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Original Article

High Mobility Group Box1 (HMGB1) released from cancer cells induces the expression of pro-inflammatory cytokines in peritoneal fibroblasts

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High Mobility Group Box1 protein (HMGB1), one of the mediators of inflammation, is associated with tumorigenesis. The HMGB1-Receptor for advanced glycation end-products (RAGE) in gastric adenocarcinoma tissues promoted gastric cancer growth, however, there are no reports concerning the relationship between the expression of HMGB1 in gastric cancer and cancer-related inflammation. Fibroblasts exist most abundantly on cancer tissue where inflammation occurs. So, we studied the effects of HMGB1 released from cancer cells on the fibroblasts. The expression of HMGB1 in cancer cells and nuclear factor-kappa B (NF-kB) in fibroblasts were evaluated immunohistochemically in human gastric cancer specimens. Cytoplasmic HMGB1 expression in the cancer cells and nuclear translocation of NF-kB in fibroblasts were detected at deeper invasion. To determine whether HMGB1 released from cancer cells induces the expression of pro-inflammatory cytokines in fibroblasts, we analyzed the activation of Toll-like receptor (TLR) signaling. Fibroblasts stimulated by recombinant HMGB1 and the HSC44PE-conditioned medium showed the phosphorylation of Interleukin-1 receptor associated-kinase 4 (IRAK4), nuclear translocation of NF-kB, and enhanced pro-inflammatory cytokine expression. Treatment with HSC44PE-conditioned-medium transfected with siRNA-HMGB1 reduced the expressions of pro-inflammatory cytokines in the fibroblasts. We propose that HMGB1 released from cancer cells induces the expression of pro-inflammatory cytokines in peritoneal fibroblasts through the HMGB1-TLR2/4 pathway.

Key word: cancer, inflammation, HMGB1, peritoneal fibroblasts, TLR-2, TLR-4

High Mobility Group Box1 (HMGB1) is a highly conserved nuclear protein, acting as a chromatin-binding factor that bends DNA and promotes access to transcriptional protein assemblies on specific DNA targets such as p53 and nuclear factor kappa B (NF-kB).[1,2] HMGB1 is released from necrotic cells and secreted by inflammatory cells, and has been implicated in the pathogenesis of inflammatory diseases.[3–5] Previous reports have shown that HMGB1 and the receptor for advanced glycation end-products (RAGE) are detected in gastric cancer and are associated with cancer growth.[6,7] Neutralizing antibodies to HMGB1 decrease cancer incidence and size in colitis-associated cancer models,[8] and targeting HMGB1 ligands or its receptors represents an important potential application in cancer therapeutics.[9] When released or secreted from the cells, HMGB1 binds to Toll-like receptors (TLR)-2 and -4 expressed on immune cells such as macrophages and lymphocytes. TLRs have also been shown to be expressed on fibroblasts in some specific locations such as the gingiva and dental pulp and their expressions are enhanced also in response to bacterial infection, gingivitis and chronic periodontitis.[10,11] Activation of the HMGB1-TLR2/4 signaling pathway has been demonstrated in inflammatory diseases such as rheumatoid arthritis, arteriosclerosis and sepsis.[12] The cancer stroma is composed of various types of cells including immune cells, endothelial cells and fibroblasts. Fibroblasts are the most abundantly occurring cells in the cancer stroma, and promote cancer cell growth, invasion and metastasis.[13,14] Fibroblasts maintain the structural integrity of cells by producing extracellular matrix, and play important roles in tissue remodeling as well as in wound healing in

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inflammation.[15] It has been reported that expression of alpha-SMA is detected on myofibroblasts and activated fibroblasts, which secretes pro-inflammatory cytokines upon stimulation such as LPS and PMA.[16] An array of cytokines and chemokines that are mitogenic and/or chemoattractants for granulocytes, mast cells, macrophage/monocytes and fibroblasts are produced by the interaction between cancer cells and inflammatory cells. In gastric cancer, serum IL-6 in patients is correlated with gastric cancer invasion and liver metastasis, and IL-6 induces gastric cancer cell invasion via c-Src/ RhoA/Src pathway.[17] High level of serum IL-6 is a poor prognosis factor of disease reoccurrence and survival in gastric cancer patients.[18] Cytokine production, one of the inflammation responses between cancer cells and cancer microenvironment, would be involved in gastric cancer promotion and progression. Studies about the role of fibroblasts conducted to date now have been focused mainly on the formation of cancer stroma. As mentioned above, pro-inflammatory cytokines production through the TLR2/4-HMGB1 pathway has been reported only in immune cells.[19] Fibroblasts exist most abundantly on cancer tissue where inflammation occurs, and they may also be involved in producing pro-inflammatory cytokines in the cancer microenvironment. However, there is no report concerning the relationship between the expressions of HMGB1 in gastric cancer and cancer-related inflammation by fibroblasts. In this study, we showed that fibroblasts derived from the human peritoneum express TLR-2 and -4, and HMGB1 released from cancer cells activates the TLR-2 and -4 expressed on the fibroblasts by examining the phospholyration of IL-1 receptor associated kinase (IRAK4), nuclear translocation of NF-kB and expression of pro-inflammatory cytokines.

