The IL-8 Production by Streptococcal Pyrogenic Exotoxin B

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We have previously identified $\alpha_v\beta_3$ and Fas as receptors for the streptococcal pyrogenic exotoxin B (SPE B), and G308S, a mutant of SPE B with RSD motif, which interacts with Fas only. This study aims to evaluate how SPE B interacts with cells to induce the production of IL-8. Our results showed that following exposure to SPE B or G308S, the levels of IL-8 protein and mRNA were increased and the increase was inhibited by the addition of anti-Fas antibody, suggesting that the increased production of IL-8 by SPE B is mediated through Fas receptor. In the presence of G308S, the association of FADD and procaspase 8, and activation of NF-κB were also detected. The application of siRNA of FADD and of procaspase 8 could inhibit the NF-κB activity. The proteolytic activity of caspase 8 was required for the NF-κB activity. Further studies showed that G308S could increase the phosphorylation of ERK and the translocation of NF-κB into the nucleus, and the inhibition of ERK phosphorylation decreased the IL-8 production, mRNA expression and activation of NF-κB. In addition, siRNA of procaspase 8 could inhibit the G308S-induced cleavage of MEKK1, binding of MEKK1 to caspase 8, activation of ERK and the NF-κB activity. Taken together, the production of IL-8 by SPE B in A549 cells is mediated by Fas, and followed by the activation of FADD, caspase 8, MEKK1, ERK and NF-κB. Exp Biol Med 234:1316–1326, 2009

Key words: SPE B; IL-8; Fas; NF-κB; A549 cells

Introduction

In response to various stimuli, epithelial cells play an important role in innate immunity through the activation of nuclear factor κB (NF-κB) to upregulate the inflammatory molecules (1–4). After stimulation, the NF-κB inhibitor (I-κB) is phosphorylated by I-κB kinase (IKK) and leaves NF-κB. The active NF-κB is translocated into the nucleus to enhance cytokine gene expression (5). On the other hand, some evidence also indicates MAP kinase is involved in the regulation of cytokines. For example, the up-regulated levels of IL-6 and IL-8 are eliminated in the presence of MAP kinase inhibitors after *S. pyogenes* infection (4). Pretreatment of cells with p38 kinase inhibitor can down-regulate the level of IL-8 in response to *S. typhimurium* infection (1). The activation of ERK is involved in thrombin-induced IL-8 release from dermal fibroblasts (6). From these studies, we suggest that NF-κB and the MAP kinase signaling pathway may have an important role in the molecular mechanism of innate immunity of SPE B-infected epithelial cells.

Streptococcus pyogenes (group A streptococcus, GAS) is a gram-positive bacterial pathogen that causes many severities of infection. The GAS-infected respiratory epithelial cells will induce the early innate immunity responses to increase the production of IL-8 and IL-6 (4). Streptococcal pyrogenic exotoxin B (SPE B) is secreted by all GAS and has been demonstrated to be an important factor involved in streptococcal infections. *In vivo* study indicates that GAS can induce the secretion of cytokine as well (7). However, the molecular mechanism of SPE B-induced IL-8 production is not clear. Our previous study has identified $\alpha_v\beta_3$ and Fas as receptors for the streptococcal pyrogenic exotoxin B (SPE B) (8). Fas (apo-1/CD95) is well known as a cell death receptor by triggering apoptotic signal pathways in various types of cells. Upon stimulation, Fas leads to the recruitment of cytoplasmic molecules like Fas-associated death domain protein (FADD), which then

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attracts procaspase 8. Aggregation of procaspase 8 causes subsequent autoproteolytic activation of caspase 8 (9). In addition to its role in apoptosis, Fas also mediates nonapoptotic signaling. Fas ligand induces cell-autonomous NF-κB activation and IL-8 expression in HEK293 cells by a mechanism different from that of TNF-α (10). FADD, caspase 8 and RIP are also involved in the activation of NF-κB (11). For mitogen-activated protein kinases, JNK, but not ERK or p38, is required for FasL-induced AP1 activation and IL-8 expression in HEK293 cells (12). However, the IL-1 β-induced IL-8 production in cystic fibrosis lung epithelial cells requires ERK1/2, and p38, but not JNK, for the activation of NF-κB (13), while the protease-activated receptor-induced IL-8 production in A549 cells requires activation of ERK1/2 and JNK (14).

In this study, experiments were designed to explore how SPE B interacts with cells to increase the IL-8 production and what MAPK is involved in the activation of NF-κB. Both SPE B and G308S were used for this study. The Circular Dichroism analyses showed that SPE B and G308S had 23.8% and 23.3% α-helix, 53.5% and 52.6% β-structure, and 22.7% and 24.1% coil, respectively. Basically G308S remains a similar conformation to SPE B. G308S cannot interact with integrin due to the conversion of glycine residue at 308 to serine. Thus, SPE B can bind to $\alpha_v\beta_3$ and Fas, while G308S binds only to Fas. Our results showed that in the presence of SPE B or G308S, IL-8 production was increased through the activation of Fas, FADD, caspase 8, MEKK1, ERK and NF-κB.

Materials and Methods

Purification of Recombinant SPE B and Its Mutant G308S. The expression and purification of recombinant SPE B (rSPE B) are described elsewhere (15). The gene encoding ProSPE B was amplified using a polymerase chain reaction (PCR) with six Histidine tags and a BamH1 recognition site, and cloned into the BamH1 site of the pET21a vector (Novagen), which was then transferred into *Escherichia coli* BL21 pLYs. A wild-type construct was used to produce a G308S mutation using overlap extension PCR (16). An inoculum (250 μl) of stock culture was added to 250 ml of LB/AMP medium (Sigma Chemical, St. Louis, MO) and allowed to grow to an optical density (590 nm) of 0.5–1.0. An aliquot of 250 μl of isopropyl-β-D-thiogalactopyranoside (IPTG, 100 mg/ml, MDBio) was added to induce protein expression. For rSPE B, cells were grown at 33°C overnight, while for G308S, cells were grown at 37°C overnight. After induction, cells were pelleted using a centrifuge at 5,000 rpm for 15 min; pellets were resuspended in 80 ml of buffer A (20 mM Tris, 200 mM NaCl (pH 7.0)) and broken three times using a French press at 1500 kg/cm^2 for 30 s. The entire content was centrifuged at 12,000 rpm at 4°C for 10 min to collect supernatant, which was then separated on a Ni-chelated column (Amersham Biosciences, UK) and eluted using a 0 to 200 mM imidazole gradient. The collected fractions were concentrated using Amicon ultrafiltration with a 10-kDa cutoff membrane (Millipore Corporation, Billerica, MA) and dialyzed with PBS buffer. The purity was verified using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). rSPE B was expressed as a 42-kDa zymogen and converted to a 28-kDa active enzyme during the course of purification. G308S was expressed as a 42-kDa zymogen and converted to a 28-kDa active enzyme during the course of purification. The purified rSPE B and G308S were passed through Detoxi-Gel (Pierce) to remove lipopolysaccharide contamination.

