Differential regulation of ERK1/2 and p38 MAP kinases in VacA-induced apoptosis of gastric epithelial cells

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Submitted 20 June 2007; accepted in final form 19 December 2007

VacA and recombinant VacA (rVacA). Activation of p38 MAPK and Bax dimerization by rVacA were increased in a dose-dependent manner. rVacA-induced ERK1/2 MAPK activation was maximal at 30 min and 4 h and 1–4 μg/ml of rVacA. rVacA-induced SOD-1 expression was considerably diminished by inhibiting ERK1/2 MAPK and it was slightly increased by inhibiting p38 MAPK. rVacA increased or decreased villin expression depending on dose and exposure time and its expression was mainly appeared in the contractile actin ring of the dividing cells. Despite its cytoprotective effect, SB-203580, a p38 inhibitor, was unlikely to reduce VacA-induced Bax dimerization and rather inhibited villin and Bcl2 expression, indicating that p38 may also play a role in cell proliferation or differentiation for survival after VacA intoxication. Furthermore, p38 inhibitor accelerated rVacA-induced cell death after exposure of AGS cells to H2O2 but ERK1/2 inhibitor protected cells from H2O2 insult. These results suggest that SOD-1 and villin are expressed differentially upon VacA insult depending on dose and exposure time via ERK and p38 MAP kinases; decrease in SOD-1 and villin expression coupled with Bax dimerization leads to apoptosis of gastric epithelial cells.

Helicobacter pylori; VacA; SOD-1; villin; MAP kinase signaling

VacA and recombinant VacA (rVacA) strains, induces large cytoplasmic vacuoles in epithelial cells with structural and functional changes, leading to apoptosis that is, in part, responsible for H. pylori-induced epithelial cell damage (13, 31, 48). Purified VacA and recombinant VacA (rVacA) induced apoptosis (21, 40) that was via a mitochondria-dependent pathway, stimulating cytochrome c release (28, 65). To the contrary, Caputo et al. (18) reported that VacA was associated with H. pylori-induced VEGF upregulation, which depends on the activation of the EGFR and MAP kinase cascade. The exclusive role of VacA in apoptosis, therefore, may be questionable.

Epidemiological studies have shown that gastric cell damage is associated with disruption of cytoskeletal structure (26, 62) and reactive oxygen species (ROS) (10, 16). The protection of cells against them is a pivotal mechanism for cell survival. In the meanwhile, the gastritis induced by H. pylori infection stimulates the generation of ROS by the inflammatory cells present in the mucosa, resulting in gastric cell apoptosis which is strongly associated with atrophic gastritis and gastric cancer (20, 39, 44, 57, 71). By multivariate logistic regression analysis, the association of VacA with risk of glandular atrophy and intestinal metaplasia was increased in H. pylori-positive mucosa in our unpublished data. Thus we need to investigate how VacA contributes to the pathogenesis of gastric mucosa after H. pylori infection in relation to MAP kinase pathways that mediate proliferation, differentiation, apoptosis, and stress (9, 17, 23, 36, 72).

Superoxide dismutases (SOD) are essential enzymes that eliminate superoxide radical and hence protect cells from damage induced by free radicals (33). Loss of SOD-1 leads to severe damage of mitochondria in neuroblastoma cell (5), senescence in cultured fibroblasts, and apoptotic cell death in HeLa cells (12) as well as in spinal neurons (59). Villin, an actin-binding protein, is an important marker of the preneoplastic cell type and induces enhanced motility and remodeling of the actin cytoskeleton contributing to the wound healing and cell proliferation (56). Deficiency for the villin gene in mice can organize microvilli in brush border but limits the severing of actin filaments in response to cellular injury, resulting in a deficiency in wound repair and cell motility, therefore leading to cell death in response to chronic injury (8, 51).

Herein, we show that the activation of the MAP kinase cascade on VacA stimuli could subsequently trigger changes in SOD-1 and villin expression and affect cellular apoptosis and proliferation.

MATERIALS AND METHODS

Helicobacter pylori strains and culture conditions. Helicobacter pylori ATCC 49503 (strain 60190) and ATCC 43504 (NCTC 11637), wild-type, cytotoxic, cagA-positive strains with the vacA genotype s1/m1, and ATCC 51932 (T30a), a wild-type, cagA-negative with the vacA genotype s2/m2 (6), were purchased from American Type Culture Collection (Manassas, VA). H. pylori were inoculated on Mueller-Hinton agar plates supplemented with 5% horse serum. The plates were incubated for 2–3 days at 37°C in a humidified 5% CO2 incubator.

