

# Induction of cytokine production in cholesteatoma keratinocytes by extracellular high-mobility group box chromosomal protein 1 combined with DNA released by apoptotic cholesteatoma keratinocytes

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**Abstract** High-mobility group box chromosomal protein 1 (HMGB-1), a nuclear DNA binding protein, was recently rediscovered as a new proinflammatory cytokine. The purpose of this study was to determine HMGB-1 expression in vivo and to identify the effect of extracellular HMGB-1 in inflammatory process associated with bone destruction in cholesteatoma. We investigated the expression and location of HMGB-1 in the cholesteatoma and healthy skin using an immunofluorescence assay. We also detected apoptosis and DNA fragments in the cholesteatoma by TUNEL staining. HMGB-1 concentration in apoptotic supernatants from UV light-treated cells, culture supernatants and its translocation in cholesteatoma keratinocytes stimulated by supernatants from UV light-treated cells were measured by immunoblot analysis and immunofluorescence assay. Cultures of human cholesteatoma keratinocytes were exposed to CpG-DNA, HMGB-1, or CpG-DNA complexed to HMGB-1 for 24 h. Cytokines in the culture supernatant were measured by ELISA. In addition, levels of proinflammatory cytokines released by cholesteatoma keratinocytes stimulated by supernatants from UV light-treated cells with or without anti-HMGB-1 antibodies and supernatants from UV light-treated cells with DNase 1 were measured by enzyme-linked immunosorbent assay. The expression of HMGB-1 in cholesteatoma increased and it translocated both to the cytoplasm and extracellular space. Furthermore, the HMGB-1 concentration in supernatants increased significantly after addition of supernatants from UV light-treated cells. TNF- $\alpha$  and IL-1 $\beta$  can be induced by purified HMGB-1 combined

with CpG-DNA in the cholesteatoma keratinocytes. In addition, supernatants of apoptotic cells containing HMGB-1–DNA were effective in inducing TNF- $\alpha$  and IL-1 $\beta$  secretion. This study suggested that persistent expression of extracellular HMGB-1 and DNA fragments in cholesteatoma leads to TNF- $\alpha$  and IL-1 $\beta$  production, causing bone resorption and destruction. Thus, we have implicated that HMGB-1–DNA complexes might act as a key molecule involved in bone resorption associated with cholesteatoma.

**Keywords** High-mobility group box chromosomal protein 1 · Interleukin-1 $\beta$  · Tumor necrosis factor- $\alpha$  · Primary cholesteatoma · Bone resorption

## Introduction

Cholesteatoma of the middle ear is characterized by invasion of the middle ear cavity by epithelium of the external auditory meatus and drum as well as resorption of the auditory ossicles and surrounding bone tissues in the invaded subepithelial region [1]. Accumulation of keratin debris is caused by the decreased migration ability of keratinized epithelial cells. Infection is thought to accelerate this process. The histopathological changes include hyperproliferation and differentiation of the epithelium and accompanying bone destruction.

Cholesteatoma may cause bone destruction, resulting in intratemporal and intracranial complications, which have high morbidity and mortality [2]. There are numerous theories to explain the destructiveness of cholesteatomas, including physical pressure due to accumulated keratin and other waste, which was initially proposed as the cause of bone destruction. A biochemical theory was also

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postulated, in which enzymes and cytokines released by cholesteatomas could cause bone lysis and destruction.

Recent studies have focused on the role of cytokines such as tumor necrosis factor (TNF)- $\alpha/\beta$ , interleukin (IL)-1 $\alpha/\beta$ , epidermal growth factor, and transforming growth factor- $\beta$  in cholesteatoma bone destruction and discussed their relative importance in bone destruction and the inflammatory process [3–7]. Akimoto et al. [8] reported that IL-1 and TNF- $\alpha$  levels were higher in congenital and acquired cholesteatomas compared with skin of the external auditory meatus. However, the exact source of TNF- $\alpha$  and IL-1 in cholesteatoma is still unknown.

High-mobility group box protein 1 (HMGB-1) is a structural nuclear protein that binds DNA and is involved in the organization of chromatin [9]. Recently, it was shown to function as a proinflammatory mediator when released from cells [10]. HMGB-1 can be actively secreted by macrophage/monocytes via inflammatory stimuli [11] or passively released from the nuclei of necrotic or damaged cells. In contrast, cells undergoing apoptosis are poor HMGB-1 secretors [12]. However, some studies have investigated the direct involvement of HMGB-1 in the induction of apoptosis. Bell et al. [13] reported HMGB-1 release occurred during the course of apoptosis and necrosis in Jurkat cells. Binding of HMGB-1 to the receptor for advanced glycation end products (RAGE) and Toll-like receptor (TLR)2 and TLR4 leads to the recruitment of inflammatory cells and the release of proinflammatory cytokines including IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 [14–17].

Here, we analyzed the expression and subcellular localization of HMGB-1 in cholesteatomatous specimens and the source of extracellular HMGB-1 in cholesteatoma. In addition, we also investigated the role of HMGB-1 in inflammatory process associated with bone destruction in cholesteatoma.

## Materials and methods

### Patients and tissues

All cholesteatomatous specimens including cholesteatomatous matrix, perimatrix, and contents were obtained from 10 patients with acquired primary cholesteatoma undergoing tympanoplasty in the Department of Otorhinolaryngology at Eye and ENT Hospital, Fudan University, Shanghai, China. The diagnosis was based on clinical, radiological, and intraoperative findings. Prior to surgery, written consent was obtained from all patients to obtain tissue samples of the resected cholesteatoma. The study was approved by the Institutional Ethics and Review Committee of the Faculty of Clinical Medicine, Eye and ENT Hospital, Fudan University, Shanghai, China and was

performed in accordance with the 1964 Declaration of Helsinki and its later amendments. In addition, microbiopsies obtained from the external canal skin of the deep meatus in five patients undergoing myringoplasty for dry perforation served as a control group.

### Tissue preparation and immunofluorescence

Specimens were obtained from 10 patients (five patients with acquired primary cholesteatoma and five healthy external canal skins) and used for immunofluorescence. Each specimen was fixed overnight in 10 % buffered formalin at room temperature and embedded in paraffin. Serial sections were cut at 7  $\mu$ m thickness and then placed onto 3-aminopropyltriethoxysilane-coated glass slides. Paraffin sections were deparaffinized with toluene and rehydrated with serially graded ethanol solutions. After rinsing in PBS, the sections were preincubated with 10 % normal goat serum for 1 h to block nonspecific reactions with the primary antibody. Then, the sections were incubated overnight with rabbit monoclonal anti-HMGB-1 antibody (Abcam, Cambridge, MA, USA) at 250-fold dilution. After washing with PBS, the sections were incubated for 1 h at room temperature with Alexa 546-labeled goat anti-rabbit antibody. The slides were washed with PBS and the nuclei were counterstained with blue fluorescent 4',6-diamidino-2-phenylindole (DAPI). Finally, the sections were examined with an Axioskop microscope (Carl Zeiss, Oberkochen, Germany).

