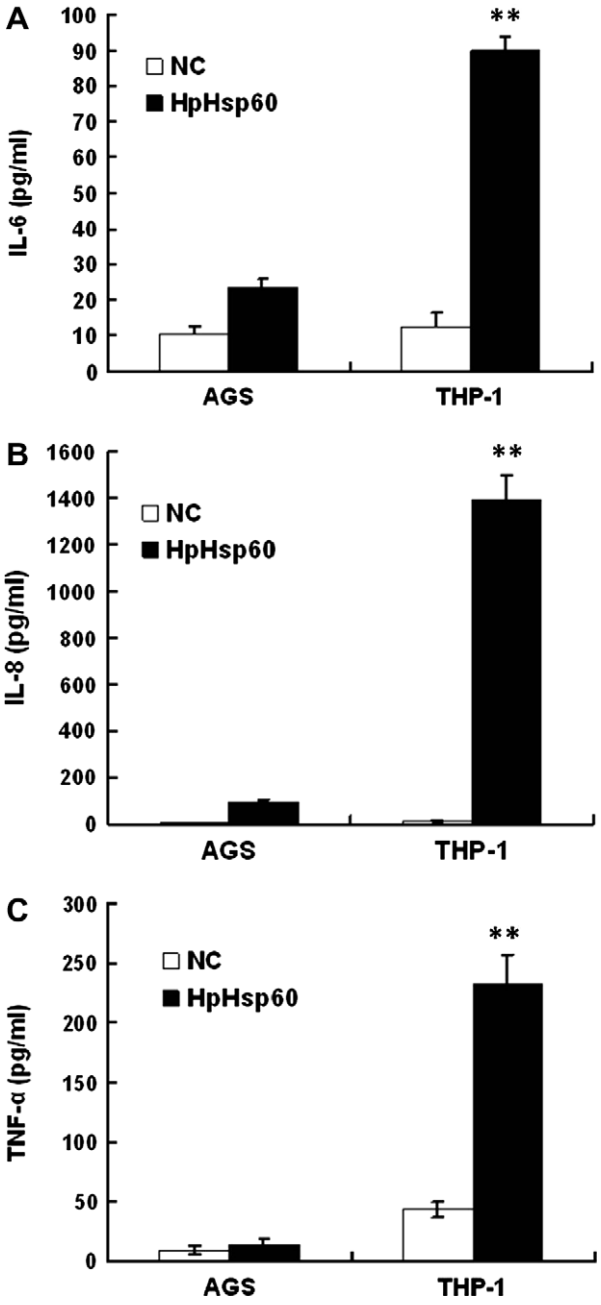

Fig. 4. Pro-inflammatory cytokine production induced by HpHSP60 in AGS and THP-1 cells. Data are expressed as means ± SEM for three replicates ($n = 9$, $^{**}p < 0.01$).

Discussion

In this study, we demonstrated the role of HpHSP60 in gastric tumor progression. This seems to occur by promoting the migration ability of cancer cells (Fig. 3A), by increasing the angiogenic activity of endothelial cells (Fig. 3B), and by inducing inflammatory cytokines production by both gastric epithelial cells and monocytes (Fig. 4). These results suggest that HpHSP60 is able to accelerate tumorigenesis by multiple mechanisms.

Microbial HSP60s are known as immunodominant antigens because they usually induce a powerful immune response after infection [22]. The HSP60 derived from *Chlamydia pneumoniae* causes diffuse pneumonia with a massive infiltration of neutrophils into the lung tissue that increases IL-18 production in the bronchoalveolar lavage fluid [23]. HSP65 of *Mycobacterium leprae* has been found to stimulate the release of TNF-α, IL-6, and IL-8 from human monocytic cells [24]. Recently, *H. pylori* HSP60 has been reported to induce pro-inflammatory cytokines such as IL-6 and IL-8 from human monocytic cells and/or gastric epithelium cells through the TLR-2 and TLR-4 pathway [16,17,21]. Based on this earlier research, we observed that HpHSP60 could trigger the releases of IL-6, IL-8, and TNF-α from THP-1 cells (Fig. 4). This verifies that *H. pylori* HSP60 is indeed a dominant factor in the induction of host inflammation.