Cell line culture conditions. AGS human gastric epithelial cells (KCLB 21739) purchased from Korean Cell Line Bank (KCLB) were grown in RPMI 1640 medium supplemented with 10% FBS (Invitro-
gen), 1% penicillin-streptomycin, and 1% antibiotic-antimycotic at 37°C in a 5% CO2 incubator.

Polymerase chain reaction and plasmid construction. Primers were designed to amplify a vacA fragment encompassing the mature VacA (43) from *H. pylori* strain ATCC 49503 (GeneBank accession no. U05676). The sequences of sense primer with an *Nde* site and antisense primer with a *Xho* site were 5' - ggaattcCATATGTTTTT-TACAAACCGTGATCA-3' and 5' -cgctCGAGACTCTAGCATGGA-3', respectively. The parameters for PCR were 94°C for 2 min, × 1; 94°C for 30 s, 45°C for 30 s, 68°C for 2 min, × 10; 94°C for 30 s, 45°C for 30 s, 68°C for 2 min + 5 s increase per cycle, × 20; 68°C for 7 min, × 1. The resulting PCR product was digested with *Nde* and *Xho* and ligated to the pET 41b (+) digested with the above-mentioned restriction enzymes to create plasmid pVAC953. The recombinant plasmid contains the vacA fragment encoding the mature *VacA* cytotoxin (amino acids 34 to 854, including the A34M mutation) in-frame with a 5' TAATACGACTCACTATAGGG-3' or T7 terminator (5' -GCTAGTTATGTCAGGCG-3') by a sequencing analyzer (Genotech, Daejeon, Korea).

Production of recombinant *VacA*. *E. coli* BL21 (DE3) was transformed by 1 ng of pVAC953 and kanamycin-resistant clones were selected. One colony bearing pVAC953 was inoculated into 5 ml of Luria Bertani broth containing 30 μg/ml of kanamycin and grown at 37°C overnight with shaking. The overnight cultures were diluted 1:100 into Terrific broth supplemented with 0.5% glucose and 0.02% NP-40, 0.2% glycerol, and 0.1 M NaCl, 5% glycerol, 0.05% NP-40, protease inhibitor cocktail (Complete mini, Roche Germany), 1 mM PMSF (Roche), and 0.5 mg/ml egg white lysozyme (Sigma). The bacterial suspensions were incubated for 30 min at room temperature and then treated with freezing at −70°C and thawing at 37°C three times. After three successive rounds of freeze-thaw, DNase, RNase, and MgCl2 were added to a final concentration of 20 μg/ml, 10 μg/ml, and 10 mM, respectively. The bacterial suspensions were incubated on ice for 30 min and then sonicated for 1 min (Sonic's & Materials, Vibra Cell, pulse: 2 s, Amp: 30 W). The bacterial lysate were centrifuged at 12,000 g for 10 min at 4°C to remove insoluble cell debris. The supernatant was used for purification using His-Bind affinity chromatography (Novagen). The purification of rVacA toxin was performed according to the manufacturer's protocol. Briefly, the first pool of fractions eluted with 0.5 M imidazole by His-Bind column chromatography were reloaded into another His-Bind column equilibrated with 20 mM Tris-HCl (pH 7.9) supplemented with 0.5 M NaCl, 0.02% NP-40, 0.2% glycerol, and 0.1 M imidazole. The eluate with 0.5 M imidazole by the second chromatography was dialyzed against PBS and used in this study as a recombinant vacuolating cytotoxin.

Production of the polyclonal anti-rVacA antibodies raised in rabbit. New Zealand White rabbits were immunized with intradermal injections of 50 μg of rVacA, mixed with Freund's adjuvant. Immunoglobulins were purified by using rProtein G-Agarose (GIBCO-BRL). This immunoglobulin recognized VacA in immunoblot not only with the ~89 kDa s1/m1 type but also with the ~92 kDa s2/m2 type.