### TUNEL staining

Paraffin sections of the acquired primary cholesteatoma and healthy skin were stained for TUNEL with In situ Cell Death Detection Kit (Roche, Indianapolis, IN, USA). Briefly, each slide was deparaffinized and rehydrated, and then treated with citrate buffer (10 mM, pH 6) for 10 min. After rinsing in PBS, the sections were incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and TMR-dUTP for 1 h. The TdT reaction was carried out in a humidified chamber at 37 °C. DAPI was used as counterstaining. For the negative control, TdT was omitted from the reaction mixture. For positive controls, some sections were treated with DNase I (2,000 U/ml; Thermo Fisher) before staining.

### Cell culture, validation, and stimuli

Tissue from the acquired primary cholesteatoma was hand carried to the lab 2 h after resection and transferred into 5 ml Hanks balanced salt solution (HBSS). The tissue was then cut into small pieces with scissors, and digested with

200 U/ml collagenase IV (Sigma Aldrich, St. Louis, MO, USA) at 4 °C overnight. The digested cells were washed twice with HBSS and then centrifuged for at 1,500 rpm for 5 min. The pellet was removed, added to 10 ml Keratinocyte serum-free medium (KSFM; Invitrogen, Carlsbad, CA, USA) with 100 µl Pen Strep (Invitrogen, Carlsbad, CA, USA), and cultured in humidified CO<sub>2</sub> at 37 °C. The KSFM media and antibiotics were changed every 3 days. Cells were passaged when they reached confluence (>50 % coverage of culture dish).

The cultured cells were validated for phenotype by immunofluorescence analysis. In this study, aliquots of the cultured cell were grown on 35-mm collagen-coated glass bottom dishes and labeled for immunofluorescent confirmation of specific marker anti-pankeratins for cholesteatoma keratinocytes.

The cholesteatoma keratinocytes were placed at a density of  $2 \times 10^4$  cells per well in 96-well plates and were stimulated for 24 h with CpG oligodeoxynucleotides alone (2 µM/l, Invivogen, San Digo, CA, USA), HMGB-1 (3 uM/l, R&D systems, Minneapolis, MN, USA) alone, or HMGB-1 plus CpG-DNA. Cytokines in the supernatant were measured by ELISA according to the manufacturer's instructions (Neobioscience, China).

#### Induction of cell death

The cholesteatoma keratinocytes were resuspended at  $5 \times 10^7$  cells/ml in KSFM and irradiated with UV light as described previously [18]. Briefly, the UV light irradiation was conducted in 2 ml of cell suspension in 9.4-cm petri dishes (BD Falcon) using 500 mJ (GS gene linker UV chamber; Bio-Rad, Hercules, CA, USA). Cells treated with UV light were cultured for 16–24 h at 37 °C with 7 % CO<sub>2</sub>. The cells were removed by centrifugation (400×g for 5 min), the supernatants were tested for cytokine-inducing capacity, and the remaining cells were analyzed by flow cytometry for cell death by staining with FITC-labeled annexin V (BD Biosciences) and propidium iodide (PI; BD Biosciences).

#### Immunoblot analysis of HMGB-1 in supernatants from UV light-treated cells

Supernatants from UV light-treated cells were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and HMGB-1 levels were determined by immunoblot analysis with reference to the standard curve for purified human-recombinant HMGB-1 (Prospec, East Brunswick, NJ, USA), obtained by serial dilutions. The concentrations of purified human-recombinant HMGB-1 standard ranged from 0 to 400 ng/ml.

Supernatants from UV light-treated cells were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane (PVDF membrane) for Western blotting analysis.

#### Anti-HMGB-1 antibodies neutralize HMGB-1 in the supernatants of UV light-treated cells and enzyme treatments in vitro

Anti-HMGB-1 neutralizing antibody (mouse anti-HMGB-1 monoclonal antibody) was obtained from Biologend Corporation (San Diego, USA). A solution of the supernatants of UV light-treated cells was mixed with equal volume of the anti-HMGB-1 antibody and incubated at 37 °C for 12 h. Neutralization was confirmed by electrophoresis on a 12 % SDS-PAGE. RNase-free DNase I (Thermo Fisher) was used at a final concentration of 20 Kunitz units/ml. This enzyme was added to the supernatants of UV light-treated cell and incubated at 37 °C for 1 h. Then the neutralized supernatants and supernatants treated with enzyme were added to the cultures of the cholesteatoma keratinocytes. After being stimulated for 0, 1, 4, 8, 12, 24, or 48 h, the culture supernatants were collected. IL-1β and TNF-α levels in culture supernatants were assayed using commercial human IL-1β and TNF-α enzyme-linked immunosorbent assay (ELISA) test kits according to the manufacturer's instructions (Neobioscience, China).

#### Western blotting for HMGB-1 detection in culture supernatant

Cholesteatoma keratinocytes were washed twice with HBSS and 1.0 ml was introduced into each well of a six-well culture plate at a concentration of  $1 \times 10^6$  cells/ml. The supernatant from UV light-treated cells was added into each well at a final volume of 1.3 ml. After the plates were stimulated for 0, 4, 8, 12, 24, 36, or 48 h, the conditioned media was collected and the cells were obtained and prepared for extraction of total cellular protein. Conditioned media from cells were centrifuged through Centricon (Millipore, Billerica, MA, USA) 10-kDa filter devices for 1 h at 3,000×g. Supernatants were collected from the top reservoir of the filter device and spun through an Ultrafree-MC (Millipore) 100-kDa filter device for 15 min at 4,000×g. Equal volumes of elution were fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PVDF membrane) for Western blotting analysis. Total cellular protein extracts were prepared using Whole Cell Lysis/Extraction Buffer (WCLB, Applygen, Beijing, China) according to the manufacturer's instructions.

### Measurement of IL-1 $\beta$ and TNF- $\alpha$ in culture supernatants and preparation of cytoplasmic extracts

Using the same method as described above, the cholesteatoma keratinocytes were cultured and stimulated in six-well culture plates. After being stimulated for 0, 1, 4, 8, 12, 24, 36, or 48 h, the culture supernatants were collected and the cells were obtained and stored at  $-80^{\circ}\text{C}$  until used. IL-1 $\beta$  and TNF- $\alpha$  levels in culture supernatants were assayed using commercial human IL-1 $\beta$  and TNF- $\alpha$  enzyme-linked immunosorbent assay (ELISA) test kits according to the manufacturer's instructions (Neobioscience, China). To prepare the cytoplasmic fraction, cells were lysed using a nuclear and cytoplasmic protein extraction kit according to the manufacturer's instructions (Beyotime Institute of Biotechnology, China). Briefly, cells were washed in ice-cold phosphate-buffered saline (PBS) and resuspended by pipetting up and down 10 times in 100  $\mu\text{l}$  of ice-cold cell lysis buffer (10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 1 mM DTT, 0.4 % I-GEPAL with the following protease inhibitors: Aprotinin 2  $\mu\text{g}/\text{ml}$ , PMSF 1 mM, benzamidin 250  $\mu\text{g}/\text{ml}$ , leupeptin 2  $\mu\text{g}/\text{ml}$ ). After being placed on ice for 15 min, cell lysates were spun in a microcentrifuge at 12,000 rpm for 5 min at  $4^{\circ}\text{C}$  and supernatants were collected as the cytoplasmic fraction and stored at  $-80^{\circ}\text{C}$  for Western blot analysis.