MATERIALS AND METHODS

Cell lines

Three human gastric (MKN7, HSC44PE and HSC60) and two human colon (Caco2 and DLD1) cancer cell lines were used in the study. MKN7 was obtained from Immuno-Biological Laboratories Company (Gunma, Japan), and HSC44PE, HSC60 were provided by Dr. Kazuyoshi Yanagihihara.[20] Caco2 and DLD1 were obtained from American Type Culture Collection (Manassas, VA, USA). MKN7, Caco2 and DLD1 were maintained in DMEM medium (Life Technologies, Carlsbad, NM, USA) containing 10% heat-immobilized fetal bovine serum (FBS, Life Technologies), supplemented with penicillin/streptomycin (P/S, Life Technologies). HSC44PE and HSC60 were maintained in RPMI 1640 medium (Life Technologies) containing 10% heat-immobilized FBS, supplemented with P/S.

Human primary fibroblast cells (hPFCs)

Non-cancerous serosal tissues (at least 5 cm away from the cancer lesion) were obtained from the surgically resected specimens of four gastric cancer patients. The procedure for isolating hPFCs was as previously described.[21] hPFCs were maintained in alpha-MEM medium (Life Technologies) containing 10% heat-immobilized FBS, supplemented with P/S. Written informed consent was obtained from the patients for use of their tissues for study purpose. The study protocol was approved by institutional review board of the National Cancer Center East.

Recombinant protein and antibodies

Recombinant HMGB1 (rHMGB1) was purchased from Chrondrox Company (Redmond, WA, USA). The rabbit-polyclonal antibody against HMGB1 (ab18256) and mouse-monoclonal antibodies against TLR2 (TL2.1) and TLR4 (76B357.1) were obtained from Abcam (Cambridge, UK). The mouse-monoclonal antibody against alpha smooth muscle actin (1A4) was purchased from DAKO (Glostrup, Denmark). The rabbit-polyclonal antibodies against NF-kB p65, IRAK4 and phosphorylated IRAK4 were procured from Cell Signaling Technology (Danvers, MA, USA). Alexa Fluor 546-conjugated goat-anti-rabbit IgG and Alexa Fluor 488-conjugated goat-anti-mouse IgG were purchased from Invitrogen (Carlsbad, NM, USA). Horse radish peroxidase-linked species–specific whole antibody was obtained from GE healthcare (Fairfield, CT, USA).

Collection of the cancer- and fibroblast- conditioned media

On Day0, all cancer cell lines (5.0×10^6 per dish) and hPFCs (5.0×10^5 per dish) were incubated in DMEM containing 10% FBS. The culture media were replaced with DMEM without FBS on Day2 and the conditioned media were collected and filtered with a Vacuum Filtration System (Millipore, Billerica, MA, USA) and dispensed in 1 mL increments into each tube on Day3. The collected specimens were frozen and stored at –80°C until use.

Western blot analysis (WB)

All cancer cell lines and hPFCs were lysed. 20 μL of the conditioned media and a total of 10 μg of protein from the cancer cells and hPFCs were used for the WB, performed as previously described.[22] Goat-polyclonal anti-actin (Santa

Table 1 si-RNA sequences

siRNA-ID		Sequences (5′-3′)
s6645	Sense	GGAUUAUUAGAAUCAAACAtt
	Antisense	UGUUUGAUUCUAAUAAUCCca
s6646	Sense	CUUUCAUAUAGUUAGCUAAtt
	Antisense	UUAGCUAACUAUAUGAAAGga
s6647	Sense	AGAUAGUUUUCAUCCAUAAtt
	Antisense	UUAUGGAUGAAAACUAUCUca

Cruz Biotechnology, Dallas, TX, USA) was used as the loading control.

RNA interference

The RNA interference experiment was performed using the Silencer Select Pre-designed siRNA (Invitrogen) according to the manufactur's instructions. The siRNA sequences were in Table 1. Silencer Select Negative Control#2 siRNA was used as the negative control. Briefly, HSC44PE cells (5.0×10^5 per well) were transfected with siRNA. Twelve hours after the transfection, the conditioned media were replaced with DMEM containing 10% FBS. Twenty-four hours after the transfection, the conditioned media were replaced with DMEM not containing FBS. Forty-eight hours after the transfection, conditioned media were collected and stored at −80°C until use.

Real-time reverse transcriptase-polymerase chain reaction (qRT-PCR)

All cancer cell lines and hPFCs at the designated times were washed with PBS twice, and suspended in 1 mL of TRIzol. Total RNA was purified according to the manufacturer's instructions, and cDNA was synthesized by PrimeScript RT reagent Kit (Takara, Shiga, Japan). RT-PCR was carried out in a Lightcycler48 (Roche, Basel, Switzerland). The primers used are listed in Table 2.