Cell Culture. A549 cells are routinely maintained in the laboratory in defined minimal essential medium (DMEM; Sigma) supplemented with 10% heat inactivated fetal calf serum (Gibco Life Technologies), 2 mM of L-glutamate (Sigma), and 50 μg/ml of gentamycin (Sigma). The cells were incubated in a CO_2 incubator at 37°C and 5% CO_2 in a humidified atmosphere. For incubation, cells (8×10^4 cells/well) were seeded into a 24-well plate for 24 h. The cells were then washed with PBS and incubated in the complete medium containing specific agents for the period of time as indicated in each experiment.

IL-8 Production. The secreted protein levels of IL-8 in the supernatants of A549 cells were measured by using ELISA kit from R&D Systems.

Immunoblotting. A549 cells at a density of 2×10^5/well were seeded into a 6-well plate 1 day before treatment. Cells were washed with cold phosphate-buffered saline (PBS) and treated with new medium containing agents as described for each experiment for various times. The supernatants of cell lysate were collected and boiled in sample buffer for 5 min at 100°C. Denatured proteins were separated by SDS-PAGE. Following SDS-PAGE, proteins were transferred to PVDF membrane, and blocked with 5% low fat milk at 4°C overnight. PVDF-membranes were probed with one of the following primary antibodies diluted in PBST (PBS containing 0.05% Tween-20) or TBST (Tris-Buffered Saline Tween-20): a 1/100 dilution of anti-procaspase 8 (Cell Signaling, #9746), 1/200 dilution of anti-NF-κB p65 subunit (Cell Signaling, #3031), 1/200 dilution of anti-FADD (Cell Signaling, #2782), 1/500 dilution of anti-ERK (Cell Signaling, #4695) and 1/500 dilution of anti-MEKK1 (Santa Cruz Biotechnology, SC-449). The membranes were washed four times with PBST or TBST and probed with 0.1 μg/ml of the corresponding Horseradish peroxidase (HRP)-conjugated secondary antibody. The protein bands were visualized with a Liquid DAB-black Substrate Kit. Control of protein loading was achieved by anti-β-actin Ab (1/10000) (Sigma) or anti-PCNA Ab (1/500) (Oncogene). In the immunoprecipitation assay, the cell lysates were preincubated with 2.5 μg of anti-FADD antibody (Santa Cruz Biotechnology, SC-56093) overnight at 4°C. The cell lysates were incubated with protein A-agarose at 4°C for 3 h. Protein A beads were washed with cold PBS four times, then resuspended in

Laemmli buffer (25 μl; 0.0625 M Tris-Hcl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue) and boiled. Proteins were detected using anti-procaspase 8 antibody.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from A549 cells and 5 μg of total RNA was subjected to an oligo dT primed reverse transcriptase reaction. Complementary deoxyribonucleic acid (cDNA) was subjected to a polymerase chain reaction (PCR). The primers used for IL-8 amplification were: 5′-GCAGCTCTGTGTGAAGGTGCA-3′and 5′-GAATTCTCAGCCCTCTTCAA-3′. The conditions for PCR amplification of IL-8 were as follows: starting at 94°C for 5 min, followed by 28 cycles at 94°C for 30 s, 58.5°C for 30 s, 72°C for 40 s and a final elongation step at 72°C for 10 min. Before PCR amplification with each primer pair, an aliquot of the cDNA was amplified with β-actin primers to determine the equivalent amounts of RNA. All PCR products were separated by electrophoresis on 2% (wt/vol) agarose gels and quantified by Quantity One analysis software (Bio-Rad Laboratories, Richmond, CA).

Nuclear Extraction. Nuclear extracts from treated A549 cells were prepared at different times. Cells were scraped from the culture dish and washed with cold PBS. Pellets were resuspended in 500 μl of cell lysis buffer (10 mM Hepes-KOH (pH 7.9) at 4°C, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF and 10 mM KCl), incubated on ice for 15 min, and then vortexed for 15 s. Cell lysates were centrifuged at 15000 × *g* at room temperature for 10 s to collect supernatants which were considered as constituting cytosol. The pellet was resuspended in 100 μl of cold nuclear lysis buffer (20 mM Hepes-KOH (pH 7.9), 25% glycerol, 420 nM NaCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF and 1.5 mM $MgCl_2$), incubated on ice for 20 min and then centrifuged at 15,000 × *g* for 10 s to remove the nuclear debris. Both cytosolic and nuclear supernatants were used to detect NF-κB using Western blotting.

Short Interfering RNA (siRNA) Preparation. The expression of short hairpin RNA is described elsewhere (17). The pSUPER/enhanced green fluorescent protein (EGFP) vector was kindly provided by R. Agami (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The pSUPER/EGFP vector was digested with BglII and HindIII and the oligonucleotides (5′-gatccccGTTCCTGAGCCTGGACTACttcaagagaGTAGTCCAGGCTCAGGAACtttttggaaa-3′ and 5′-agcttttccaaaaaGTTCCTGAGCCTGGACTACtctcttgaaGTAGTCCAGGCTCAGGAACggg-3′; the lower case letters at 5′ and 3′ ends represent restriction enzyme cutting sites for construction and the lowercase letters in the middle sections of the sequences represent the spacers for siRNA to form a loop structure) (17) were ligated into the vector. For transfection, cells (5×10^6) were washed with serum-free DMEM and incubated in a medium containing 2.5 μg DNA and 5 μl of Lipofectamine 2000. After 24 h incubation, the medium was removed and cells were maintained in DMEM supplemented with 10% heat inactivated fetal calf serum for an additional 24 h before the assay. The EGFP-positive cells were sorted by FACSAria (Becton Dickinson) with a purity ranging from 72% to 76% in different experiments.

For the FADD and MEKK1 silencing assay, cells were transfected with control siRNA (Santa Cruz Biotechnology), FADD siRNA (Santa Cruz Biotechnology) or MEKK1 siRNA (Santa Cruz Biotechnology), according to the manufacturer's protocol. A549 cells at a density of 2×10^5/well were seeded into medium without antibiotics 1 day before transfection. siRNA was mixed with siTransfection medium (Santa Cruz Biotechnology) in one tube and siTransfection reagent (Santa Cruz Biotechnology) to siTransfection medium in another tube and incubating the contents of the tubes at room temperature for 10 min. After incubating, the mixtures were combined and incubated at room temperature for 30 min. After 30 min, the medium covering the cells was replaced with fresh medium without antibiotics and the mixtures were added to the cells. The cells were incubated for 24 h. To verify that the siRNA was reducing the expression of protein, Western blotting was performed as described above.