### Western blot analysis

Protein concentration was measured using a BCA protein assay kit (Beyotime Institute of Biotechnology, China). For Western blot assay, the cytoplasmic fraction, concentrated culture supernatants, and total cellular protein extracts were added to Laemmli sample buffer and boiled for 5 min. Subsequently, protein from each sample was subjected to 12 % SDS-PAGE and transferred onto a PVDF membrane. The membrane was washed with Tris-Buffered Saline/Tween-20 (TBST; 20 mM Tris, 500 mM NaCl, and 0.1 % Tween-20) and blocked with 5 % milk in TBST for 1 h. After washing with TBST three times, the membrane was incubated with primary antibody overnight at  $4^{\circ}\text{C}$ . The following antibodies were used: monoclonal anti-HMGB-1 antibody was purchased from Abcam and monoclonal anti- $\beta$ -actin antibody was from Sigma. The membrane was washed three times with TBST and incubated with HRP-conjugated secondary antibody in blocking buffer for 1 h. After washing three times with TBST, immunoreactive bands were visualized by incubation with ECL plus detection reagents (GE Healthcare) for 5 min and exposure to ECL Hyperfilm (GE Healthcare). The densitometry of bands was quantified with NIH Image 1.63 software. Protein expression was normalized to the quantity of  $\beta$ -actin. The gray values were expressed in relation

to that of control and presented as mean  $\pm$  standard deviation ( $M \pm SD$ ).

### Immunofluorescence assay for HMGB-1 translocation

To investigate the translocation of HMGB-1 in the cholesteatoma, cholesteatoma keratinocytes were cultured in a Lab-Tech chamber slide (Nalge Nunc International, Cambridge, MA, USA) and then stimulated with supernatants from UV light-treated cells. After stimulation, cells were fixed with 4 % paraformaldehyde solution for 30 min. The chamber slide was then incubated with a blocking buffer containing 1 % BSA and PBS-0.02 % Tween (PBST) for 1 h at  $25^{\circ}\text{C}$  and incubated with anti-HMGB-1 rabbit monoclonal antibody (Abcam) at 250-fold dilution overnight at  $4^{\circ}\text{C}$ . The chamber slide was then washed with PBST, followed by 1 h incubation with Alexa 546-labeled anti-rabbit IgG diluted 1:200 with PBST. Finally, the chamber was examined using an Axioskop microscope (Carl Zeiss, Oberkochen, Germany) after washing three times. Nuclear counterstaining was performed with DAPI.

### Quantitative RT-PCR

To examine the influence of supernatants from UV light-treated cells on mRNA expression for HMGB-1, IL-1 $\beta$ , and TNF- $\alpha$ , the cholesteatoma keratinocytes were cultured and stimulated in six-well plates for 0, 4, 8, 12, 24, 36, or 48 h. After stimulation, the cells were used for total RNA extraction using Trizol (Invitrogen) according to the manufacturer's instructions. After purification, complementary DNA (cDNA) was synthesized from 1  $\mu\text{g}$  total RNA using the first-strand cDNA synthesis kit (Takara, Dalian, China) according to the manufacturer's instructions. HMGB-1, TNF- $\alpha$ , IL-1 $\beta$ , and GAPDH were then amplified by quantitative real-time PCR (qRT-PCR) using the primer sequences and PCR parameters shown in Table 1. qRT-PCR was performed using the SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> GC (Takara, Dalian, China) and run in a LightCycler (Roche Diagnostics, Indianapolis, IN, USA). Individual RT-PCR tests were repeated on at least three independent occasions. Melting curve analysis was performed to verify that only one product was amplified. Data were analyzed for fold change ( $2^{-\Delta\Delta\text{CT}}$ ) value calculation using GAPDH for normalization.

### Statistical analysis

Statistical analysis was performed using the statistical software package SPSS (Version 11.5) and were evaluated using one-way ANOVA followed by a LSD multiple comparison test for comparison of three or more groups. Statistical significance was defined as a p value less than

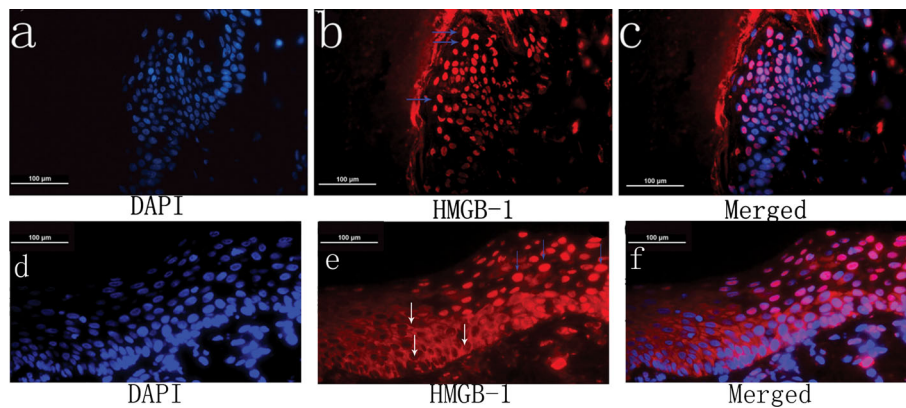


**Table 1** Sequences of primers used in qRT-PCR

Genes	Sequences	Accession number	PCR conditions <sup>a</sup>	Size (bp)
HMGB-1	F:5'-TGATGTTGCGAAGAACTGG-3' R:5'-TTCAGCCTTGACAACCTCCCT-3'	DI170360	58 (45)	169
IL-1 $\beta$	F:5'-AATGATGGCTTATTACAGTGGCAA-3' R:5'-AAGCCCTTGCTGTAGTGGTGG-3'	AF124819	58 (45)	153
TNF- $\alpha$	F:5'-TCTTCTCCTTCTGATCGTGG-3' R:5'-CTACAGGCTTGCTACTCGGG-3'	X02910	58 (45)	165
GAPDH	F:5'-GGAGTCCACTGGCGTCTTC-3' R:5'-GCTGATGATCTTGAGGCTGTTG-3'	JN613429	58 (45)	157

*F* forward primer, *R* reverse primer

<sup>a</sup> Annealing temperature in °C (number of cycles)



**Fig. 1** Localization of HMGB-1 in normal skin (a–c) and primary cholesteatoma (d–f). Immunofluorescence analysis demonstrated HMGB-1 that was localized in the cytoplasm and nucleus of epithelial cells situated mostly in the metrical layer (e). The sections were incubated with anti-HMGB-1 rabbit polyclonal antibody. Skin

tissues were used as controls. The nucleus was labeled with DAPI (blue, a, d). Blue arrows depict nuclear HMGB-1. White arrows depict cytoplasmic HMGB-1 and extracellular HMGB-1. (Color figure online)

0.05 for all analyses and the data were presented as mean  $\pm$  standard deviation ( $M \pm SD$ ).