Assay for vacuolating activity and cell viability. Vacuolating activity was assessed according to Cover et al. (22). Briefly, cells were seeded at 2–5 × 10^5 cells per well into 96-well plates and were grown overnight. The grown cells were washed with PBS and overlaid with a serum-free culture medium in the presence or absence of 10 mM NH4Cl to which H. pylori saline extract or H. pylori culture broth was added. Then cells were incubated for varying time at 37°C in a 5% CO2 incubator. If necessary, the cells were incubated with rVacA for varying time or with varying concentrations. After incubation for...
indicated time, cell vacuolation was examined by inverted light microscopy and quantified by a neutral red uptake assay. Cell viability was determined by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium, inner salt] assay (Promega) or the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay (Roche), according to the manufacturer’s protocols.

HSE and VacA enrichment by partial purification as nVacA sample. Saline extracts were prepared by harvesting bacteria (ATCC 49503) in 0.9% of NaCl from blood agar culture for 36 to ~48 h. The suspensions were gently vortexed and centrifuged at 4,000 rpm for 15 min at 4°C and then filtered with a 0.22-μm syringe filter. In some experiments, H. pylori saline extract (HSE) was preincubated for 1 h on ice with neutralizing anti-VacA antibody before addition to the cells. Alternatively, HSE was immunodepleted by preincubation for 1 h on ice with neutralizing anti-VacA sera or nonimmunized sera and protein A-Sepharose (Invitrogen). After removal of immune complexes by centrifugation, the supernatant was tested for residual activity. For partial purification of VacA, HSE was precipitated with ammonium sulfate at 50% saturation, centrifuged for 20 min, 4°C, at 12,000 rpm. The pellet was dissolved in 50 mM Tris-HCl (pH 8.0) and then dialyzed against two changes of 50 mM sodium phosphate (pH 6.0) at 4°C; reprecipitated materials were removed by centrifugation and loaded on CM-Sephadex (Pharmacia) preequilibrated in phosphate buffer (pH 6.0). After a column chromatography, VacA was mainly existed in unbound fractions which was devoid of albumin and used for native VacA, designated as “nVacA.” nVacA was immunodepleted of CagA by incubation with anti-CagA antibody C-300 bound to Protein A Sepharose (Santa Cruz Biotechnology) and then filter sterilized via a 0.2-μm filter.

AGS cells exposed to various H. pylori or VacA samples. Subconfluent monolayer of AGS cells on a six-well plate was cocultured for 5 h with H. pylori in the presence or absence of anti-rVacA antibodies in a serum-free medium containing 10 mM NH4Cl and then the cells were lysed in a 2 × SDS sample buffer. The effect of rVacA was assessed by AGS cells treated with indicated amounts of rVacA for various incubation periods in the presence or absence of anti-rVacA antibody or NH4Cl. If necessary, anti-VacA antibody, 5-nitro-2-(3-phenylpropyl-amino) benzoic acid (NPPB), PD-98059, or SB-203580 was pretreated for 30 min prior to addition of rVacA. The lysed cells were subjected to SDS-PAGE and immunoblot.

Immunoblot analysis. Samples were separated by SDS-PAGE, transferred to Immobilin-P membrane (Millipore). The membranes were blocked for 1 h with 3% BSA in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) followed by incubating with primary antibody at a appropriate dilution in TBST containing 3% BSA overnight at 4°C. After being washed three times with TBST for 10 min each, the membranes were incubated for 1 h with secondary antibody conjugated with horseradish peroxidase. Signals were amplified by the enhanced chemiluminescence system (Pierce) and exposed to X-ray film (Kodak).