Stimulation by the gastric cancer cell-conditioned media

On Day0, hPFCs (1.0×10^5 per well) were incubated in DMEM containing FBS. The corresponding conditioned medium or recombinant HMGB1, as control, was added to the hPFCs on Day2, followed by incubation for the indicated periods. The total and phosphorylated IRAK4 amounts were evaluated by WB and the expressions of pro-inflammatory cytokines were measured by qRT-PCR. hPFCs stimulated with DMEM were used as the control.

Table 2 qRT-PCR primer sequences

Name		Sequences (5′-3′)
TLR2	Forward	CAGGGAGCTCTTAGTGACCAAGTGAA
	Reverse	CACAAAGTATGTGGCATTGTCCAG
TLR4	Forward	AGGATGATGCCAGGATGATGTC
	Reverse	TCAGGTCCAGGTTCTTGGTTGAG
HMGB1	Forward	AGGATCCCAATGCACCCAAG
	Reverse	CGCAACATCACCAATGGACAG
IL1B	Forward	CCAGGGACAGGATATGGAGCA
	Reverse	TTCAACACGCAGGACAGGTACAG
IL6	Forward	AAGCCAGAGCTGTGCAGATGAGTA
	Reverse	TGTCCTGCAGCCACTGGTTC
IL8	Forward	ACACTGCGCCAACACAGAAATTA
	Reverse	TTTGCTTGAAGTTTCACTGGCATC
GAPDH	Forward	GCACCGTCAAGGCTGAGAAC
	Reverse	ATGGTGGTGAAGACGCCAGT

Immunocytochemistry

hPFCs (1.0×10^4 per well) were stimulated with 10 μg/mL rHMGB1or HSC44PE-conditioned medium for the indicated periods and fixed with 10% neutral buffered formalin solution (Wako, Osaka, Japan), followed by permeabilization by 0.1% TritonX-100. Then, the hFPCs were incubated with rabbit-polyclonal anti-NF-kB p65 (1:500) and mouse-monoclonal anti-alpha-SMA (1:100), followed by incubation with Alexa Fluor 546 goat-anti-rabbit IgG and Alexa Fluor 488 goat-anti-mouse IgG. Nuclear counterstaining was performed with DRAQ5 (Biostatus, Shepshed, UK, 1:500). The cancer cell lines (3.0×10^4 per well) were fixed with 10% neutral buffered formalin solution (Wako) followed by permeabilization by 0.1% TritonX-100. Then, the cancer cell lines were incubated with rabbit-polyclonal anti-HMGB1 (1:1000) followed by that with Envision+ System-HRP Labbeled Polymer anti rabbit (Dako). Nuclear counterstaining was performed by Mayer's Hematoxylin Solution.

Immunohistochemistry

In order to detect the expression level and the intracytological localization of HMGB1 and NF-kB in the gastric cancer tissues, immunohistochemistry was performed on paraffin-embedded tissues obtained from 20 surgically resected human gastric cancer specimens (10 specimens, moderately differentiated type; 10 specimens, poorly differentiated type). After deparrafinization and antigen retrieval, the samples were incubated with rabbit-polyclonal anti-HMGB1 (1:1000), rabbit-polyclonal anti-NF-kB p65 (1:500) and mouse-monoclonal anti-alpha-SMA (1:100) followed by that with Envision+ System-HRP labeled polymer anti rabbit (Dako). The tissues were then incubated with diaminobenzidine peroxidase substrate. Nuclear counterstaining was performed with Mayer's Hematoxylin solution. Gastric cancer cells

showing translocation of HMGB1 to the cytoplasm were counted in two different areas according to the gastric cancer cell depth: (i) shallow invasion areas, cancer invasion level at the submucosal layer; and (ii) deeper invasion areas, cancer invasion level at the subserosal layer. One thousand cancer cells were counted from 10 randomly selected fields in each area.[23] The rates of HMGB1 cytoplasmic translocation in the gastric cancer cells and NF-kB nuclear translocation in fibroblasts were calculated in 10 selected fields from each area. alpha-SMA-positive area for fibroblasts per one field was calculated in 10 selected fields from each area by Axio Vision Rel4.7 (ZEISS, Oberkohen, Germany). The total area of one field was 2037600 pixels. Immunofluorescence analysis of the gastric cancer tissues was also performed using mouse-monoclonal anti-alpha-SMA (1:100) and rabbit-polyclonal anti-NF-kB p65 (1:500) followed by Alexa Fluor 546 goat-anti-rabbit IgG and Alexa Fluor 488 goat-anti-mouse IgG as the second antibodies. Nuclear counterstaining was performed with DRAQ5 (Biostatus, 1:500).

Statistical analysis

The statistical significance of differences between any two groups of interest was evaluated by Student's *t* test. Differences were considered significant at $P < 0.05$. The correlation between the cytoplasmic localization of HMGB1 in the gastric cancer cells and the localization of the gastric cancer cells was evaluated by the χ^2-test using STAT Flex Ver5.0. The error bars show the mean ± SD.