## Results

### Expression of HMGB-1 in cholesteatomatous tissue

HMGB-1 expression was evaluated in acquired primary cholesteatoma and normal skin by immunofluorescence. Nuclear HMGB-1 was expressed in both primary cholesteatomatous specimens (Fig. 1d–f) and normal external canal skin (Fig. 1a–c). Control staining with irrelevant isotype-matched control antibody was negative (data not shown). Translocation of HMGB-1 to the cytosol was detected in primary cholesteatoma (Fig. 1e). Staining without obvious restriction by cell membranes, suggestive of extracellular presence of HMGB-1, was also detected in acquired primary cholesteatoma (Fig. 1e). In normal external canal skin, no cytosol and extracellular staining for HMGB-1 was observed.

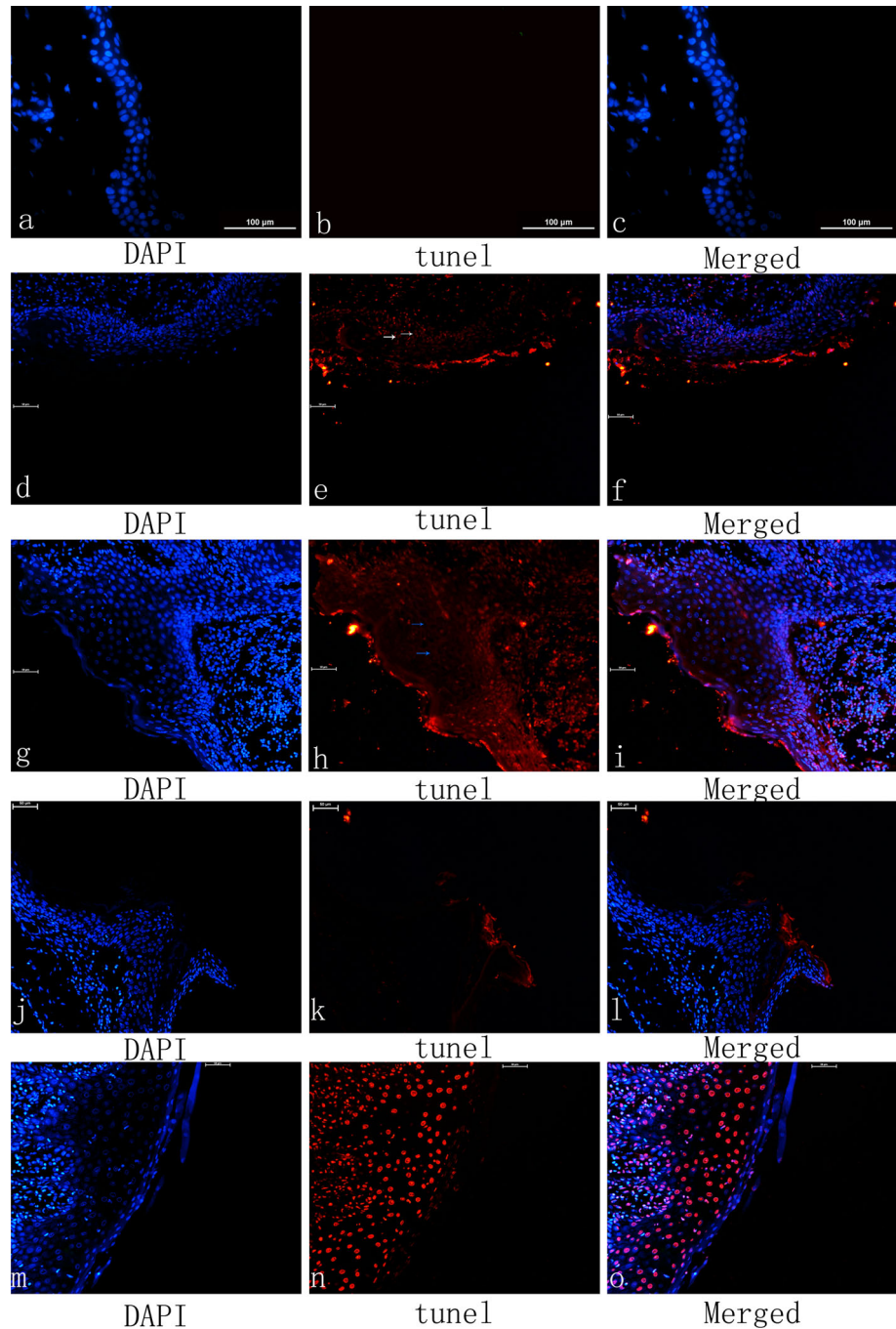
Apoptosis and DNA fragment is present in cholesteatoma

To detect apoptosis and DNA fragments in cholesteatoma, we stained paraffin sections using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL). A great number of apoptotic cells was observed in the cholesteatoma epithelium (Fig. 2d–f) but not in healthy skin (Fig. 2a–c). At the same time, DNA fragments were also detectable in the cytoplasm and extracellular space of keratinocytes in all patients with cholesteatoma (Fig. 2g–i).

### Validation of cholesteatoma keratinocytes

To validate the primary cultures, the expression of pancytokeratins (CKs) which was widely used as positive marker for keratinocytes was analyzed. The immunofluorescence analysis showed that the cultured cells were characterized by a positive staining for cytokeratins (Fig. 3).

**Fig. 2** TUNEL staining in normal skin (a–c), acquired secondary cholesteatoma (d–i). Detection of large numbers of apoptotic cells (d–f) and DNA fragments in cytoplasm and extracellular space of keratinocytes in the cholesteatoma (g–i). (j–l) is for the negative control. DNase I-treated cholesteatoma tissue served as a positive control (m–o). *White arrows* depict apoptotic cells. *Blue arrows* depict DNA fragments in cytoplasm and extracellular space. (Color figure online)



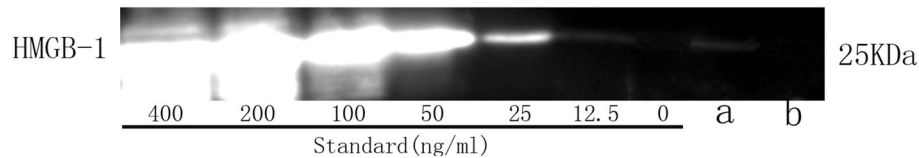
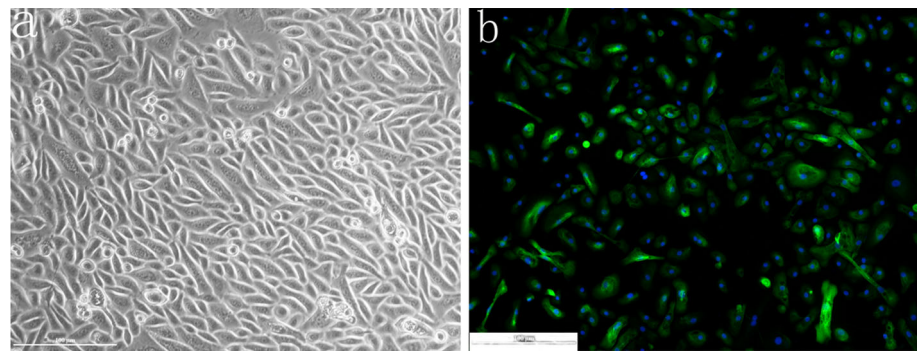
HMGB-1 levels in the supernatants of UV light-treated cells treated with or without anti-HMGB-1 antibodies

Using immunoblot analysis, we measured the HMGB-1 concentrations in the supernatants of UV light-treated cells treated with or without anti-HMGB-1 antibodies. We could detect expression of HMGB-1, a 25-kd protein, in the supernatants of UV light-treated cells, but not in that treated with anti-HMGB-1 antibodies (Fig. 4).

