Acid-induced cell injury and death in lung epithelial cells is associated with the activation of mitogen-activated protein kinases

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Abstract. Gastric hydrochloric acid (HCl) has been regarded as a causative factor of acute lung injury (ALI). The activation of mitogen-activated protein kinases (MAPKs) has been suggested to be a mechanism involved in the pathogenesis of ALI *in vivo*. However, the effects of HCl on MAPK activation in lung epithelial cells remain to be fully elucidated. Further investigation into the role of MAPK activation in acid-induced cell injury and death is also needed. In the present study, BEAS-2B cells were treated with HCl (pH 4.0 medium) for 5, 15 and 30 min, and the acidified medium was then removed. Cell viability and death were detected by MTT assay and trypan blue exclusion staining, respectively. The activation of MAPKs [c-Jun N-terminal kinase (JNK), p38 MAPK and extracellular signal-regulated kinase (ERK) 1/2] was analyzed by western blot analysis. Cytotoxicity was assessed by lactate dehydrogenase (LDH) release, and IL-8 levels in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA). Cell apoptosis was detected as changes in the levels of capase-3, Bad and fas by western blot analysis and the number of apoptotic cells by using Annexin V/propidium iodide (PI) staining. Following pre-treatment with the JNK inhibitor II (10 μmol/l), the p38 inhibitor SB202190 (10 μmol/l) or the ERK inhibitor U0126 (10 μmol/l) for 30 min, BEAS-2B cells were exposed to HCl for 30 min. Cell viability, cytotoxicity, IL-8 levels and apoptosis were detected 4 h following acid stimulation. The viability of BEAS-2B cells was inhibited and cell death was increased in the presence of HCl. HCl stimulation induced activation of MAPKs in a time-dependent manner. HCl exposure increased the levels of IL-8 and the release of LDH, and induced apoptosis in BEAS-2B cells. JNK and p38 inhibitors increased cell viability and decreased cytotoxicity and cell apoptosis, while ERK inhibitor had no effect on cell viability, cytotoxicity or apoptosis. These results indicate that acid exposure induced epithelial cell injury and death. The activation of JNK and p38 is involved in HCl-induced epithelial lung cell injury and death.

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Introduction

Acute respiratory distress syndrome (ARDS) is a multifactorial, heterogeneous disease associated with high rates of mortality and disability in critically ill patients (1-3). Acid aspiration-induced lung injury accounts for ~11% of all clinical ARDS cases and is a major cause of morbidity in cases of critical illness (4,5). Acid aspiration-induced ARDS is characterized by pulmonary edema from increased vascular and epithelial permeability, as well as alveolar and interstitial inflammation with leukocyte infiltration (6). Cell death has been demonstrated in the lung during the pathogenesis of acute lung injury (ALI)/ARDS. However, the mechanism of acid-induced cell injury and death in ALI has yet to be fully elucidated.

Mitogen-activated protein kinases (MAPKs), including the c-Jun NH2-terminal kinase (JNK), p38 MAPK and extracellular signal-regulated kinase (ERK) 1/2, are commonly involved in the pathogenesis of ALI. JNK and p38 MAPK modules are involved in cell survival and apoptosis, while the ERK module has been shown to play a crucial role in cell proliferation. Lipopolysaccharide (LPS) has been shown to induce the activation of MKK3/p38 signaling pathways and epithelial/endothelial cell apoptosis in animal models of ALI (7,8). However, changes in JNK, p38 and ERK1/2 expression following exposure of lung epithelial cells to acid remain to be fully elucidated. Further investigation is also needed with regard to the effects of MAPK pathway blockade on cell injury and death.

The present study investigated the effects of acid exposure on MAPK pathways, cell injury and death in lung epithelial cells using JNK, p38 and ERK1/2 inhibitors. The main pathways involved in acid-induced epithelial cell injury and death were identified.