RESULTS

Correlation of extracellular HMGB1 in the gastric cancer cells with nuclear NF-kB on the fibroblasts in the gastric cancer tissues

When the cancer areas were divided according to the depth of invasion into: shallow invasion areas, cancer invasion level at the submucosal layer; and deeper invasion areas, cancer invasion level at the subserosal layer, the alpha-SMA-positive area for fibroblasts per one field was larger in the deeper invasion area than in the shallow invasion area. Thus, fibroblasts in the deeper invasion area would be involved in the inflammation (Fig. 1a,b). Localization of HMGB1 in gastric cancer cells and NF-kB in fibroblasts was examined using gastric cancer specimens. HMGB1 was found to be mainly localized in the nuclei of the gastric cancer cells in the shallow invasion area. On the other hand, HMGB1 was more frequently observed in the cytoplasm of the gastric cancer cells in the deeper invasion area (Fig. 1c,d). In the deeper invasion area, NF-kB nuclear translocation could be observed in some fibroblasts (Fig. 1e,f).

Secretion of HMGB1 into the conditioned medium of gastric cancer cells

The expression of HMGB1 in the five cancer cell lines was examined by qRT-PCR. The mRNA expression level of HMGB1 was higher in all the five cancer cell lines than in the hPFCs (Fig. 2a). To examine whether the cancer cells produced and secreted HMGB1 into the cell culture medium, WB was performed. HMGB1 protein was detected in the conditioned medium of all the cancer cell lines, whereas no HMGB1 was detected in the conditioned medium of the hPFCs (Fig. 2b). The HMGB1 mRNA expression and protein expression levels in the conditioned media were not parallel. The cancer cell lines were divided into two groups in accordance with the secretion level of HMGB1, namely, the HMGB1-high group (high levels of secretion; HSC44PE, DLD1) and the HMGB1-low group (low levels of secretion; MKN7, HSC60, Caco2). The HMGB1 mRNA expression level was higher in HSC60 than in HSC44PE, while the HSC44PE-conditioned medium contained more HMGB1 protein than the HSC60-conditioned medium. All cancer cell lines expressed HMGB1 in the nucleus and along with the expression in the nuclear membrane, cytoplasmic localization was also detected in the HSC44PE and DLD1, the conditioned media of both of which showed high concentration of HMGB1 (Fig. 2c). HSC44PE and HSC60 were established from pleural fluid or ascitic fluid of patients with scirrhous gastric carcinoma. When inoculating these cell lines into abdominal cavity of mice, the mouse inoculated HSC44PE could cause cancerous peritonitis but the inoculated HSC60 could not.[20] A colorectal cancer, DLD1, was known to cause cancerous peritonitis.[24] This result suggests that HMGB1 would be involved in the cancerous peritonitis which is cited as one of the inflammatory response in cancer.

Expression of TLR-2 and -4 on the human peritoneal fibroblasts

The mRNA expressions of TLR-2 and -4, receptors for HMGB1, on four primary cultures of fibroblasts were examined by qRT-PCR and WB. Three of the cultured fibroblasts expressed TLR-2 and -4 at both the mRNA (Fig. 3a) and protein level (Fig. 3b). However, protein expression of TLR-2 and -4 was not found in case #2 (Fig. 3a,b). Thus, the mRNA expression levels of TLR-2 and -4 were not correlated with the corresponding protein expression levels. Cell surface expressions of TLR-2 and -4 were confirmed in two fibroblasts (cases #3 and #4) by FACS analysis (data not shown).