HMGB-1 levels change in culture supernatants over time

HMGB-1 concentrations in culture supernatants of cholesteatoma keratinocytes stimulated with supernatants from UV light-treated cells for 0, 4, 8, 12, 24, 36, or 48 h were measured by immunoblot analysis. Combined PI and annexin V staining and flow cytometric analysis of UV light-treated cells revealed a pattern consistent with late

**Fig. 3** Production and validation of the cholesteatoma keratinocytes. **a** Image of cultured cholesteatoma keratinocytes at the third passage. **b** Cholesteatoma keratinocytes double-labeled for pancytokeratins (green) and nuclei (blue). (Color figure online)

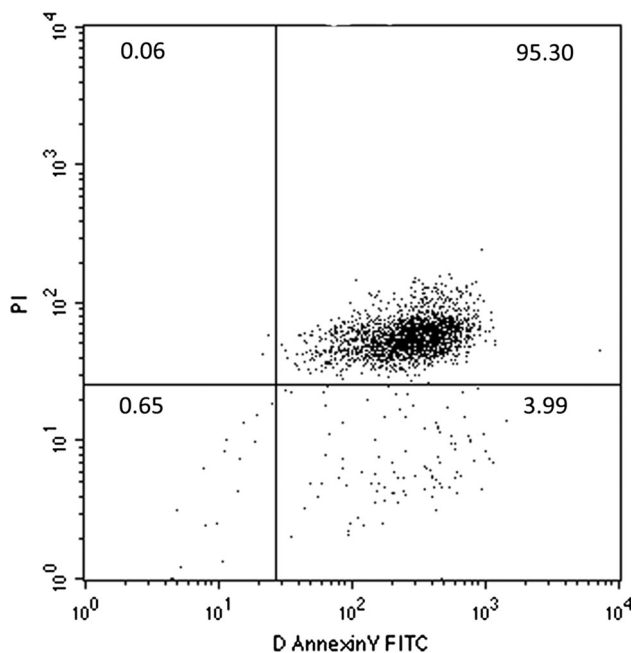


**Fig. 4** High-mobility group box chromosomal protein 1 (HMGB-1) concentrations in the supernatants of UV light-treated cells treated with or without antibodies. HMGB-1 concentrations were determined by immunoblot analysis. HMGB-1, a 25-kd protein, was expressed in

the supernatants of UV light-treated cells (a) but not in supernatants of cells treated with anti-HMGB-1 antibodies (b). Lanes on the left indicate the range of standard HMGB-1 concentrations (0–400 ng/ml)

apoptosis (Fig. 5). The expression of HMGB-1, a 25-kd protein, was detected in culture supernatants and the supernatants from UV light-treated cells. This indicated that cholesteatoma keratinocytes could release HMGB-1 during the course of apoptosis (Fig. 6). Concurrently,

HMGB-1 concentrations in culture supernatants were high at 4 h, had slightly declined by 12 h, and then slightly increased, reaching a peak at 48 h (Fig. 6), suggesting UV light-treated cells can release an unknown substance that induces HMGB-1 release. Although the nature of the substance is unknown, we demonstrated that it could be released into the culture medium.



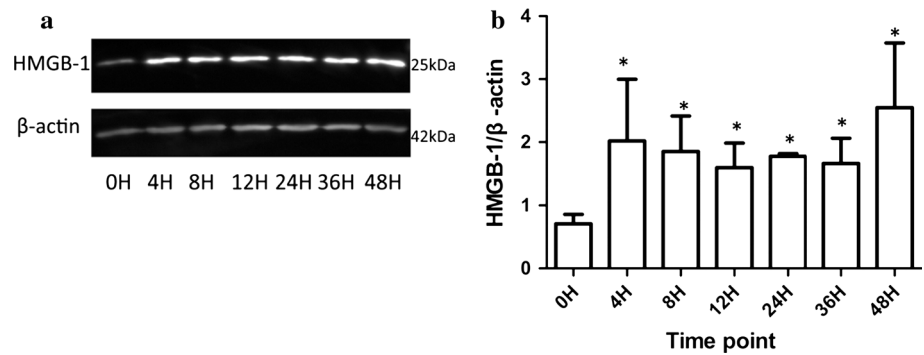
**Fig. 5** Characteristics of cell death of UV light-treated cholesteatomatous keratinocytes. Cells were stained with propidium iodide and annexin V-fluorescein isothiocyanate (FITC) and analyzed by flow cytometry. Results of representative experiments are shown

#### Translocation and expression of HMGB-1 in cholesteatoma keratinocytes

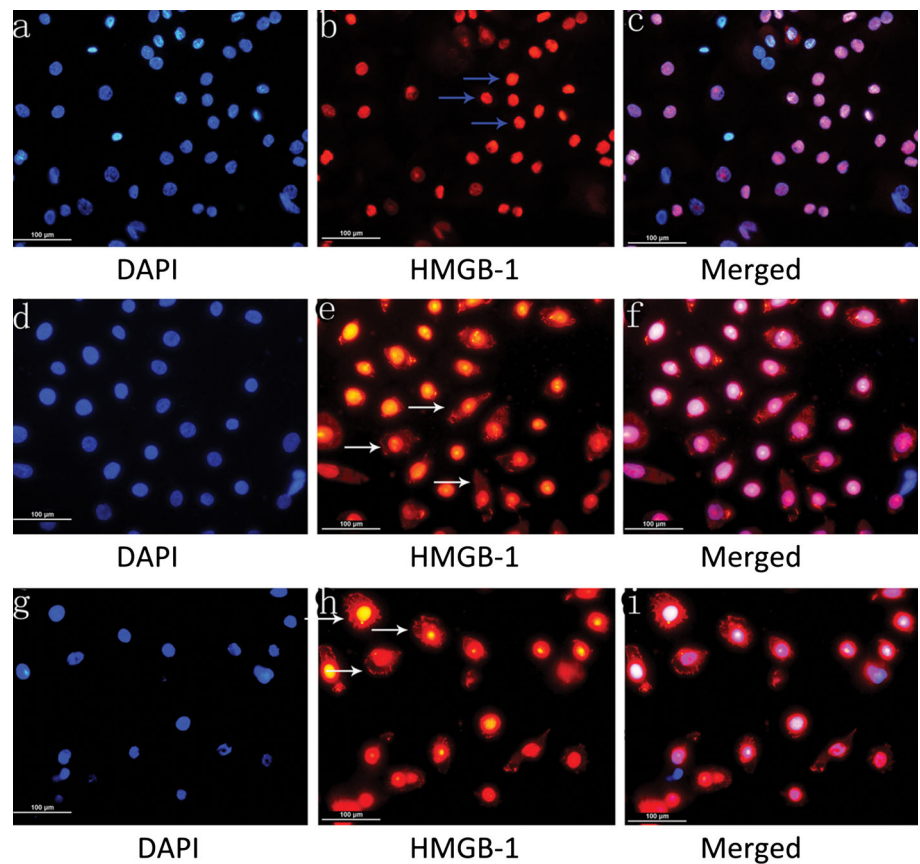
To examine the translocation of HMGB-1, cholesteatoma keratinocytes were cultured in a Lab-Tech chamber slide and stimulated with supernatants from UV light-treated cells for 1 and 24 h. Immunofluorescence assay demonstrated that in the control group without stimulation, HMGB-1 staining was observed only in the nucleus (Fig. 7a–c). However, translocation of HMGB-1 from the nucleus to the cytosol was observed in cholesteatoma keratinocytes at 1 h (Fig. 7d–f) and it was maintained for 24 h (Fig. 7g–i).