Materials and methods

Materials. All of the reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), BD Biosciences

(Bedford, MA, USA) and Corning Costar (Cambridge, MA, USA) unless otherwise specified. JNK inhibitor II, SB202190 (p38 inhibitor) and U0126 (ERK1/2 inhibitor) were purchased from Calbiochem (San Diego, CA, USA). The cell proliferation (MTT) and cytotoxicity detection kits [lactate dehydrogenase cytotoxicity (LDH)] were obtained from Roche GmbH (Mannheim, Germany). Mouse anti-phospho-JNK, mouse anti-JNK, mouse anti-phospho-p38 MAP kinase, mouse anti-p38 MAP kinase, mouse anti-phospho-ERK1/2, mouse anti-ERK1/2, mouse anti-fas, mouse anti-Bad and mouse anti-GAPDH antibodies were obtained from Santa Cruz Biotechnology,Inc.(SantaCruz,CA,USA).Rabbitanti-capase-3 antibody was purchased from Abcam (Cambridge, UK). Goat anti-mouse, goat anti-rabbit and donkey anti-goat IgGs were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). An electrochemiluminescence (ECL) kit was obtained from Pierce Biotechnology, Inc. (Rockford, IL, USA). Electrophoresis reagents and equipment were purchased from Bio-Rad (Hercules, CA, USA).

BEAS-2B epithelial cell culture. Human lung epithelial BEAS-2B cells [American Type Culture Collection (ATCC), Manassas, VA, USA] were grown as a monolayer in 5% CO_2 at 37˚C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 U/ml penicillin and 50 mg/ml streptomycin (complete medium). BEAS-2B cells were seeded in 6-, 12- or 24-well culture plates for each experiment.

Acid exposure. BEAS-2B cells were plated at densities of $4x10^5$ cells/well in 6-well plates. After 24 h of incubation, the medium was changed to serum-free DMEM followed by a 24-h culture. The cells were then treated with HCl (pH 4.0 DMEM) for 5, 15 and 30 min at 37˚C in 5% CO_2. Negative control cells were exposed to phosphate-buffered saline (PBS) at the same volume of HCl used in the acid-exposed group. After incubation with HCl, the acidified medium was discarded, and the cells were washed three times with complete medium to confirm neutralization of the culture medium (9). BEAS-2B cells were then cultured with serum-free DMEM for an additional 4 h.

Cell pre-treatment with JNK inhibitor II, SB202190 or U0126. In the MAPK inhibitor pre-treatment experiments, BEAS-2B cells were incubated with JNK inhibitor II, SB202190 or U0126 at 10 μM for 30 min prior to HCl exposure (10-12). Pre-treatments were initiated by adding concentrated solutions to reach the final concentrations in each well. JNK inhibitor II, SB202190 and U0126 stock solutions were prepared by dissolution in DMSO as the vehicle.

Cell viability measurements using the MTT assay. Cell viability was measured using the MTT reduction assay as previously described (13). BEAS-2B cells ($2.5x10^4$/well) were grown in 100 μl of DMEM containing 10% FBS using 96-well plates. Briefly, after exposure to HCl (pH 4.0 DMEM) for 5, 15 or 30 min, the supernatant was removed, and the cells were gently washed with complete medium and incubated with 100 μl of fresh culture medium and 10 μl MTT (5 mg/ml). After 4 h of incubation, 100 μl of the solubilization solution was added to dissolve the formazan crystals overnight, and the absorbance was read using a microplate reader (SpectraMax M5, Molecular Devices, CA, USA) at a wavelength of 570 nm. Using the control wells to determine optimum cell growth, cell viability was defined as the percentage of the average absorbance of treated wells divided by the absorbance of control wells.

Cell death assessment by trypan blue staining. Cell death was determined after treatment with HCl using a trypan blue dye exclusion assay, as previously described (14). BEAS-2B cells ($4x10^5$/well) were grown in 6-well plates. Following HCl exposure, the cells were detached by trypsinization and collected after centrifugation. The cells were resuspended in PBS and incubated with an equal volume of 0.4% trypan blue for 5 min at room temperature. The number of dead cells (stained cells) was counted using a hemocytometer and expressed as a percentage of the total cells counted.

Detection of cytotoxicity by the LDH assay. LDH is a stable cytoplasmic enzyme present in most cells and is released into cell culture supernatant when the cell membrane is damaged. LDH assays were performed using a non-radioactive alternative to the (^{3}H)-thymidine-release assay and the (^{51}Cr)-release assay (catalogue no. 11644793001). BEAS-2B cells were grown in 6-well plates until confluent, serum starved for 24 h and exposed to previously defined experimental conditions (3 wells each). At 4 h, the medium was collected, centrifuged at 4˚C for 5 min and incubated with the appropriate kit reagents. The absorbance was then measured with a spectrophotometer at 490 nm and compared with the appropriate control cells.