Chronic inflammation has been suggested to be highly associated with tumor development [20]. Persistent infection with *H. pylori* may cause continuous inflammation by expressing HpHSP60 and this could result in the initiation of tumors. Therefore, it would be a logical inference that *H. pylori*-infected patients with lower titers of neutralized anti-HpHSP60 antibodies may have a higher incidence of developing gastric tumors. Our results seemed to support this inference since gastric cancer patients were found to have significantly lower antibody titers (Fig. 1).

Our results also show that HpHSP60 is able to enhance tumor cell migration and vessel angiogenesis (Fig. 3). These effects might be connected with the ability of HpHSP60 to stimulate inflammation. *H. pylori*-associated gastric cancers have been proposed to be related with chronic inflammation [25]. A previous study has suggested that *H. pylori*-induced persistent inflammation is associated with promoting the angiogenesis process [26]. Ruegg et al. also specified that macrophages play an important

role in prompting chronic inflammation within the tumor microenvironment [27]. In addition, the up-regulation of COX-2 expression and increased production of pro-inflammatory cytokines such as IL-1, IL-6, TNF-α, and IFN-γ, which are released from inflammatory cells during *H. pylori* infection, could also affect tumor cell behavior, perhaps by the induction of motility, and organization of the tumor's vascular network [28,29]. In this (Fig. 4) and our previous studies [18], HpHSP60 have been shown to stimulate secretion of the angiogenic factor IL-8 and expression of GRO in AGS and monocytic cells. IL-8 and GRO are both ELR^+ CXC chemokines and have high affinity to engage to CXCR2, which would cause the increase in cytoplasmic calcium concentration [30]. The CXCR2 inhibitor, SB225002, and intracellular calcium chelator BAPTA abolished the HpHSP60-induced increases in the tube formation of HUVEC (unpublished results) that indicated the CXCR2 signal pathway played an important role for HSP60-enhanced tube formation.

In fact, evidence supporting the hypothesis that HSP60s can act as a cancer promoter has been presented previously in a number of reports. The intracellular bacterium *C. trachomatis*, which is highly associated with the development of cervical and ovarian cancer [31], is able to produce large quantities of HSP60, which forms complexes with caspase-3, Bax, and Bak from the host cells; these complexes inhibits apoptosis [13,32]. The same mechanism was also found to occur with human HSP60, which can inhibit cell death [33]. This protein also shows a higher expression level in neoplastic tissue from various tumors during carcinogenesis [34]. Similarly, canine HSP60 has been shown to be a tumor marker and is involved in the carcinogenesis of canine tumors [35]. These results all support the idea that HSP60s indeed participate in tumorigenesis.

In a manner that is different to HSP60s from other species, HpHSP60 did not enhance cell proliferation or protect the cells from apoptosis. Two reagents with different mechanisms were used to initiate apoptosis in this study, Mitomycin C damages DNA by cross-linking bases to induce apoptosis [36] while curcumin induces apoptosis via inhibiting the NF-κB pathway [37] or interfering with the microtubule activity [38]. However, HpHSP60 failed to rescue either type of cell from chemical-mediated cell death. Thus, we suggest that HpHSP60 is not able to retard apoptosis in cancer cells that is induced by damage during DNA replication or by interference with the cell cycle. This discrepancy may be due to the place where HSP60 acts. Both the human and the intracellular *Chlamydial* HSP60 are located within cells and can easily interact with the apoptosis-related or proliferation-associated factors. Notwithstanding this, the extracellular pathogen *H. pylori*-derived HSP60 will have difficulty crossing the cell membrane to react with such molecules.

Taking all the above results together, we consider that HpHSP60 is able to activate proinflammtory responses that enhance tumor cell migration and the angiogenic process. By promoting angiogenesis and metastasis, HpHSP60 accelerates tumor malignancy. *H. pylori* has been defined as a carcinogen and therefore the role of the bacterium's HSP60 in cancer progression is worthy of further elucidation.

Acknowledgments

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