Fig. 2. Comparative analysis of cytotoxicity of various VacA A: the effects of various VacA preparations on AGS cells were investigated. S2m2 VacA and s1m1 VacA were HSEs of ATCC 51932 and ATCC 49503, respectively. To get rid of VacA from HSE, s1m1 vacA HSE was immunodepleted by preincubation for 1 h on ice with neutralizing anti-VacA or nonimmunized rabbit antibody bound to protein A-Sepharose. After removal of immune complexes by centrifugation, immunodepleted HSE with anti-VacA antibody (VacA-reduced s1m1 HSE) or nonimmunized sera (s1m1 HSE+serum) was tested for residual activity. B: immunoblot analysis of phospho-ERK1/2 with 20 μg of AGS cells lysates after incubation for 2 h with the indicated VacA sample in the absence or presence of anti-VacA antibody. C: vacuolating activity depending on incubation time was measured by a neutral red dye uptake assay. D: a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation colorimetric assay was used to quantify living cells. Values represent relative cell viability as a percentage of cells treated with PBS and the means and SD from quadruplet samples. AGS cells were treated for 4, 8, and 20 h with PBS as control, PBS plus anti-VacA, s1m1 vacA HSE, s1m1 vacA HSE plus anti-VacA, s2m2 vacA HSE, VacA-reduced s1m1 vacA HSE (immunodepleted with anti-sera against rVacA), or s1m1 HSE+serum (immunodepleted with nonimmunized sera) in the presence of 10 mM NH4Cl. *P < 0.05, †P < 0.01 compared with control at indicated incubation time without anti-VacA antibody.
Immunoblot analysis of β-actin, Bax, Bcl-2, pERK1/2, ERK2, pp38, p38, SOD-1, β-tubulin, and villin were performed to determine expression levels of various H. pylori strains. The effect of H. pylori infection on the expression of β-actin, Bax, Bcl-2, pERK1/2, ERK2, pp38, p38, SOD-1, β-tubulin, and villin were assessed by AGS cells treated with various VacA samples with respective antibodies: anti-β-actin, anti-Bax, anti-Bcl-2, anti-ERK2, and anti-p38 (Santa Cruz Biotechnology), anti-villin (Chemicon), anti-Cu/Zn SOD (Stressgene), anti-pERK1/2, anti-pp38 (Cell Signaling), and anti-β-tubulin (Sigma).

DAPI staining. The induction of apoptosis of cells by rVacA was assessed by DAPI (4',6-diamidino-2-phenylindole) (Roche-Boehringer, Mannheim, Germany) staining. In brief, the cells on a slide glass were fixed with 4% paraformaldehyde followed by washing with PBS and staining with a 1 μg/ml DAPI solution for 10 min. The slides were then viewed under a fluorescence microscope (Nikon).

FITC-immunofluorescence staining of AGS cells. rVacA with or without anti-VacA antibody-treated AGS cells were washed in PBS, fixed with cold acetone for 10 min at room temperature, and then washed in PBS three times. Anti-villin antibody (1:50) was incubated for 1 h at room temperature and washed in PBS three times followed by incubation with the FITC-conjugated anti-mouse secondary antibodies (National Veterinary Research & Quarantine Service, Korea) for 30 min at 37°C. After the washings, the coverslips were mounted on the glass microscope slide with a drop of 50% glycerol PBS solution (pH 8.0). The cells were analyzed with an Olympus fluorescence microscope.

Microscopic imaging. Cells were visualized with TMS phase-contrast inverted microscopes (Nikon). Images were edited via Adobe Photoshop 7.0 and Power Point.

RESULTS

Comparative analysis of the activity of native and recombinant VacA inducing vacuolation and apoptosis. After incubation for 8 h, rVacA increased neutral red uptake in AGS cells in a dose-dependent manner up to 0.8 μg/ml but decreased at higher concentration (Fig. 1A). After AGS cells were incubated with indicated concentrations of rVacA for 24 h in serum-free media, cell viability was measured according to the ability of living cells to reduce the uncolored MTS substrate to a colored product (Fig. 1C). As shown in Fig. 1C, rVacA induced mild dose-dependent apoptosis in AGS cells. To compare the activity of rVacA to that of the native version, the vacuolation and apoptosis by native protein with HSE were assessed (Fig. 1, B and D). The concentration of VacA in HSE was determined from the rVacA standard by antigen-capture ELISA. The concentration of VacA in HSE corresponded to ~1% of the total protein of HSE (data not shown). As shown in Fig. 1B, however, the vacuolating activity of 50 μg/ml of HSE coincided with that of 0.8 μg/ml of rVacA. From the result, the activity of rVacA was supposed to be ~60% of that of nVacA. While the vacuoles induced by nVacA were formed less than 4 h after addition of HSE and maintained during incubation for 10–20 h, and filled the entire cytoplasm, those induced by rVacA were formed slowly, somewhat defective and localized at the late endosomes. Furthermore, the perinuclear vesicles aggregates appeared to be collapsed, implying rVacA might disrupt actin cytoskeleton structure (Fig. 1E).