Detection of apoptosis by Annexin V-FITC/propidium iodide (PI) staining. Annexin V-FITC/PI staining was performed to detect apoptotic cells following acid exposure. BEAS-2B cells were plated into 24-well plates and treated with HCl for 30 min. BEAS-2B cells were then gently washed, and stained with Annexin V-FITC (green fluorescence) and PI (red fluorescence) for 15 min in the dark at room temperature. The cells were observed using a dual wavelength fluorescence microscope (Nikon, Tokyo, Japan).

Western blot analysis. Total proteins were extracted from BEAS-2B cells in lysis buffer (0.1% Triton X-100) and separated by 12% SDS-PAGE under reducing conditions. The samples were then transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in TBS (0.01 mM Tris buffer, pH 7.5, and 0.4 mol/l NaCl) containing 0.1% Tween-20 and 5% skimmed milk for 1 h and incubated with 2 μg/ml mouse anti-human p-JNK, JNK, p-p38, p38, p-ERK, ERK, fas, Bad or capase-3 in a primary antibody dilution buffer at 4˚C overnight. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse, goat anti-rabbit or donkey anti-goat IgG (dilution, 1:15,000), and the signals were detected using an ECL kit.

IL-8 assay. IL-8 levels in the supernatants were measured using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions.

Statistical analysis. All the data are expressed as the means ± SD. Statistical analysis was performed using SPSS 16.0 (SPSS, Inc., Chicago, IL, USA). Group comparisons were performed using one-way ANOVA, followed by the Student-Newman-Keuls test for pairwise multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

HCl exposure reduces BEAS-2B cell viability and induces BEAS-2B cell death. We examined whether exposure to HCl affects the viability of BEAS-2B cells and induces cell death. As shown in Fig. 1A, the viability of BEAS-2B cells decreased in a time-dependent manner after stimulation with acid. BEAS-2B cell death increased with treatment time following HCl stimulation (P<0.05); 19% cell death was observed when the cells were exposed to HCl for 30 min (Fig. 1B).

HCl exposure activates MAPK family proteins in a time-dependent manner. To identify mechanisms underlying HCl-mediated apoptosis, the expression of MAPK proteins was examined by western blot analysis. After exposure to HCl (pH 4.0 DMEM), the levels of phosphorylated JNK, p38 and ERK1/2 markedly increased in BEAS-2B cells compared with control BEAS-2B cells in 3 independent experiments, indicating that these three MAPK pathways were activated after HCl stimulation. Furthermore, after HCl exposure for 5, 15 and 30 min, JNK, p38 and ERK1/2 phosphorylation was found to be significantly increased in a time-dependent manner (Fig. 2). Based on these results and the literature, pH 4.0 DMEM was used to stimulate BEAS-2B cells for 30 min.

HCl exposure induces cell injury and apoptosis. Cytotoxicity was observed when the cells were exposed to HCl, which resulted in increased levels of LDH (P<0.05; Fig. 3B). HCl exposure was also found to enhance the production of IL-8 (P<0.05), indicating that HCl induces cell injury and inflammation (Fig. 3C).

BEAS-2B cell apoptosis was determined by the assessment of cleaved caspase-3 (active form of caspase-3), fas and Bad expression by western blot analysis and the number of apoptotic cells using Annexin V/PI staining. Caspase-3, a key member of the caspase family, is widely accepted as a reliable indicator for cell apoptosis (15). Fas is an important molecule in the regulation of apoptosis (16). Bad is a pro-apoptotic protein in the Bcl-2 family (17). The upregulation of fas, caspase-3 and Bad constitutes the hallmark of cell apoptosis. In the present study, the levels of fas, caspase-3 and Bad were found to be significantly increased in the presence of HCl. Furthermore, the number of positive cells following Annexin V/PI staining was significantly increased compared with the number of control cells (Fig. 4A).