We quantified the expression of HMGB-1 in cytosol of cholesteatoma keratinocytes by immunoblot analysis. Treatment with supernatants from UV light-treated cells for 0, 1, 4, 8, 12, 24, 36, and 48 h demonstrated the levels of HMGB-1 in the cytoplasm of cholesteatoma keratinocytes was time-dependent. HMGB-1 concentrations reached a first peak at 1 h, slightly declined at 24 h and then sharply increased, reaching a second peak at 48 h (Fig. 8).

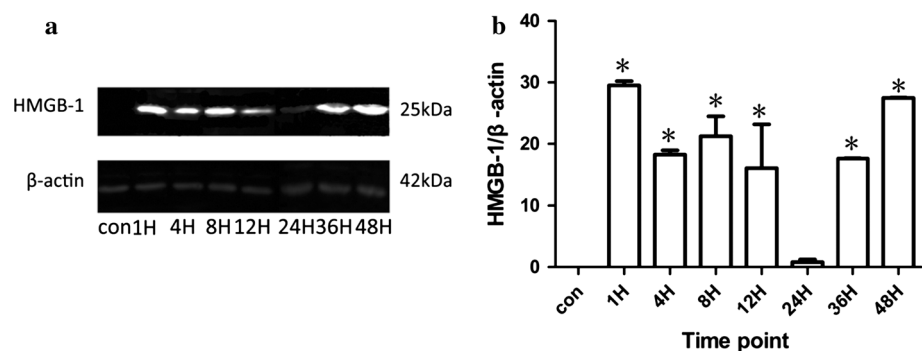
**Fig. 6** Immunoblot analysis showing the expression of high-mobility group box-1 (HMGB-1) in supernatants (a). Graphs indicate the relative expression of bands compared with  $\beta$ -actin (b) ( $*P < 0.05$  vs. 0 h)



**Fig. 7** Translocation of high-mobility group box chromosomal protein 1 (HMGB-1) in cholesteatoma keratinocytes after stimulation with supernatants from UV light-treated cells. Immunofluorescence assay indicated that HMGB-1 was translocated from the nucleus to the cytosol following stimulation. Cholesteatoma keratinocytes were stimulated for 1 h (d–f) or 24 h (g–i) and then compared with controls (a–c). The control group only exhibited HMGB-1 staining in the nucleus (a–c). Cholesteatoma keratinocytes were incubated with anti-HMGB-1 rabbit polyclonal antibody after stimulation. Nuclei were stained with DAPI (blue). Blue arrows depict nuclear HMGB-1. White arrows depict cytoplasmic HMGB-1. (Color figure online)



**Fig. 8** Immunoblot analysis shows the expression of high-mobility group box-1 (HMGB-1) in the cytoplasm of cholesteatomatous keratinocytes (a). Graphs indicate the relative expression of bands compared with  $\beta$ -actin (b) ( $*P < 0.05$  vs. 0 h)





HMGB-1 complexed to CpG-DNA induces expression of cytokine in cholesteatoma keratinocytes

HMGB-1 and DNA were thought to form a complex to induce cytokines release in pDCs [16]. We next evaluated whether the presence of HMGB-1 could influence cytokines expression in cholesteatoma keratinocytes. Cultures of human cholesteatoma keratinocytes were exposed to CpG-DNA, HMGB-1, or CpG-DNA complexed to HMGB-1 for 24 h. Keratinocytes cultured with CpG-DNA complexed to HMGB-1, but not CpG DNA alone or LL-37 alone, led to the release of IL-1 $\beta$  (Fig. 9b) and TNF- $\alpha$  (Fig. 9a).

Induction of cytokines by substance released from UV light-treated cells

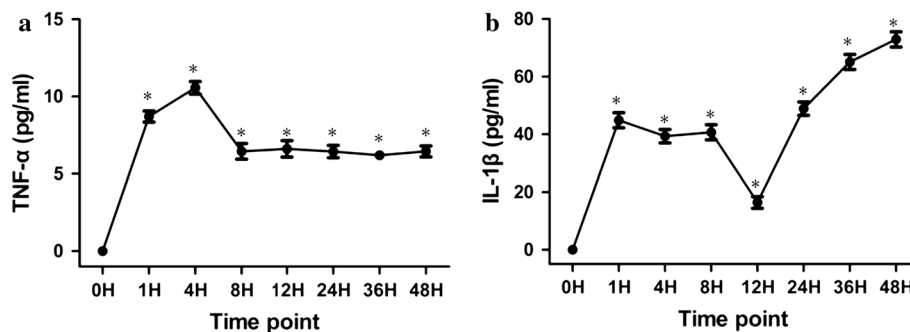
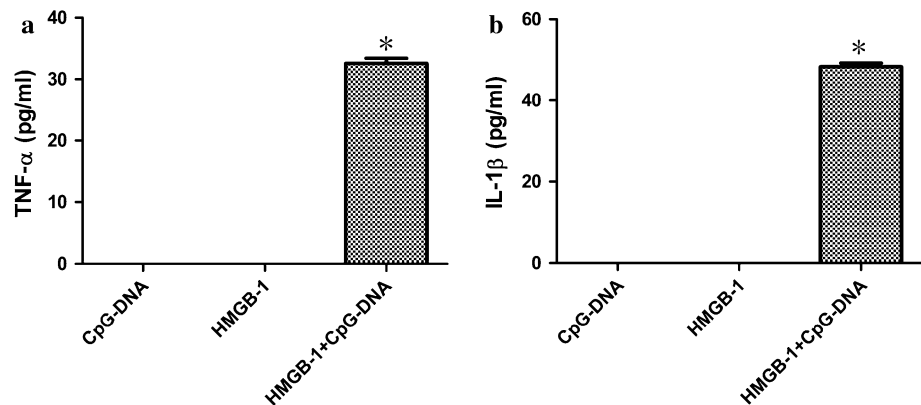
To determine whether the supernatant from UV light-treated cells had a proinflammatory effect, we measured the concentration of IL-1 $\beta$  and TNF- $\alpha$  in culture supernatants by ELISA. Supernatants from UV light-treated cells were potential TNF- $\alpha$  and IL-1 $\beta$  inducers. However, the secretion patterns for these cytokines were different. The

peak TNF- $\alpha$  level after stimulation with supernatants occurred at 4 h and then tended to decrease (Fig. 10a). However, except at 12 h, IL-1 $\beta$  levels increased in a time-dependent manner (Fig. 10b).