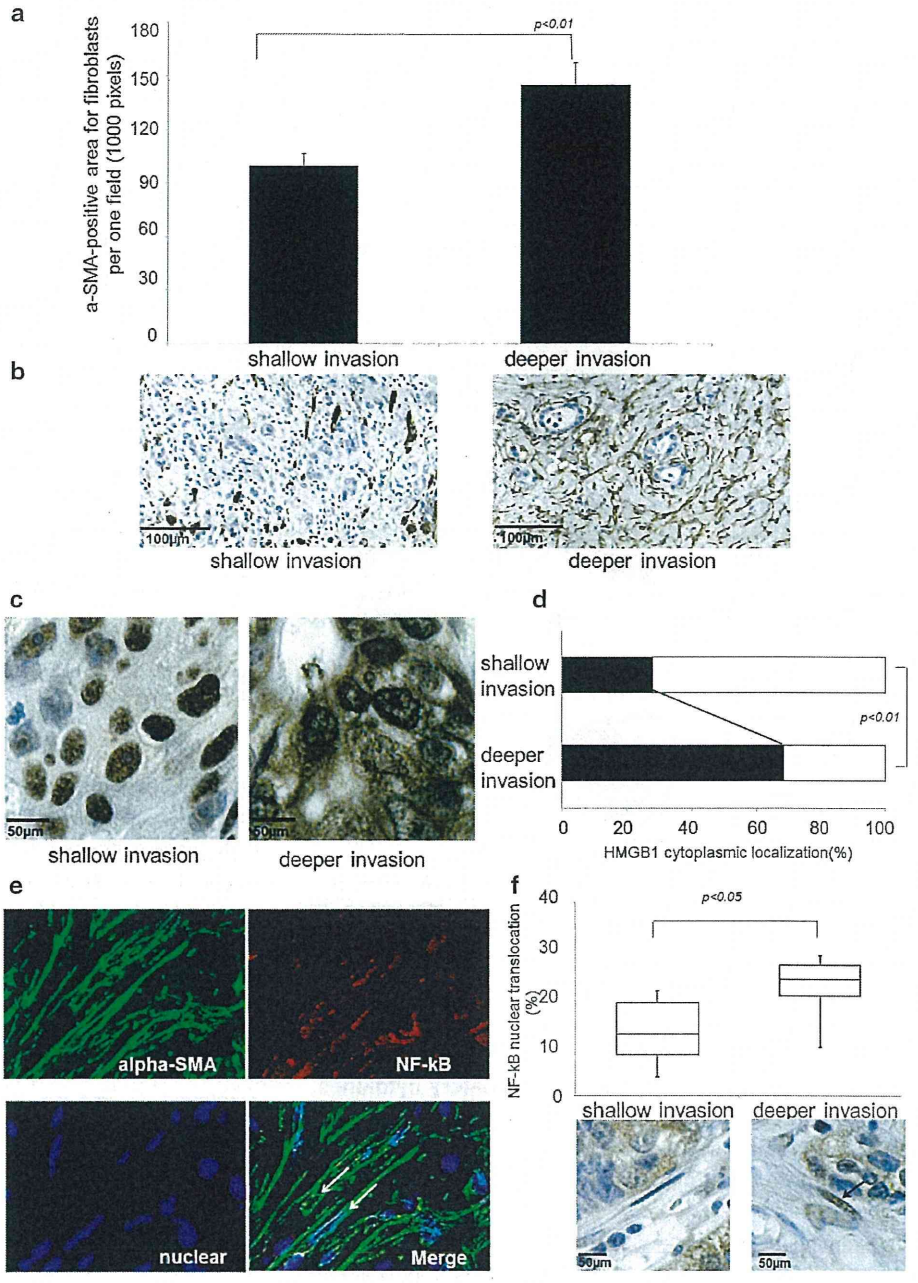

Figure 1 Localization of HMGB1 in the gastric cancer cells and of NF-kB in the fibroblasts surrounding the gastric cancer cells. (**a**) alpha-SMA-positive area for fibroblasts in the shallow and deeper invasion areas (n = 20). (**b**) Immunohistochemistry for alpha-SMA in human gastric cancer specimens (n = 20). (**c**) Immunohistochemistry for HMGB1 in human gastric cancer specimens (n = 20). (**d**) Rate of HMGB1 cytoplasmic localization in gastric cancer cells (n = 20). (**e**) Translocation of NF-kB in the fibroblasts surrounding the gastric cancer cells in the deeper invasion areas (arrows; NF-kB nuclear translocation). (**f**) Rate of NF-kB intracellular localization in the fibroblasts surrounding the gastric cancer cells in the shallow and deeper invasion areas (n = 20). □, negative; ■, positive.

Induction of expression of pro-inflammatory cytokines, nuclear translocation of NF-kB, and phosphorylation of IRAK4 in human peritoneal fibroblasts by recombinant HMGB1

In order to examine whether HMGB1 secreted from the cancer cells could induce the expressions of pro-inflammatory cytokines in fibroblasts, the mRNA expressions of cytokines were examined in the fibroblasts after rHMGB1 stimulation. The mRNA expressions of the pro-inflammatory cytokines, interleukin (IL)1-beta, IL6 and IL8 were induced in a dose-dependent manner in the fibroblasts after 24 hours' stimulation with rHMGB1 (Fig. 4a), while those of IL12A, IL12B and tumor necrosis factor-alpha (TNF-alpha) were not induced. Phosphorylation of IRAK4 and nuclear translocation of NF-kB were observed after 15 and 120 minutes' stimulation with HMGB1, respectively (Fig. 4b,c). These results indicate that HMGB1 activated the TLR/IRAK/NF-kB axis to induce the expressions of pro-inflammatory cytokines in the fibroblasts.

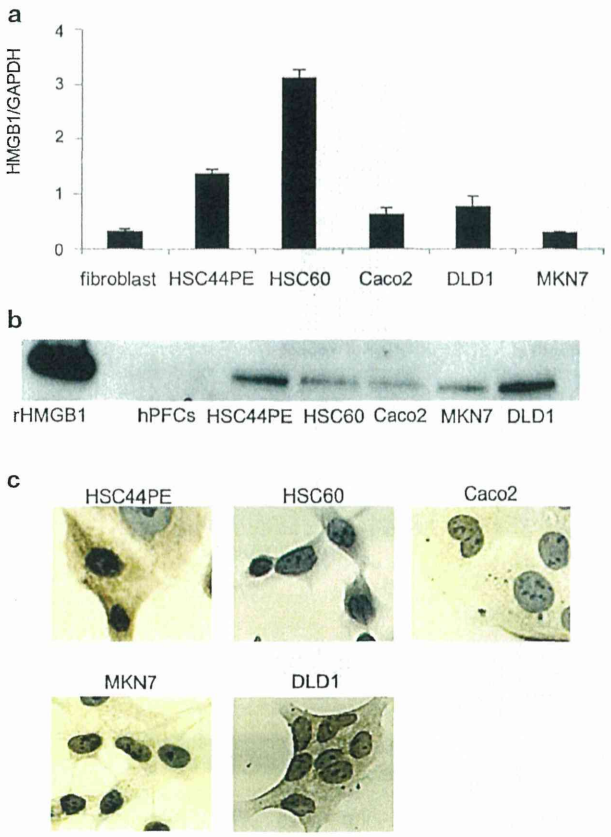

Figure 2 Expression and secretion of HMGB1 in cancer cell lines. (**a**) mRNA expression levels of HMGB1 in hPFCs and cancer cell lines (n = 3). (**b**) Secretion of HMGB1 into the conditioned media of the cancer cell lines and hPFCs. (**c**) Immunocytochemistry of HMGB1 in the cancer cell lines.