Effects of MAPK inhibition on cell injury in BEAS-2B cells. To further investigate the association between MAPK activation and cell injury, BEAS-2B cells were pre-treated with JNK inhibitor II, SB202190 (p38 inhibitor) or U0126 (ERK1/2 inhibitor) in the presence of HCl. Pre-treatment with SB202190 and JNK inhibitor II reversed the decrease in BEAS-2B cell viability (Fig. 3A) and significantly inhibited the increase in LDH (Fig. 3B; P<0.05) and IL-8 expression (Fig. 3C; P<0.05). However, pre-treatment with U0126 had no effect on cell viability, cytotoxicity or IL-8 production (Fig. 3A-C).

Effects of MAPK inhibition on BEAS-2B cell apoptosis. Pre-treatment with JNK inhibitor II or SB202190 decreased the levels of caspase-3, Bad and fas. However, pre-treatment with U0126 did not inhibit the expression of caspase-3, Bad or fas, suggesting that U0126 had no effect on cell apoptosis (Fig. 3D). These results indicate that HCl effectively induces apoptosis via the JNK and p38 pathways, as observed in the pre-treatment profiles of MAPK inhibitors.

Discussion

The present study demonstrated that: i) HCl exposure induces BEAS-2B cell injury and death; ii) the activation of JNK, p38 and ERK1/2 in BEAS-2B cells after HCl stimulation is time dependent; iii) the inhibition of JNK or p38 inhibits BEAS-2B cell injury and apoptosis and iv) the inhibition of ERK1/2 does not affect the level of IL-8, the release of LDH and the expression of apoptotic proteins. Furthermore, the JNK and p38 pathways were identified to be involved in HCl-induced epithelial cell injury and death.

Acid-induced epithelial cell injury and death in vitro. Acid aspiration-induced lung injury is a common disease in the intensive care unit and is correlated with the prognosis of patients. The main characteristics of acid-induced ALI are increased permeability of the alveolar-capillary interface and alveolar as well as interstitial inflammation with edema. The intratracheal instillation of HCl is an *in vivo* model of ALI (18,19). HCl has also been used to replicate the ALI model *in vitro* (9). The results of the present study indicated that the viability of BEAS-2B cells decreased and the number of dead cells increased with HCl treatment time. Furthermore, the release of IL-8 and LDH was found to be increased in BEAS-2B cells after HCl exposure. These data showed that acid exposure induces epithelial cell injury and inflammation, and stimulates cell death.

Apoptosis, necrosis, and oncosis have been associated with cell death. Apoptosis has been suggested to be a mechanism underlying the pathogenesis of ALI/ARDS and other lung diseases (20,21). Apoptosis is an active form of cell death, the timing of which is genetically determined during the course of development. Various biochemical features of apoptosis have been identified and are frequently used as an indication for apoptosis, such as caspase activation, DNA fragmentation and the externalization of phosphatidylserine, a cell surface marker for phagocytosis (22). Additional studies have demonstrated that alveolar epithelial injury in humans with ALI or ARDS is associated with the local upregulation of the Fas/FasL system and activation of the apoptotic cascade. The present study found that caspase-3, fas and Bad were increased after acid stimulation of BEAS-2B cells. Imai *et al* (23) demonstrated epithelial cell apoptosis in rabbit lungs after intratracheal administration of HCl. Taken together, acid exposure *in vivo* and *in vitro* is suggested to induce epithelial cell apoptosis in ALI. Apoptosis is an important cellular mechanism in HCl-induced cell injury.