Reporter Assay. Cells were transfected with firefly luciferase reporter plasmids 2xTRE-Luc and −133-Luc, 100 ng or NF-κB-Luc, 50 ng and pRL-TK carrying Renilla luciferase cDNA (10 ng as an internal control) using the Lipofectamine 2000 (Invitrogen). In other assays, cells were cotransfected with siRNA plasmids. The total amount of transfected DNA per culture was kept constant within each experiment. After 24 h transfection, cells were treated with SPE B or G308S for 4 h and then harvested for luciferase activity using the Dual-Luciferase Reporter Assay (Promega) in a LB9501 Lumat luminometer (Berthold, Bad Wildbad, Germany). Luciferase activity was normalized with the Renilla luciferase activity.

Statistical Analysis. Statistical analysis was done using Student's *t* test or one way ANOVA and then Tukey's post test. Statistical significance was set at $P < 0.05$.

Results

The Production of IL-8. ELISA assay was used to measure the level of IL-8 in the conditioned medium of A549 cells treated with various concentrations of SPE B or G308S for 6 h. The level of IL-8 secretion was significantly elevated in the medium in the presence of SPE B (Fig. 1A) or G308S (Fig. 1B) in a similar pattern, reaching a peak at 10 μg/ml and then decreasing slightly at 20 μg/ml. The maximal IL-8 production stimulated by SPE B or G308S was about 4 times that of the control. We have previously reported that there are two receptors for SPE B, $\alpha_v\beta_3$ and Fas (8). Antibodies against these two receptors were then used to evaluate which receptor is responsible for IL-8 production by SPE B. The results showed that the IL-8 production induced by SPE B or G308S was inhibited by anti-Fas antibody but not by anti-$\alpha_v\beta_3$ antibody.

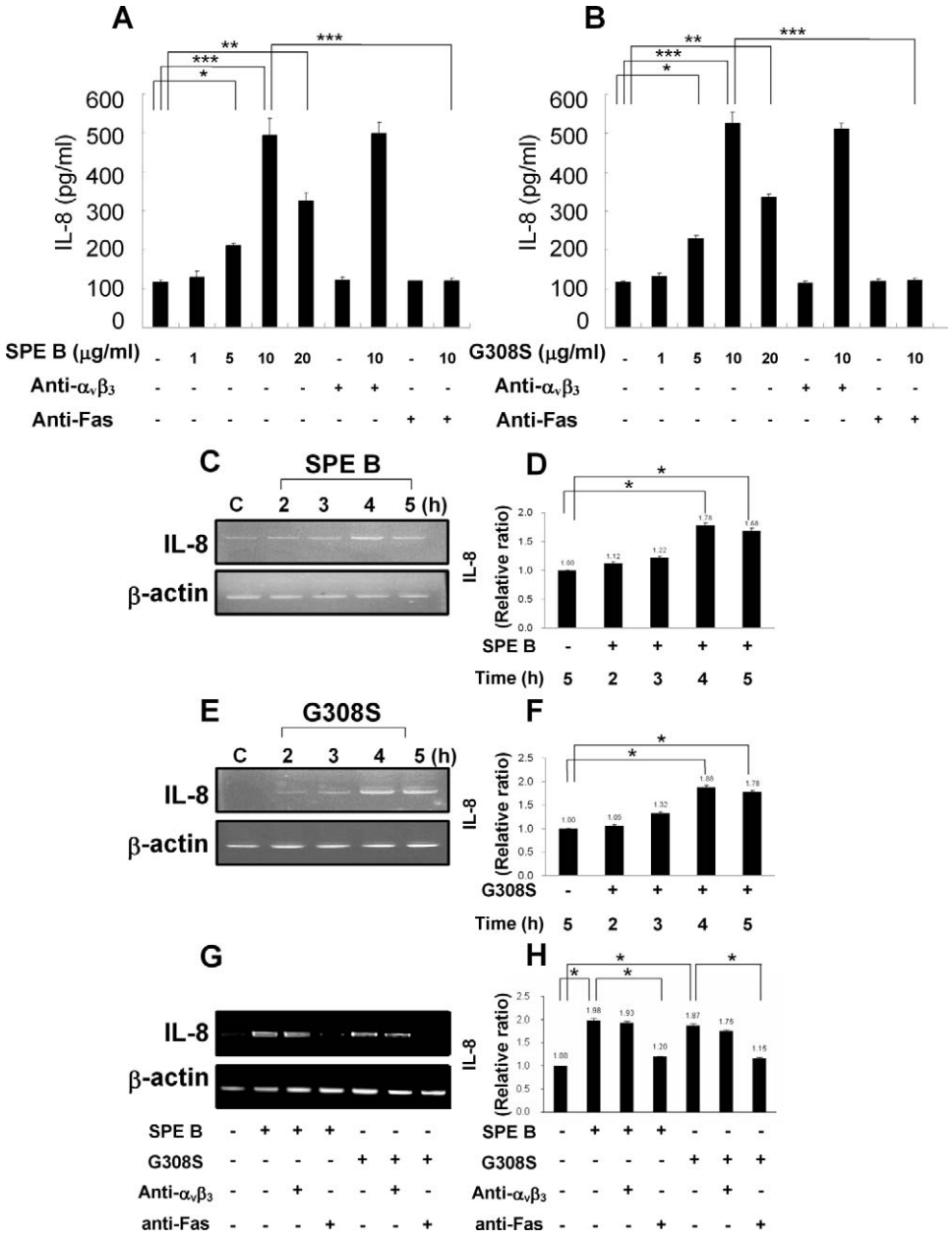

Figure 1. SPE B/G308S induces IL-8 production through FAS receptor. Cells were treated with different concentrations of SPE B (A) or G308S (B) in the presence or absence of anti-$\alpha_v\beta_3$ antibody (4 μg/ml) (Santa Cruz Biotechnology, SC-7312) or anti-Fas antibody (0.5 μg/ml) (Santa Cruz Biotechnology, SC-715) for 6 h, and then the conditioned medium was collected and used for the analysis of IL-8. The level of IL-8 was determined by ELISA kit. Results are the mean ± SEM of three independent experiments. *, **, *** denote significance levels of 0.05, 0.01 and 0.001, respectively. Cells (3×10^6) were treated with SPE B (10 μg/ml) (C) or G308S (10 μg/ml) (E) for 2, 3, 4 and 5 h or with PBS for 5 h. Cells (3×10^6) were also treated with SPE B or G308S (10 μg/ml) in the presence of anti-Fas antibody (0.5 μg/ml) or anti-$\alpha_v\beta_3$ antibody (4 μg/ml) (G) for 5 h. Total RNA was prepared from A549 cells and subjected to RT-PCR analysis of IL-8 mRNA expression. The cDNA was amplified with specific oligos for IL-8 and β-actin. Each band was quantified by densitometric analysis, a quantitative result of IL-8 vs. β-actin is expressed as relative ratio (D, F and H); the ratio of IL-8 to β-actin in control sample is defined as 1, and the ratios of each IL-8 to β-actin in other time points are compared to control, and their ratios are presented. Results are the mean ± SEM of three independent experiments. * denotes significance level of 0.05.