To determine whether HMGB-1 is a mediator of cytokine production, neutralizing anti-HMGB-1 antibodies were added to the cultures of the cholesteatoma keratinocytes. Treatment with anti-HMGB-1 antibodies eliminated the cytokine-inducing capacity of supernatants from UV light-treated cells, indicating that the induction of increased IL-1 $\beta$  (Fig. 11b) and TNF- $\alpha$  (Fig. 11a) synthesis by supernatants from UV light-treated cells requires HMGB-1.

To examine whether treatment of apoptotic cell material with DNase affected its ability to induce cytokine production, apoptotic supernatants from UV light-treated cells treated with DNase were added to the cultures of the cholesteatoma keratinocytes. Treatment with DNase decreased the cytokines-inducing ability of supernatants from UV light-treated cells (Fig. 12). This study demonstrated the importance of DNA fragments in the apoptotic cell material for induction of IL-1 $\beta$  (Fig. 12b) and TNF- $\alpha$  (Fig. 12a).

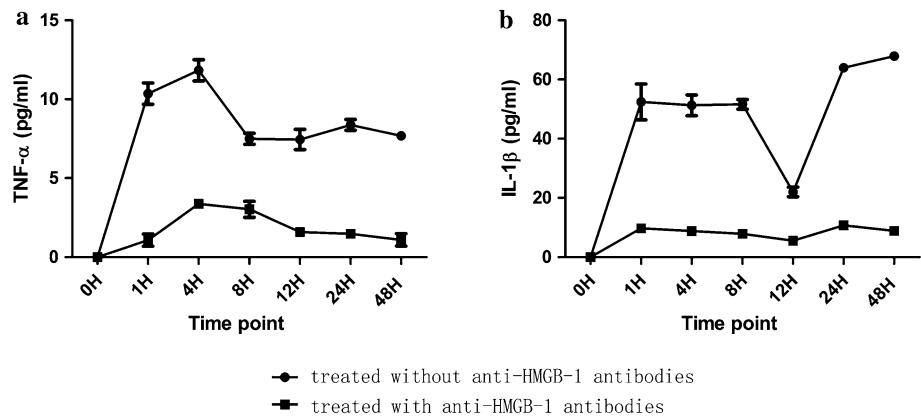
**Fig. 9** Human cholesteatomatous keratinocytes were stimulated with CpG DNA (2  $\mu$ M/l), HMGB-1 (3  $\mu$ M/l), or their combinations. Supernatants were collected after 24 h of culture, and levels of TNF- $\alpha$  (a) and IL-1 $\beta$  (b) were determined by immunoassay (\* $P$  < 0.05 vs. CpG-DNA or HMGB-1)



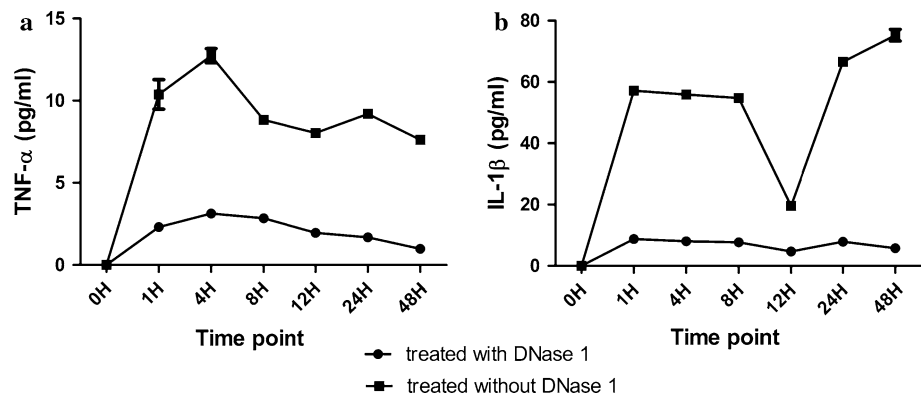
**Fig. 10** Secretion patterns of TNF- $\alpha$  (a) and IL-1 $\beta$  (b). Keratinocytes were stimulated with supernatants from UV light-treated cells, and levels of TNF- $\alpha$  and IL-1 $\beta$  released into culture medium were measured by enzyme-linked immunosorbent assay. Values are the

mean  $\pm$  SD. TNF- $\alpha$  levels peaked at 4 h and tended to decrease (\* $P$  < 0.05 vs. 0 h). IL-1 $\beta$  levels increased in a time-dependent manner, except at 12 h, and were all significantly higher than those at 0 h (\* $P$  < 0.05 vs. 0 h)

**Fig. 11** Effects of anti-HMGB-1 antibodies on the cytokine-inducing capacity of supernatants from UV light-treated cells. Supernatants were obtained 16–24 h after UV light treatment. They were incubated for 12 h at 37 °C with or without anti-HMGB-1 antibodies and then tested for the ability to induce TNF- $\alpha$  (a) and IL-1 $\beta$  (b) production in cultures of the cholesteatoma keratinocytes



**Fig. 12** Effects of DNase on the cytokines-inducing capacity of supernatants from UV light-treated cells. Supernatants were obtained 16–24 h after UV light treatment. They were incubated for 1 h at 37 °C with or without DNase and then tested for the ability to induce TNF- $\alpha$  (a) and IL-1 $\beta$  (b) production in cultures of the cholesteatoma keratinocytes



HMGB-1, TNF- $\alpha$ , and IL-1 $\beta$  mRNA expression levels in cholesteatoma keratinocytes