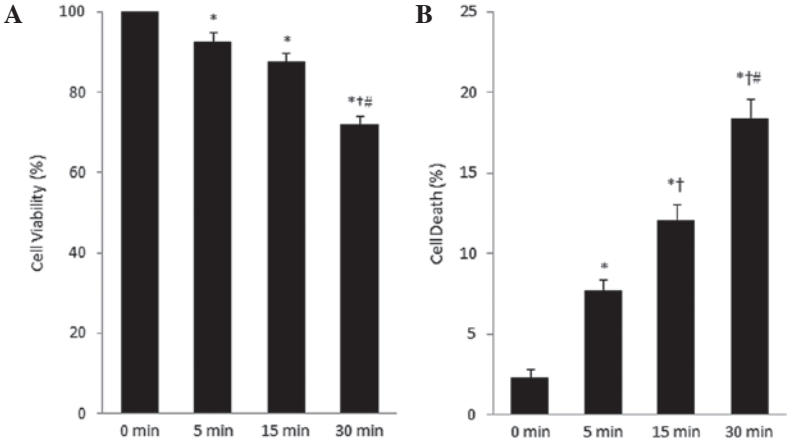

Figure 1. Effect of HCl on BEAS-2B cell viability and death. (A) A time-dependent effect of HCl treatment on BEAS-2B cell viability was observed. Cell viability was determined by MTT assay 4 h after HCl exposure for 0, 5, 15 and 30 min. (B) A time-dependent effect of HCl treatment on BEAS-2B cell death was observed. BEAS-2B cells were treated with HCl (pH 4.0 DMEM) for 0, 5, 15 and 30 min. The cells were then cultured in DMEM for 4 h. Cell death was determined by trypan blue staining 4 h after HCl exposure. Data are expressed as the means ± SEM; *P<0.05 vs. 0 min; †P<0.05 vs. 5 min; #P<0.05 vs. 15 min. n=9 from three experiments. HCL, hydrochloric acid.

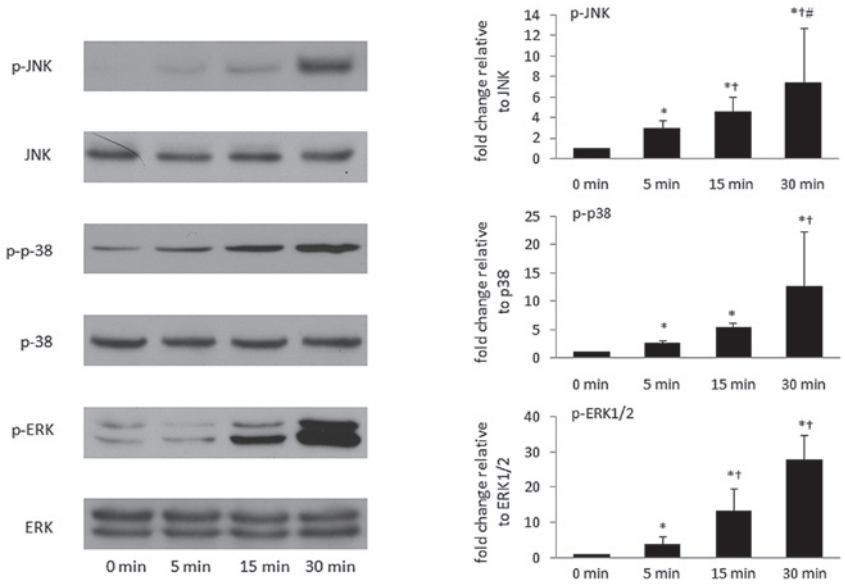

Figure 2. Effect of HCl on the activation of MAPKs in BEAS-2B cells. BEAS-2B cells were treated with HCl (pH 4.0, DMEM) for 0, 5, 15 and 30 min. The levels of MAPKs were determined by western blot analysis. For phosphorylated MAPKs, each protein level was normalized to the respective total MAPK level and is shown relative to the value for untreated control cells (normalized to 1). Data are expressed as the means ± SEM; *P<0.05 vs. 0 min; †P<0.05 vs. 5 min; #P<0.05 vs. 15 min. n=9 from three experiments. MAPKs, mitogen-activated protein kinases; HCL, hydrochloric acid.

Acid exposure activates MAPK family proteins in a time-dependent manner. The MAPK family consists of ERK, p38 MAPK and JNK. ERK is mainly activated by mitogenic stimuli, while p38 and JNK are mainly activated by stress stimuli or inflammatory cytokines (24). After acid exposure (0.1 N HCl for 3 min), a rapid increase in the activity of ERK and p38 and a delayed increase in JNK activity were shown in the Barrett's adenocarcinoma cell line SEG-1 (25). The results of the present study showed that the phosphorylation of JNK, p38 and ERK1/2 was significantly increased in a time-dependent manner after acid administration. MAPKs are activated in LPS-induced ALI, and the inhibition of pulmonary MAPK activity abrogates LPS-induced inflammation and lung injury (26). HCl exposure induced epithelial cell apoptosis and activated the MAPK pathway. Coupled with the knowledge that MAPKs regulate cell proliferation and apoptosis, we hypothesize that the activation of the MAPK pathway plays a role in acid-induced epithelial cell apoptosis.