The Level of IL-8 mRNA Is Up-Regulated in Response to SPE B or G308S Stimulation. The mRNA level of IL-8 in response to SPE B or G308S treatment was then evaluated. The time course analysis indicated that the mRNA level of IL-8 increased in both SPE B- or G308S-treated cells 4 to 5 h after treatment (Figs. 1C, 1D, 1E and 1F, respectively). Pretreatment of cells with anti-Fas antibody, but not by anti-$\alpha_v\beta_3$ antibody, decreased the mRNA level of IL-8 to the control level (Figs. 1G and 1H).

NF-κB Is Activated by G308S. Results of last two experiments (Figs. 1G and 1H) established that the SPE B-induced IL-8 production is mediated through Fas receptor. The more specific ligand G308S was used for other

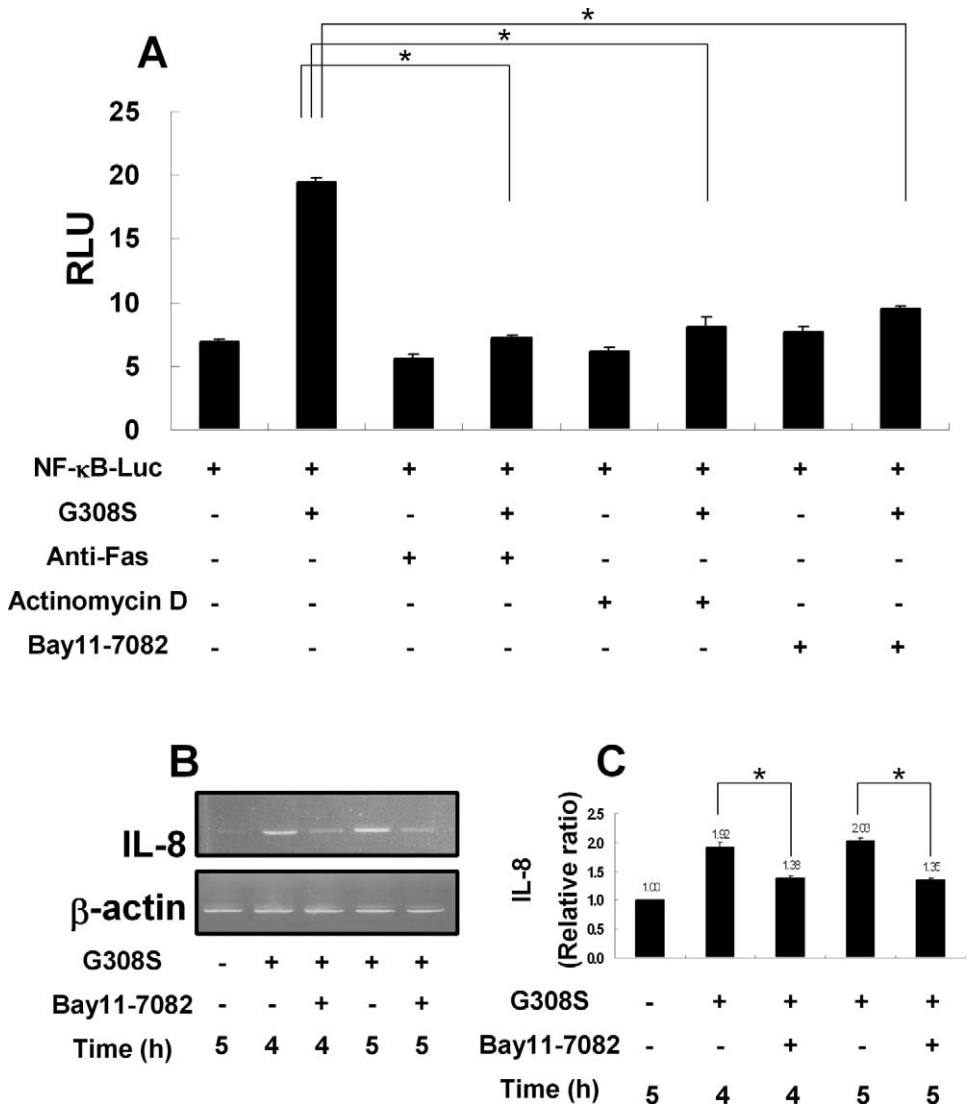

Figure 2. NF-κB activity regulates IL-8 expression in response to SPE B stimulation. Cells (2×10^5) were transfected with NF-κB reporter and treated with G308S (10 μg/ml) in the presence of NF-κB inhibitor, Bay 11–7082 (10 μM), actinomycin D (0.1 μg/ml) or anti-Fas antibody (0.5 μg/ml), and their relative luciferase activities were determined (A). Data are the means ± SEM of triplicate cultures. * denotes $P < 0.05$. Cells (3×10^6) were also treated with G308S (10 μg/ml) in the presence or absence of NF-κB inhibitor (10 μM) for 4 or 5 h (B). Total RNA was prepared from A549 cells and subjected to RT-PCR analysis. The cDNA was amplified with specific oligos for IL-8 and β-actin. Quantitative analysis of mRNA expression was carried out as described in the legend of Figure 1 and presented in (C).

experiments to evaluate the downstream pathway. Since NF-κB has been reported to have a positive role in IL-8 gene expression (10, 13), its role in SPE B-induced IL-8 was studied. The reporter assay for NF-κB showed that the luciferase activity increased in response to G308S treatment, and was inhibited by anti-Fas antibody, actinomycin D or Bay11–7082 (an IKK inhibitor) (Fig. 2A). Consistent with this result, the mRNA level of IL-8 was also down-regulated in the presence of IKK inhibitor (Figs. 2B and 2C). These experiments indicate that NF-κB is involved in G308S-induced IL-8 expression in a Fas-dependent manner.

The FADD and Caspase 8 Are Involved in G308S-Induced Expression of IL-8 mRNA. Our previous study showed that G308S could activate caspase 8 through Fas receptor (8). It has been reported that Fas-induced NF-κB signaling is a scaffolding-related function of caspase 8 in nonapoptotic Fas signaling. To study whether caspase 8 and FADD are involved in IL-8 production, A549 cells were transfected with FADD siRNA or procaspase 8 siRNA and expression of IL-8 mRNA was determined. The effectiveness of siRNA constructs was first evaluated by analyzing the expression of procaspase 8 and FADD. The result showed that the expression of procaspase 8 and FADD was significantly inhibited by their corresponding siRNAs (Fig. 3A). The immunoprecipitation showed that in the presence of G308S, FADD was associated with procaspase 8, and that this interaction was blocked by the siRNA of either FADD or of procaspase 8 (Fig. 3B), but not by their corresponding controls. When the immunoprecipitation experiment was carried out reversely—cells lysates