Epithelial cell vacuolation and apoptosis induced by VacA. To confirm the role of VacA in H. pylori-induced apoptosis, AGS cells were treated with various VacA preparations (Fig. 2A). VacA-immunodepleted HSE showed significantly reduced VacA level by ~70% of control whereas nonimmunized sera did not change the VacA level of treated HSE. ERK1/2 signal was activated differently according to the various VacA preparations, wherein ERK1/2 activation by s1m1 vacA HSE without anti-VacA antibody was higher than in any other HSE treated AGS cells during a 2-h period (Fig. 2B). Vacuolating...
activity of HSE was increased with incubation time up to 8 h and then decreased during 8–20 h of incubation (Fig. 2C). Cell viability was inversely proportional to vacuolating activity and the level of VacA (Fig. 2, C and D). The cell viability of AGS cells treated with s1/m1 vacA HSE was higher than that of AGS cells treated with VacA-depleted or PBS control at the incubation time of 4 h but it was decreased than VacA-reduced HSE or HSE plus anti-VacA antibody at the incubation of 20 h, suggesting that VacA induces not only cell proliferation at early time but also cell death of the AGS cells after prolonged exposure to it.

Comparative analysis of morphological change and protein expression of AGS cells cocultured with various H. pylori strains. Epidemiological studies have shown that gastric cell damage infected with H. pylori is associated with disruption of cytoskeletal structure (26, 62) and ROS (10, 16). To determine the role of VacA in gastric cell damage by H. pylori infection, the levels of SOD-1, a ROS scavenger enzyme and villin, an actin-binding protein, were assessed in AGS cells cocultured for 5 h with H. pylori. AGS cells infected with ATCC 49503 showed extensive cell vacuolation, disruption of cell-cell contacts and altered cell morphology (Fig. 3A). The level of VacA protein corresponded with the degree of vacuolation of AGS cells (Fig. 3, A and B). These two strains have same vacA allelic type (s1m1 type) but different cagA subtype; CagA phosphorylation site is “A-B-C” in ATCC 49503, “A-B-C-C-C” in HpKm. H. pylori induced ERK activation independently of the vacA type or the presence of cagA. But the level of ERK activation was the highest in AGS cells treated with ATCC 49503. The level of SOD-1 was slightly decreased in AGS cells with toxic VacA positive strains and the level of villin was slightly decreased in ATCC 49503 or ATCC 51932-treated AGS cells (Fig. 3, B and C).

Time-course comparison of ERK1/2, SOD-1, and villin expression upon s1m1 HSE. ATCC 49503 induced ERK activation and decreased SOD-1 and villin expression compared with negative control. Next, we investigated the effects of VacA on those protein expressions depending on incubation time using HSE of ATCC 49503. The HSE activated ERK1/2 at 1 h and lasted thereafter in a time-dependent manner over an 8-h period (Fig. 4). SOD-1 expression was increased at 1 h, reached maximal at 2 h, and decreased thereafter in a time-dependent manner. Villin was decreased in a time-dependent manner by 1 h and was then reinduced thereafter. ERK activation appeared to be associated with the increase in SOD-1 expression and the decrease in villin expression at 1 h.