Effects of MAPK inhibitors on acid-induced epithelial cell injury and apoptosis. JNK has predominantly been positively associated with stress responses, including cytokine release and apoptosis (27). In the present study, the activation of JNK by HCl in BEAS-2B cells was associated with an increase in apoptotic protein levels. The inhibition of JNK by JNK inhibitor II decreased the concentration of apoptotic proteins. However, the reduction in apoptosis was only partial, suggesting that HCl may also induce apoptosis by pathways other than JNK. Alternatively, the JNK inhibitor II may not have inhibited JNK activity completely. In the present study,

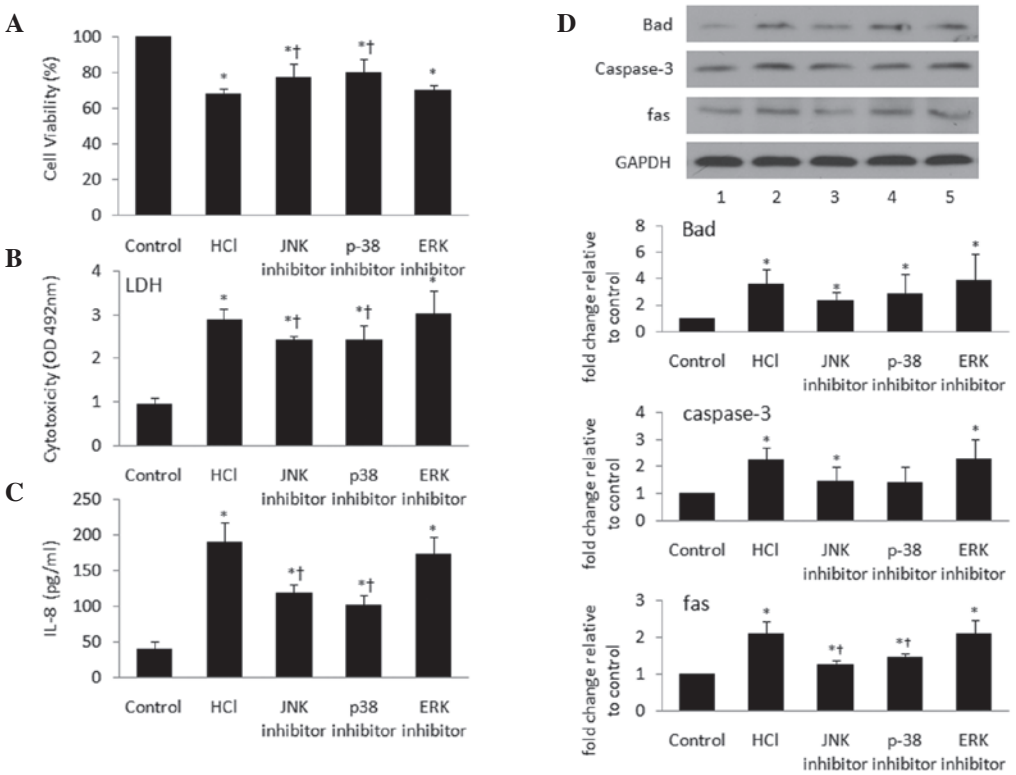

Figure 3. Effect of MAPK inhibitors on cell viability, cytotoxicity, IL-8 secretion and apoptosis in BEAS-2B cells exposed to HCl. BEAS-2B cells were pre-treated with MAPK inhibitors (10 μM) 30 min prior to HCl exposure. (A) The effects of MAPK inhibitors on BEAS-2B cell viability were detected by MTT assay 4 h following HCl stimulation. (B) The effects of MAPK inhibitors on cytotoxicity were measured as the release of LDH from BEAS-2B cells in the supernatant 4 h following HCl exposure. (C) The effects of MAPK inhibitors on the expression of IL-8 were detected by ELISA. The concentration of IL-8 in the supernatant was measured 4 h following HCl exposure. (D) Effects of MAPK inhibitors on cell apoptosis in BEAS-2B cells exposed to HCl. The levels of apoptotic proteins (Bad, caspase-3 and fas) were determined by western blot analysis. The protein levels were normalized to GAPDH and are shown relative to the value for untreated control cells (normalized to 1). 1, control; 2, HCl; 3, JNK inhibitor; 4, p38 inhibitor; 5, ERK inhibitor. Data are expressed as the means ± SEM; *P<0.05 vs. control; †P<0.05 vs. HCl. n=9 from three experiments. MAPKs, mitogen-activated protein kinases; LDH, lactate dehydrogenase; HCL, hydrochloric acid.