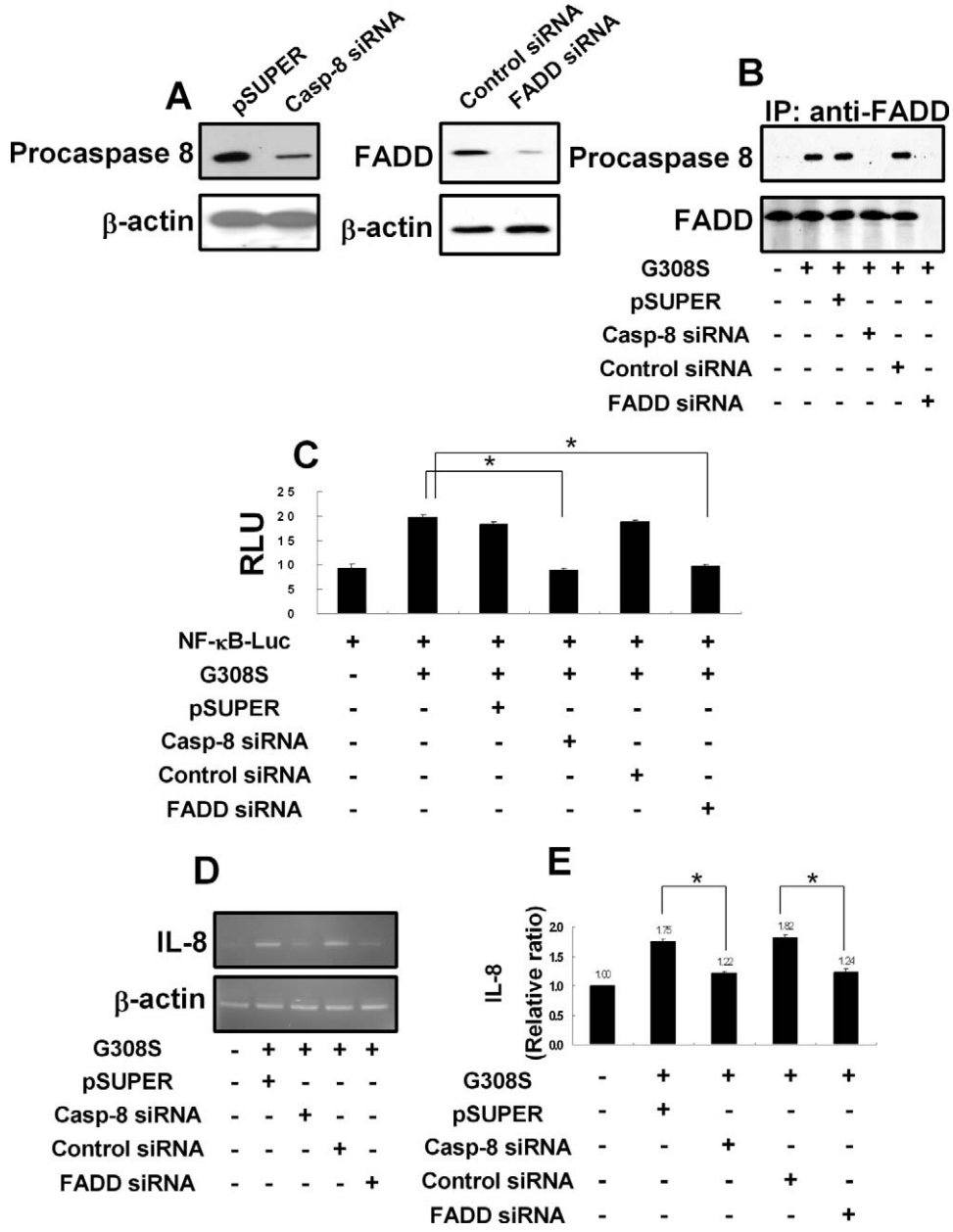

Figure 3. Inhibition of caspase 8 and FADD impairs the SPE B-induced NF-κB activation and IL-8 mRNA expression. Cells were transfected with siRNA of procaspase 8 and of FADD as described in "Materials and Methods". After transfection, the expression of procaspase 8 and FADD in A549 cells were analyzed by Western blotting (A). The transfected cells (2×10^5) were also incubated with G308S (10 μg/ml) in the presence of various siRNAs or PBS for 30 min. Total cell lysates were then immunoprecipitated with an anti-FADD antibody and immunoblotted with anti-procaspase 8 antibody. The membrane was then reprobed with an anti-FADD antibody (B). After transfection with NF-κB reporter and various siRNAs, cells (2×10^5) were treated with G308S (10 μg/ml) and the luciferase activity was measured. Data are the means ± SEM of triplicate cultures. * denotes $P < 0.05$ (C). The expression of IL-8 mRNA from transfected cells was also determined after incubation with G308S (10 μg/ml) or PBS for 5 h, total RNA was prepared from A549 cells and subjected to RT-PCR analysis of IL-8 mRNA expression. The cDNA was amplified with specific oligos for IL-8 and β-actin (D). Quantitative analysis of mRNA expression was carried out as described in the legend of Figure 1 and presented in (E).

were immunoprecipitated with an anti-procaspase 8 antibody and immunoblotted with anti-FADD antibody, similar results were observed (data not shown). The reporter assay for NF-κB showed that the luciferase activity was down-regulated in the presence of the siRNA of either FADD or of procaspase 8 (Fig. 3C). Similarly, the mRNA level of IL-8 increased in the presence of G308S, and decreased in the presence of FADD siRNA or procaspase 8 siRNA (Figs. 3D and 3E). These results reveal that FADD/caspase 8 is involved in G308S-induced NF-κB activation.

The Catalytic Activity of Caspase 8 Is Required for the G308S-Induced NF-κB Activation. We next investigated whether the catalytic activity of caspase 8 is required for G308S-induced NF-κB activation. As shown in

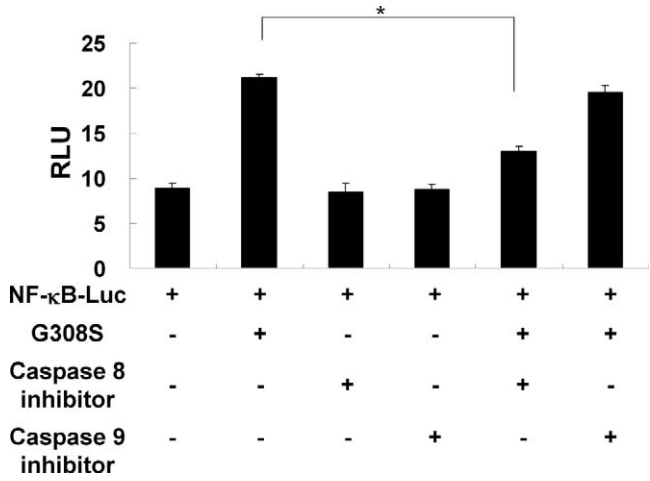

Figure 4. The catalytic activity of caspase 8 is required for G308S-induced NF-κB activation. After transfection with NF-κB reporter, cells (2×10^5) were treated with G308S (10 μg/ml) in the presence or absence of inhibitors (caspase 8 inhibitor, Z-IETD-FMK, 10 μM or caspase 9 inhibitor, Z-LEHD-FMK, 10 μM) and the luciferase activity was measured. Data are the means ± SEM of triplicate cultures. * denotes $P < 0.05$.