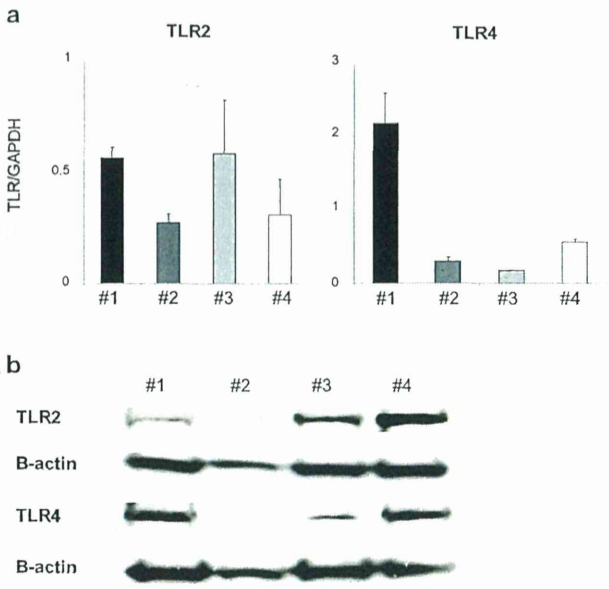

Figure 3 Expression of TLR2/4 in hPFCs. (**a**) mRNA expression levels of TLR2/4 in hPFCs (n = 3). (**b**) Protein expression levels of TLR2/4 in hPFCs.

Induction of expression of pro-inflammatory cytokines, nuclear translocation of NF-kB, and phosphorylation of IRAK4 in human peritoneal fibroblasts by the HMGB1 secreted into gastric cancer cell-conditioned media

To examine the influence of cancer cell-conditioned media on the expressions of cytokines in the fibroblasts, conditioned media of two gastric cancer cell lines, HSC44PE and HSC60, were added to the fibroblasts, and the mRNA expression levels of the cytokines were examined. The conditioned medium of HSC44PE induced the mRNA expressions of IL1-beta, IL6 and IL8 in the fibroblasts after 24 hours' stimulation, while the conditioned medium of HSC60 did not induce the expression of any pro-inflammatory cytokines (Fig. 5a). On the other hand, none of the cytokines, including IL12A, IL12B or TNF-alpha, was induced by the conditioned media of any of the other cell lines (data not shown). The conditioned medium of HSC44PE induced the phosphorylation of IRAK4 and the nuclear localization of NF-kB in the fibroblasts after 15 and 60 minutes' treatment with HSC44PE-conditioned medium, respectively (Fig. 5b,c). Thus, HMGB1 secreted into cancer cell-conditioned medium could be involved in the phosphorylation of IRAK4 and nuclear localization of NF-kB in fibroblasts. This result was consistent with the observation that the HSC44PE-conditioned medium contained more HMGB1 than the HSC60-conditioned medium.

To examine whether conditioned medium with HMGB1-knockdown cancer cells could reduce the expression of cytokines in the fibroblasts, the siRNA technique was employed to reduce the expression of HMGB1 in the HSC44PE. We examined the expression of HMGB1 in the HSC44PE-conditioned medium at 24, 48, 96 h after siRNA-HMGB1 (s6647) transfection. Decrease in the concentration of HMGB1 in the cancer cell-conditioned medium was observed at 48 h after transfection. Then, we collected and added this conditioned medium to the fibroblasts. The expression of IL8 and IL1-beta, but not of IL6, in the fibroblasts was reduced after stimulation with the conditioned medium of HSC44PE cells transfected with siRNA-HMGB1 (Fig. 5d).

DISCUSSION

Our present study showed that HMGB1 released from gastric cancer cells induced the expressions of IL1-beta, IL6 and IL8 in the stromal fibroblasts through the HMGB1-TLR2/4 signaling pathway. In human gastric cancer tissue, cytoplasmic