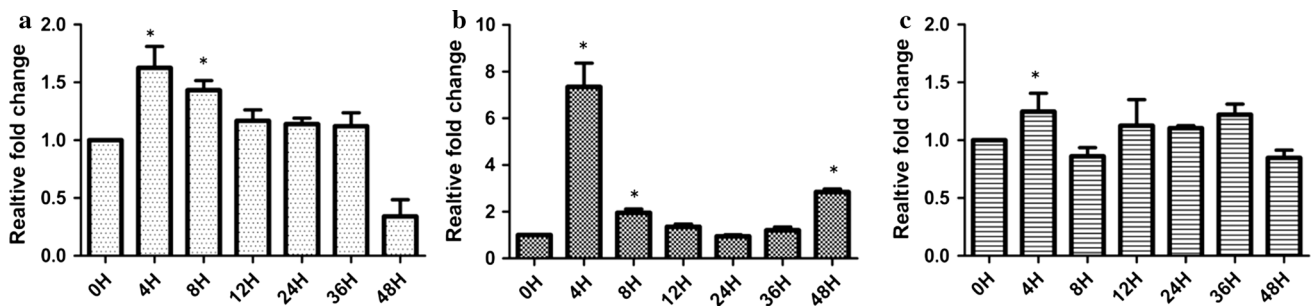
The effects of UV light-treated cell supernatants on HMGB-1, TNF- $\alpha$ , and IL-1 $\beta$  mRNA expression were analyzed by real-time PCR after exposing cholesteatoma keratinocytes to supernatants from UV light-treated cells for 0, 4, 8, 12, 24, 36, or 48 h. Supernatants from UV light-treated cells induced HMGB-1, TNF- $\alpha$ , and IL-1 $\beta$  release by cholesteatoma keratinocytes via increased gene transcription. qRT-PCR analysis revealed that HMGB-1 was up-regulated, as shown by the level of mRNA transcription after stimulation for 4 h and 8 h (Fig. 13a). TNF- $\alpha$  was also up-regulated at the mRNA level after stimulation for 4, 8, and 48 h (Fig. 13b). Up-regulation of IL-1 $\beta$  was also observed after stimulation for 4 h (Fig. 13c).

## Discussion

In this study, we found that keratinocytes in cholesteatomatous samples exhibited an increased amount of cytoplasmic and suggestive extracellular secreted HMGB-1 compared with normal skin. Previously, it was reported

that HMGB-1 was not released during apoptosis. However, Bell et al. reported that the release of HMGB-1 occurs during the course of apoptosis as well as necrosis in Jurkat cells [13]. Our study also showed that the induction of apoptosis by UV light irradiation leads to the extracellular release of HMGB-1. It was reported that proliferation and apoptosis are both present in cholesteatoma [19]. In this study, we observed that apoptotic cells were found in the cholesteatoma epithelium. This finding was in agreement with previous reports [20]. Since apoptosis is typically seen in cholesteatoma, the presence of extracellular HMGB-1 staining indicates the release of cytoplasmic HMGB-1 from apoptotic cells. In the current study, we also observed increased levels of HMGB-1 in supernatants after stimulation with supernatant from UV light-treated cells. Therefore, HMGB-1 may be actively secreted by cholesteatoma keratinocytes by stimulation of apoptotic material and may be passively released during apoptosis. Thus, it may constitute a source of extracellular HMGB-1 in cholesteatoma.

In the current study, we found that apoptotic cells released material that induced HMGB-1 translocation and release. Furthermore, such apoptotic material appeared late during the cultures, at 16–24 h after UV irradiation. This



**Fig. 13** Influence of supernatants from UV light-treated cells on mRNA expression of HMGB-1 (a), TNF- $\alpha$  (b), and IL-1 $\beta$  (c) in keratinocytes at the indicated timepoints. mRNA expression was

measured by qRT-PCR. Each set of data was expressed as the mean ratio (target/GAPDH)  $\pm$  SD. (\* $P < 0.05$  vs. 0 h group)

delayed release may be due to the retained cell membrane integrity in early apoptotic cells, which is eventually lost during the later stages of apoptosis. In normal in vivo situations, apoptotic cells are phagocytosed rapidly, while the membrane integrity is still intact. Consequently, there is less time for the release of material that can induce HMGB-1 release. However, patients with cholesteatomatous chronic otitis media have both increased apoptosis and decreased clearance of apoptotic cells and this may allow the continuous release of apoptotic material that induces extracellular HMGB-1 production in this disease.

HMGB-1 appears to have two distinct functions in cellular systems, first as an intracellular regulator of transcription, and second, it has an extracellular role where it promotes tumor metastasis and inflammation [21, 22]. An early suggestion was that HMGB-1 causes inflammatory cells to secrete TNF, IL-1a, IL-1b, IL-1RA, IL-6, IL-8, macrophage inflammatory protein (MIP)-1a, and MIP-1b, but not IL-10 or IL-12 (14). Thus, HMGB-1 might participate in a ‘cytokine storm.’ But several recent studies have found that carefully purified HMGB-1 caused little secretion of any cytokine from mouse or human macrophages [16, 17, 23]. In this study, we found that the production of TNF- $\alpha$  and IL-1 $\beta$  can be induced by purified HMGB-1 combined with CpG-DNA but not by purified HMGB-1 alone or CpG-DNA alone in the cholesteatoma keratinocytes. In addition, supernatants of apoptotic cells containing HMGB-1 were effective in inducing cytokine secretion, suggesting that the activity was due to the combined action of HMGB-1 with another material. Moreover, we also observed that DNase or anti-HMGB-1 antibody treatment of supernatants from apoptotic cells decreased their cytokine-inducing activity. Therefore, we concluded that apoptotic cells release HMGB-1 that induced TNF- $\alpha$  and IL-1 $\beta$  production when combined with DNA fragments.

Furthermore, cholesteatoma keratinocytes released TNF- $\alpha$  and IL-1 $\beta$  after stimulation with apoptotic material containing HMGB-1–DNA complexes. However, the

source of DNA fragments in cholesteatoma tissues remains unknown. It is reported that host-derived DNA can be released into extracellular environment during apoptotic cell death [24]. In this study, we observed that a larger number of apoptotic cells and DNA fragments was detectable in the cholesteatoma epithelium. These results demonstrated that extracellular DNA fragments were derived from surrounding apoptotic cells.

It is well established that the production of TNF- $\alpha$  could play an important role in bone resorption in primary cholesteatoma [25, 26]. Akimoto et al. [8] reported increased levels of TNF- $\alpha$  in primary cholesteatoma compared with normal external ear canal skin. TNF- $\alpha$  is produced by activated macrophages and mast cells [27]. However, few mononuclear cells could be detected in primary cholesteatoma. Therefore, the source of TNF- $\alpha$  in primary cholesteatomas is unclear. In our study, we found that keratinocytes stimulated by apoptotic material containing HMGB-1–DNA complexes could induce both TNF- $\alpha$  and IL-1 $\beta$ . Thus, HMGB-1–DNA complexes might act as a key molecule involved in bone resorption associated with cholesteatoma by inducing the production of TNF- $\alpha$  and IL-1 $\beta$ .

In conclusion, the activation of cholesteatomatous keratinocytes by apoptotic material containing HMGB-1–DNA complexes greatly induced the production of TNF- $\alpha$  and IL-1 $\beta$ . In addition, extracellular HMGB-1 and DNA fragments were present in the cholesteatomatous epithelium. These events suggest the persistent expression of extracellular HMGB-1 and DNA fragments in cholesteatoma leads to the production of TNF- $\alpha$  and IL-1 $\beta$ , which leads to cause bone resorption and destruction in cholesteatoma.

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**Conflict of interest** The authors declare that they have no competing interests.

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