Figure 4, luciferase activity was increased in the presence of G308S and blocked by caspase 8 inhibitor, but not by non-caspase 8 inhibitor (caspase 9 inhibitor).

The Participation of ERK in SPE B-Induced IL-8 Production. Our previous study indicated that SPE B could activate ERK and p38, but not JNK in A549 cells (unpublished data). The level of IL-8 in the conditioned medium of cells treated with G308S in the presence or absence of ERK or p38 inhibitor for 6 h was determined. The results showed that the level of IL-8 in the conditioned medium was decreased in the presence of ERK inhibitor (PD98059), but not of p38 inhibitor (SB203580) after G308S treatment (Fig. 5A). We also found that G308S stimulated the phosphorylation of p38 and ERK in A549 cells, which was inhibited by SB203580 and PD98059, respectively. Only ERK but not p38 phosphorylation was blocked by caspase 8 inhibitor (Fig. 5B), suggesting that caspase 8 is acting on the upstream of ERK. The mRNA level of IL-8 was stimulated by G308S and down-regulated by ERK inhibitor (Figs. 5C and 5D).

IκBα, while bound to NF-κB in the resting state, undergoes phosphorylation by I-κB kinase in the activation process, dissociates from NF-κB, and is finally degraded by proteasome. To determine how ERK is involved in this process, the effect of PD98059 on the status of IκBα in the cytosol and on the translocation of NF-κB from cytosol to nucleus was evaluated. As shown in Figure 5E, in the presence of G308S, IκBα disappeared gradually from cytosol and reached undetectable levels at 120 min post-treatment. The process was inhibited by PD98059. Furthermore, the translocation of NF-κB into the nucleus was also inhibited by ERK inhibitor (Fig. 5F). The reporter assay for NF-κB also showed that the luciferase activity was enhanced by G308S and inhibited by PD98059 (Fig. 5G). These results indicate that ERK is acting on the upstream of NF-κB.

MEKK1 Is Acting Between Caspase 8 and ERK in G308S-Induced IL-8 Production. Next, we investigated whether caspase 8 is responsible for the cleavage of MEKK1 in response to G308S treatment. The results showed that a cleaved fragment of MEKK1 was observed after G308S treatment and that the cleavage was blocked by procaspase 8 siRNA (Fig. 6A). To further address whether caspase 8 is binding to MEKK1, an immunoprecipitation experiment was carried out. The result in Figure 6B showed that in the presence of G308S, caspase 8 was coprecipitated with MEKK1 and this association was inhibited by MEKK1 siRNA. When the experiment was carried out in a reverse fashion by immunoprecipitating total cell lysates with an anti-caspase 8 antibody and immunoblotting with anti-MEKK1 antibody, a similar result was found (data not shown). The phosphorylated ERK induced by G308S was also blocked by the MEKK1 siRNA (Fig. 6C). These results suggest that MEKK1 is acting between caspase 8 and ERK. In addition, the luciferase assay demonstrated that G308S-induced NF-κB activation was inhibited by MEKK1 siRNA (Fig. 6D), and the mRNA level of IL-8 induced by G308S was also down-regulated by MEKK1 siRNA (Figs. 6E and 6F).

Discussion

IL-8, a central mediator of inflammation, can be produced by different cell types, including macrophages, neutrophils and epithelial cells, in response to various stimuli, such as cytokines, endotoxin and bacterial products (18). *Streptococcus pyogenes*-infected epithelial cells induce the secretion of pro-inflammatory chemokines through NF-κB and MAP kinase signaling pathways (4), and *Clostridium difficile* toxin A-stimulated intestinal epithelial cells activate AP-1 to upregulate the production of IL-8 through the Ras/MAPK cascade (19). Here we show that the SPE B-induced IL-8 production is mediated through Fas, ERK and the NF-κB pathway.

We previously identified integrin $\alpha_v\beta_3$ and Fas as receptors for SPE B-induced apoptosis (8). SPE B interacts with both integrin $\alpha_v\beta_3$ and Fas, while G308S reacts with Fas only. Although Fas is a well known death receptor, it has also been demonstrated in a wide variety of cell types that express IL-8 in response to Fas ligation. For example, the Fas/FasL pathway in bronchiolar epithelial cells leads to IL-8 expression, and promotes the inflammatory cascade (20); also, the Fas-Fas ligand system in human Glioma cells may be involved in apoptosis and IL-8 expression (21). In this study the IL-8 production and gene expression were increased to a similar extent by both SPE B and G308S, and this increase was nullified by the addition of anti-Fas antibody but not by anti-$\alpha_v\beta_3$ antibody. Therefore, it is confirmed that SPE B-induced IL-8 production is mediated only through the Fas receptor.