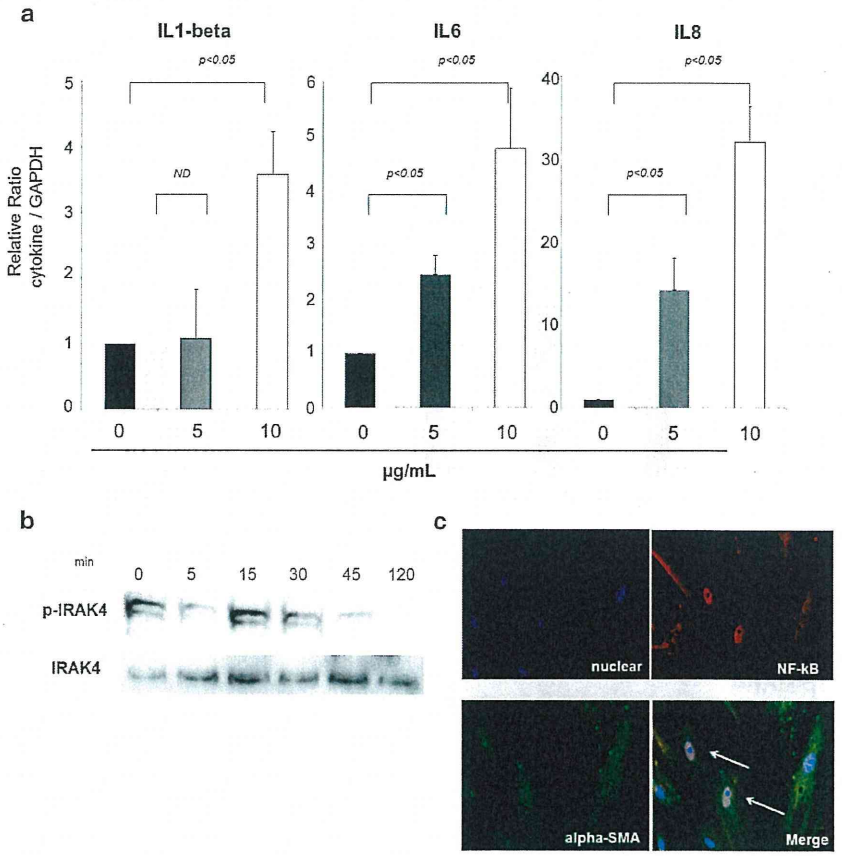

Figure 4 Induction of expression of pro-inflammatory cytokines, nuclear translocation of NF-kB and phosphorylation of IRAK4 in hPFCs by rHMGB1. (**a**) mRNA expression levels of IL1-beta, IL6 and IL8 in hPFCs after 24 h stimulation with 5 μg/mL rHMGB1, 10 μg/mL rHMGB1 or DMEM (n = 3). (**b**) Phosphorylated and total IRAK4 amounts in hPFCs at the indicated times after stimulation with 10 μg/mL rHMGB1. (**c**) Immunocytochemistry of NF-kB in hFPCs 120 min after stimulation by 10 μg/mL rHMGB1 (arrows; NF-kB nuclear translocation).

HMGB1 expression in the gastric cancer cells and simultaneous nuclear translocation of NF-kB in the fibroblasts could be detected at the deeper invasion areas. High amounts of HMGB1 were detected in the cancer-conditioned media of the HSC44PE and DLD1 cell lines, and cytoplasmic HMGB1 expression was detected in these cancer cells. Previous reports have shown that nuclear HMGB1 translocates to the cytoplasm before being released into the extracellular milieu.[4] These data suggest that HMGB1 released from the cancer cells stimulated the surrounding fibroblasts at the deeper invasion areas. The peritoneal fibroblasts also showed expression of TLR, like the synovial and gingival fibroblasts in rheumatoid arthritis and bacterial infection, respectively.[10,11] The excessive production of the pro-inflammatory cytokines TNF-alpha and IL1-beta by intra-articular macrophages is known to play a critical pathogenetic role in the development and progression of rheumatoid arthritis.[25] It is possible that HMGB1 induces the expression of these cytokines in the synovial fibroblasts through the TLR2/4 signaling pathway. In addition, the expression levels of TLR-2 and -4 were not correlated with the protein levels of TLR-2 and -4. In a previous study, the soluble form of TLR-2 resulted from the posttranscriptional modification of protein in the intracellular compartment.[26] Therefore, the inconsistency between the mRNA and protein levels of TLR-2 and -4 in the fibroblasts may be caused by posttranscriptional modification on the fibroblasts. We also demonstrated the phosphorylation of IRKA4, nuclear translocation of NF-kB and the induction of pro-inflammatory cytokines in the fibroblasts by rHMGB1 and conditioned medium of HSC44PE, which released higher amounts of HMGB1 in the cancer cell-conditioned medium. These findings suggested that fibroblasts may play a role in the inflammatory process, just like monocytes and macrophages.[19,27] HMGB1 mainly induced the expression of TNF-alpha and IL1-beta in the macrophages,[28,29] but mainly induced the expression of IL8 in fibroblasts in this study, indicating that the differential effects of HMGB1 between macrophages and fibroblasts. In inflammation, IL8 is a chemoattractant cytokine produced by a variety of cells and has distinct target specificity for the neutrophils.[30] Neutrophils play an important role in the early stages of inflammation, and also recruit inflammatory cells.[30] These findings indicate that fibroblasts induce the migration of neutrophils, and neutrophils influence inflammatory cells recruitment and activation. The neutrophils as well as inflammatory cells secretes reactive oxygen products (ROS) and cytokines, which regulate cancer cells proliferation, metastasis and angiogenesis at the deeper invasion areas.[31] Cancer cells acquired metastatic potential by cancer