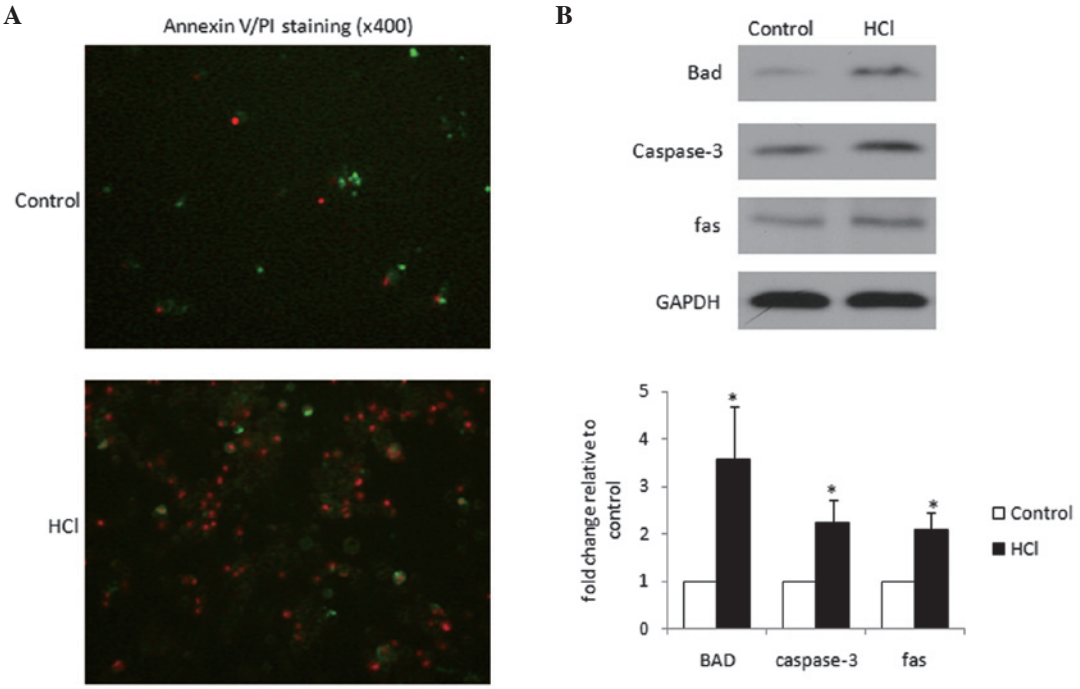

Figure 4. Effect of HCl exposure on BEAS-2B cell apoptosis. BEAS-2B cells were exposed to HCl (pH 4.0, DMEM) for 30 min. (A) Apoptosis of BEAS-2B cells was detected by Annexin V/PI staining (magnification, x400). (B) Levels of the apoptotic proteins Bad, caspase-3 and fas were determined by western blot analysis. Each protein level was normalized to GAPDH and is shown relative to the value for untreated control cells (normalized to 1). Data are expressed as the means ± SEM; *P<0.05 vs. control cells. n=9 from three experiments. HCL, hydrochloric acid.

we also found that the inhibitor of p38, SB202190, decreased cell apoptosis. Thus, the p-38 MAPK pathway is suggested to be involved in HCl-induced cell apoptosis.

Involvement of the ERK pathway in cell proliferation and apoptosis has previously been reported (28). In the present study, no change in the viability of BEAS-2B cells or the expression of apoptotic proteins was observed using ERK inhibitors. This result indicated that ERK activation in lung epithelial cells is not involved in the induction of apoptosis. Goillot *et al* (29) reported that ERK activation was transient and that JNK activation was sustained and correlated with the onset of apoptosis. This may partially explain why pre-treatment with U0126 in the present study did not affect cell viability or apoptosis after acid exposure.

In summary, acid exposure activates MAPKs in a time-dependent manner and induces lung epithelial cell injury and apoptosis *in vitro*. Moreover, the activation of p38 and JNK is involved in HCl-induced epithelial lung cell injury and apoptosis. Blockade of the JNK or p38 pathways is suggested to inhibit the apoptosis of BEAS-2B cells and reduce lung injury.

Acknowledgements

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