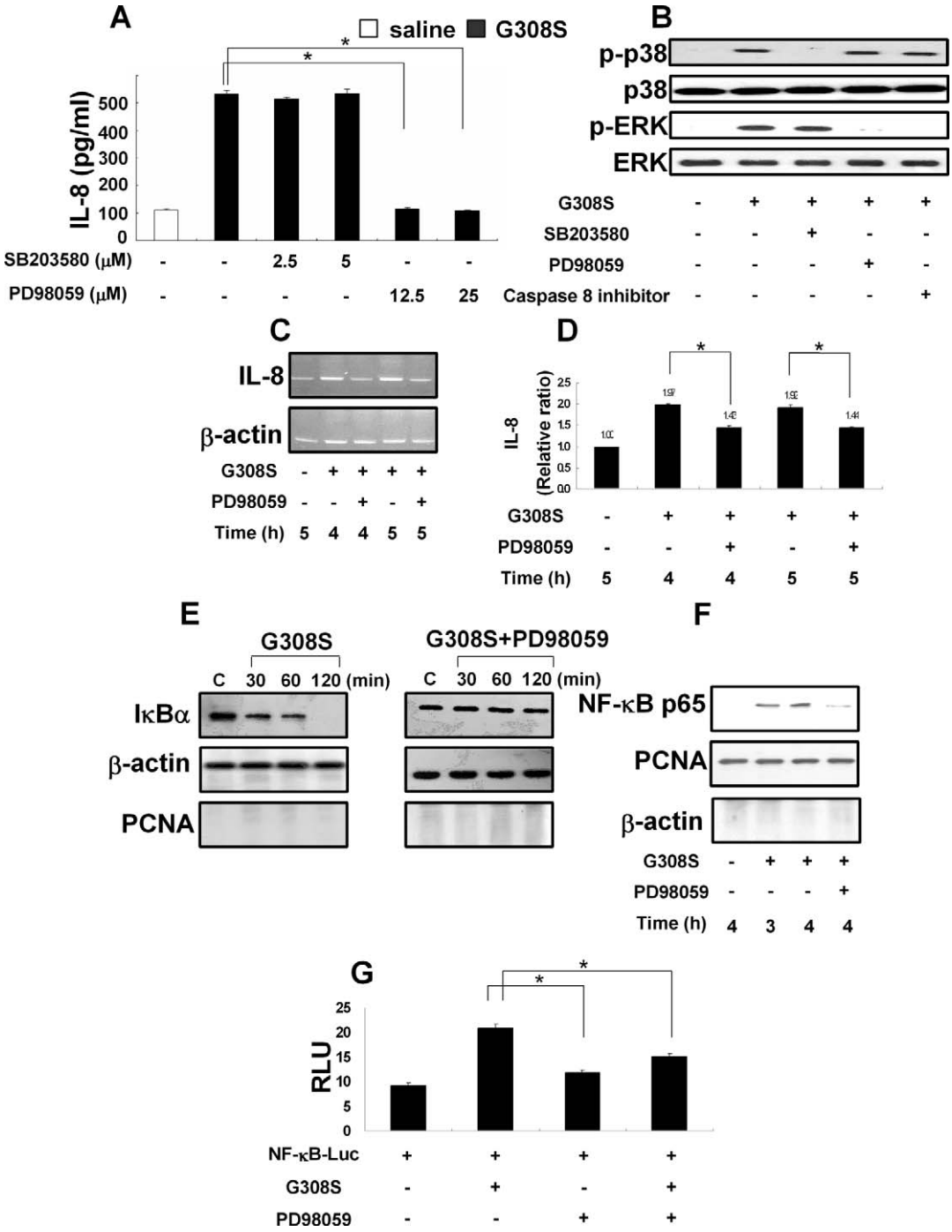

Figure 5. ERK activation by caspase 8 is involved in G308S-induced NF-κB activation. Cells were treated with G308S (10 μg/ml) in the presence or absence of inhibitors (p38 inhibitor or ERK inhibitor) for 6 h and then the conditioned medium was collected and saved for the analysis of IL-8. The level of IL-8 was determined by ELISA kit (A). Cells were treated with G308S (10 μg/ml) in the presence or absence of inhibitors (p38 inhibitor, 2.5 μM; ERK inhibitor, 12.5 μM or caspase 8 inhibitor, Z-IETD-FMK, 10 μM) for 2 h, and cell lysates were immunoblotted using antibodies against phosphorylated or total p38 and ERK (B). For the gene expression of IL-8, cells (3×10^6) were treated with G308S (10 μg/ml) in the presence or absence of PD98059 (12.5 μM) for 4 or 5 h. Total RNA was prepared from A549 cells and subjected to RT-PCR analysis. The cDNA was amplified with specific oligos for IL-8 and β-actin (C). Quantitative analysis of mRNA expression was carried out as described in the legend of Figure 1 and presented in (D). Cells (2×10^5) were incubated with G308S (10 μg/ml) in the presence or absence of PD98059 (12.5 μM) for various times. Cytosol was prepared and probed with anti-IκBα antibody (E). The nuclear extracts were probed with an anti-NF-κB p65 antibody. The membrane was then reprobed with an anti-PCNA antibody (F). After transfection with NF-κB reporter, cells (2×10^5) were treated with G308S (10 μg/ml) with or without PD98059 (12.5 μM) and the luciferase activity was measured (G). Data are the means ± SEM of triplicate cultures. * denotes $P < 0.05$.