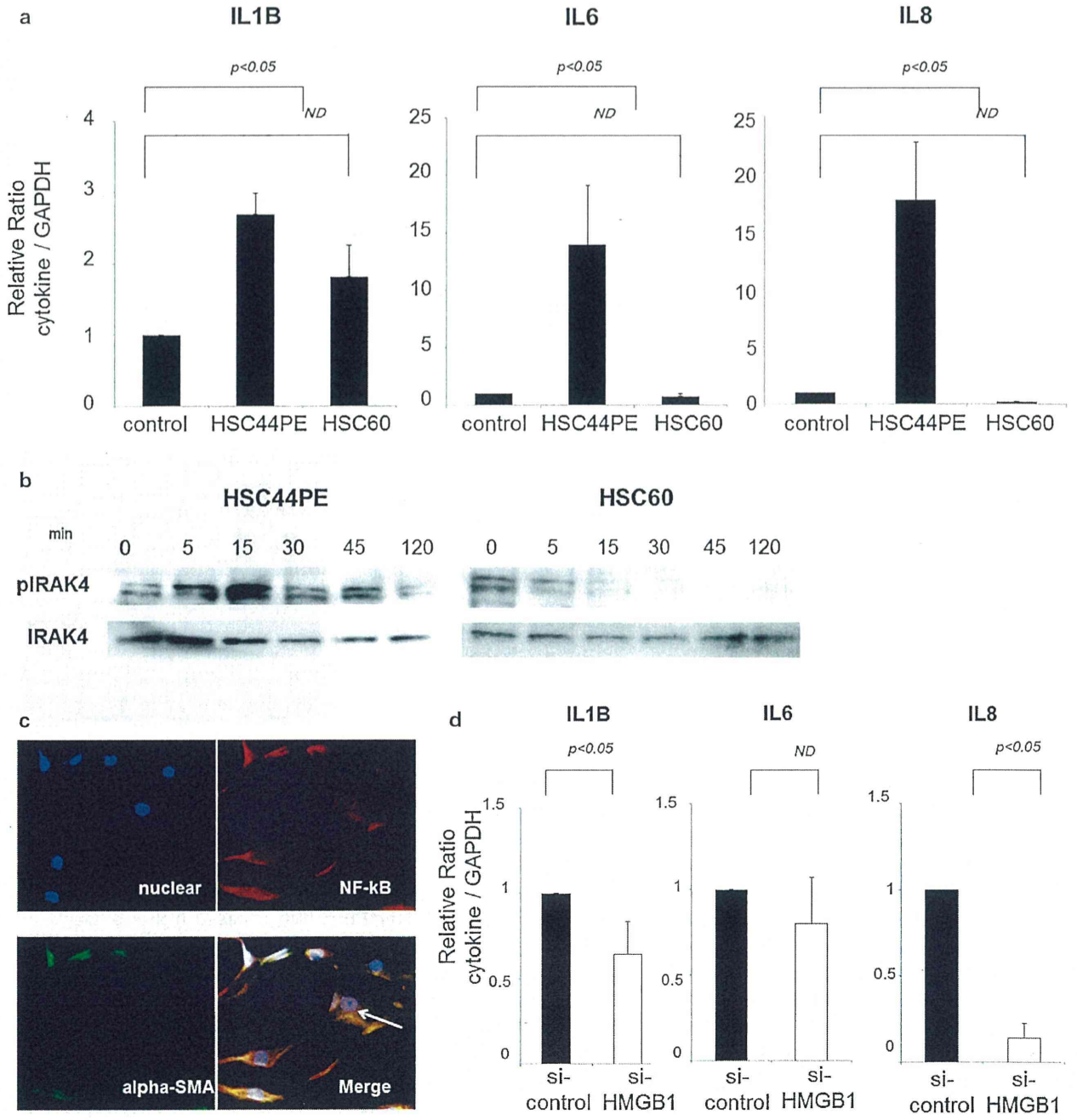

Figure 5 Induction of expression of pro-inflammatory cytokines, nuclear translocation of NF-kB and phosphorylation of IRAK4 in hPFCs by cancer cell-conditioned media. (**a**) mRNA expression levels of IL1-beta, IL6 and IL8 in hPFCs after 24 hours' stimulation with HSC44PE and HSC60-conditioned medium or DMEM (n = 3). (**b**) Phosphorylated and total IRAK4 amounts in hPFCs at the indicated time after stimulation with HSC44PE and HSC60-conditioned medium. (**c**) Immunocytochemistry of NF-kB in hPFCs after 60 minutes' stimulation with HSC44PE-conditioned medium (arrow; NF-kB nuclear translocation). (**d**) mRNA expression levels of IL1-beta, IL6 and IL8 in hFPCs after 24 h stimulation with si-HMGB1-HSC44PE-conditioned medium (n = 3).

microenvironment will cause the peritoneal metastasis. Thus, fibroblasts may trigger cancer-related inflammation and cancer progression.

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