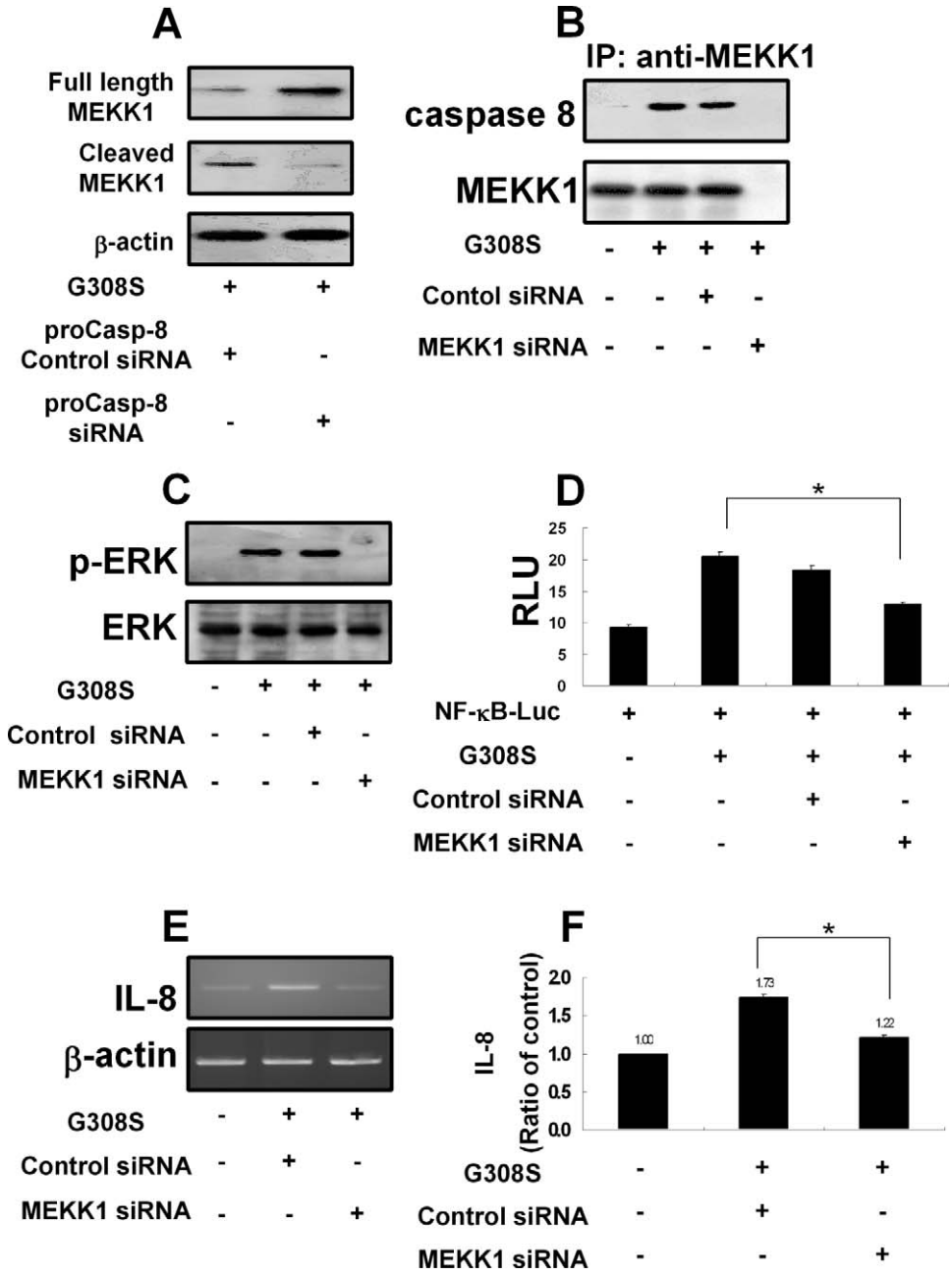

Figure 6. MEKK1 is acting between caspase 8 and ERK. Cells (2×10^5) were transfected with procaspase 8 siRNA and incubated with G308S (10 μg/ml) for 2 h. The cell lysate was probed with an anti-MEKK1 antibody (A). Cells (2×10^5) were also transfected with siRNA of MEKK1 and incubated with G308S (10 μg/ml) for 1.5 h. Total cell lysates were then immunoprecipitated with an anti-MEKK1 antibody and immunoblotted with anti-caspase 8 antibody (B). The membrane was then reprobed with an anti-MEKK1 antibody. In other experiments, cells (2×10^5) were transfected with MEKK1 siRNA and incubated with G308S (10 μg/ml) for 3 h. The cell lysate was probed with an anti-p-ERK antibody. The membrane was then reprobed with an anti-ERK antibody (C). For luciferase assay, cells (2×10^5) transfected with NF-κB vector, or MEKK1 siRNA or a combination of both were incubated with G308S (10 μg/ml) and the luciferase activity was measured (D). Data are the means ± SD of triplicate cultures. * denotes $P < 0.05$. After transfection with MEKK1 siRNA, cells (3×10^6) were treated with G308S (10 μg/ml) or PBS for 5 h. Total RNA was prepared from A549 cells and subjected to RT-PCR analysis (E). The cDNA was amplified with specific oligos for IL-8 and β-actin. Quantitative analysis of mRNA expression was carried out as described in the legend of Figure 1 and presented in (F).

The promoter of the IL-8 gene has two AP-1 binding sites and one NF-κB binding site. It has been reported that the NF-κB element is required for all cell types and that AP-1 is not necessary for transcriptional activation of IL-8 in some cells (22). In our study of A549 cells, the luciferase activity of AP-1 was not affected by the G308S treatment (data not shown), suggesting that AP-1 is not involved in the SPE B (G308S)-induced IL-8 production.

Activation of NF-κB is involved in the IL-8 production induced by Fas ligand (10). Fas-mediated non-apoptotic signaling in A549 cells was further demonstrated in our study, as SPE B was able to induce IL-8 gene expression

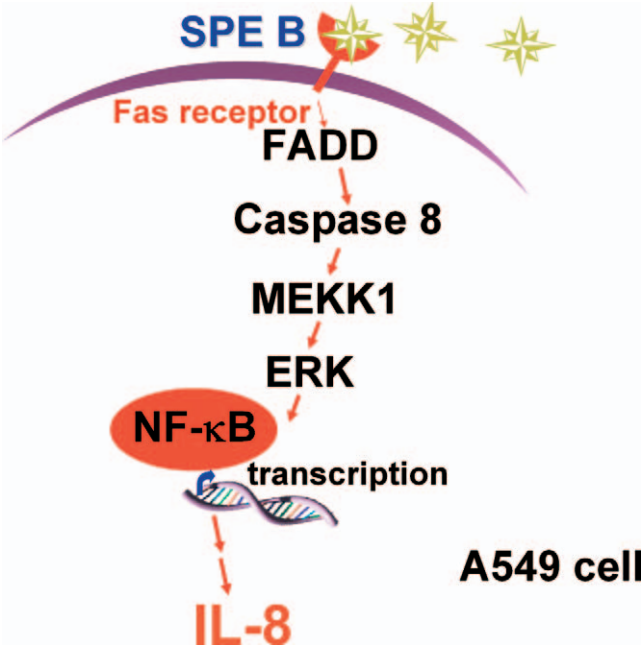

Figure 7. The SPE B-induced IL-8 secretion in A549 cell presented in this study is summarized in this figure. Binding of SPE B to Fas receptor, activated FADD, caspase 8, MEKK1, ERK and the NF-κB signal pathway, which up-regulates IL-8 production. A color figure is available in the online version of the journal.

and increase the transcriptional activity of NF-κB that was inhibited by anti-Fas antibody. These results indicated that G308S-induced expression of IL-8 mRNA in A549 cell is mediated through Fas receptor and NF-κB activation.

In the apoptotic process, the activated Fas is able to recruit the cytoplasmic death domain-containing adaptor protein FADD (Fas-associated death domain protein) that in turn recruits and activates procaspase 8 to trigger apoptosis. In addition to the apoptotic capability of Fas/FasL, FasL also possesses a nonapoptotic function in inflammatory responses. Fas-activated NF-κB, mediated through FADD and caspase 8, has been found in HT1080 cells (11), and FasL induced cell-autonomous NF-κB activation and IL-8 expression, mediated by FADD and caspase 8, have been reported in HEK 293 cells (10). In this study, A549 cells transfected with FADD siRNA or procaspase 8 siRNA could down-regulate NF-κB luciferase activity and the mRNA level of IL-8. These results suggest that FADD and caspase 8 play important roles in SPE B (G308S)-induced IL-8 gene expression. Similar results were also observed in other cell types.

It has also been reported that three MAPK pathways play important roles in promoting IL-8 gene expression (22). Inhibition of ERK and JNK, but not p38, decreases TNF-α-induced IL-8 gene expression in human airway epithelial cells (A549 cells) (23). Fas ligation on human glioma cells requires signals of ERK and p38 for IL-8 gene expression (21). Thrombin elicited IL-8 secretion is conducted by ERK and p38 signal pathways in human dermal fibroblasts (6). It is apparent that different cell types require a different MAP kinase for the IL-8 gene expression in response to different stimuli. Our previous study showed that SPE B induced the up-regulation of the active form of ERK and p38, but not JNK, in A549 cells (unpublished data). Our results in this study showed that the G308S-induced IL-8 production was inhibited by PD98059, an inhibitor of ERK phosphorylation. In addition, the G308S-induced activation process of NF-κB, including the disappearance of IκBα and the translocation of NF-κB into the nucleus, and NF-κB activity were inhibited by pretreatment of cells with PD98059. These results indicate that ERK plays an important role in the SPE B (G308S)-induced IL-8 gene expression. When cells were transfected with siRNA of MEKK1, the G308S-induced phosphorylation of ERK, and NF-κB activity were inhibited, suggesting that MEKK1 is acting on the upstream of ERK.

In conclusion (Fig. 7), we found that, following SPE B binding to its receptor Fas, the up-regulated IL-8 expression in A549 cells is mediated by FADD, the activation of caspase 8, MEKK1, ERK and the NF-κB. We hope the information on the cross-talk between MAPK and NF-κB signaling in lung epithelial cells may therefore help the understanding of interaction between SPE B and lung epithelial cells.

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