p38 MAPK and Bax mediate rVacA-induced apoptosis and requirement of ERK activation for cell survival. To confirm the significance of ERK1/2, SOD-1, and villin in VacA-induced apoptosis, we investigated cellular expression of those proteins including Bax, Bcl2, and p38 upon functional rVacA stimulation. AGS cells treated with rVacA during varying incubation times were analyzed for DNA damage, ERK and p38 activation, and Bax, Bcl2, villin, and SOD-1 expression (Fig. 5, A–C). As shown in Fig. 5A, nucleus condensation and fragmentation were found in DAPI-stained AGS cells at 16 h. The extent of DNA fragmentation was determined by a direct counting of the number of apoptotic nuclei (Fig. 5B). As shown in Fig. 5C, ERK1/2 activation induced by rVacA exhibited a biphasic time course, the early component peaking at 30 min and the late component at 4 h and then considerably reduced after a 16-h incubation. Phosphorylation of p38 was maximal at 4 h, was maintained until 8 h, and declined abruptly at 16 h. The expression of villin was increased at 1 h and declined thereafter whereas SOD-1 was decreased after 1 h, and then reincreased during 4–8 h and then declined markedly at 16 h. The results of Bax expression using anti-Bax antibody showed that the expression level of ~18-kDa band was complementary to that of a 43-kDa band. The 18-kDa band was decreased markedly at 8 h and 16 h whereas a 43-kDa band was increased abruptly at corresponding times. Bax is a proapoptotic protein and exists as a monomer in the cytosol. However, it translocates to the mitochondria, where it forms oligomers after an apoptotic stimulus (4). Thus these data indicate that an 18-kDa band is a Bax monomer and a 43-kDa band is a Bax dimer. Reportedly, the activation of p38 in response to environmental stress and inflammatory cytokines can result in apoptosis (37). Proapoptotic signals such as Bax dimerization and p38 activation were induced promptly after stimulation by rVacA. At the
same time, a defense mechanism such as antioxidant protein and a component of cytoskeletal structure were also induced. Cell viability was maintained during 8 h of incubation with rVacA with sustained phospho-ERK1/2 and Bcl2 but the balance was disrupted during 8–16 h with Bax dimerization, resulting in cell death. At 16 h, only the expression of Bax dimer was increased while those of others were decreased, which shows Bax dimerization plays critical role for apoptosis induced by rVacA. In addition, activation of p38 MAPK involved via ERK1/2 activation. Whereas NPPB did not show significant effects on cell viability, anti-VacA antibody protected cells from VacA intoxication and lasted by 20 h. rVacA, however, decreased net cell viability in a time-dependent manner and, contrary to Fig. 7C, NPPB protected moderately but not significantly cell from rVacA intoxication. Anti-VacA antibody protected cell from rVacA-induced cell death by 10 h but could not at 20 h. The differences between nVacA and rVacA may be caused by the crudity of nVacA, which might have a proliferating factor.

**rVacA induces SOD-1 via ERK activation and phosphorylation of p38 in AGS.** We investigated a possible role of MAP kinases in VacA-induced SOD-1 and villin expression using MAP kinases inhibitor. After 16 h incubation with rVacA, the expression of SOD-1 was increased compared with negative control and, however, PD-98059 inhibited that induction of SOD-1, suggesting that the induction by rVacA is via ERK1/2 activation (Fig. 8A). The level of villin remained at 1 μg/ml of rVacA and severely suppressed at 2 μg/ml rVacA thereafter, irrelevant to treatment of anti-rVacA antibody or PD-98059. The activation of p38 by rVacA was in a dose-dependent manner whereas anti-rVacA antibody attenuated the phosphorylation of p38 in AGS cells treated with 2 μg of rVacA to a similar extent of negative control. Next, the effect of rVacA on cell viability with an MTT assay was assessed. The cytotoxic effect of rVacA was unlikely to change with anti-VacA antibody. The level of villin, however, was significantly reduced under the presence of anti-VacA antibody (Fig. 7, A and B). Cell viability depending on incubation time showed that upon stimulation with 0.2 μg of VacA cell viability decreased up to 5 h, reincreased up to 10 h, and then decreased thereafter. VacA may induce cell apoptosis followed by cell proliferation to compensate for cell loss wherein SOD-1 and villin might be involved via ERK1/2 activation. Whereas NPPB did not show significant effects on cell viability, anti-VacA antibody protected cells from VacA intoxication and lasted by 20 h. rVacA, however, decreased net cell viability in a time-dependent manner and, contrary to Fig. 7C, NPPB protected moderately but not significantly cell from rVacA intoxication. Anti-VacA antibody protected cell from rVacA-induced cell death by 10 h but could not at 20 h. The differences between nVacA and rVacA may be caused by the crudity of nVacA, which might have a proliferating factor.

**Effect of NPPB and anti-VacA antibody on VacA-induced phospho-ERK1/2, SOD-1, and villin.** A nonspecific chloride channel blocker, NPPB, can inhibit the channel activity of VacA, resulting in a reduction of vacuolation and cytochrome c release caused by VacA (14). NPPB suppressed expression of phospho-ERK1/2 and villin at 0.2 μg/ml of VacA and 2 μg/ml of rVacA (Fig. 7, A and B), implying that anionic channel formation of VacA may be associated with ERK1/2 activation and villin expression. Although ERK activation by nVacA but not by rVacA was slightly decreased in the presence of anti-VacA antibody, the level of SOD-1 induced by both nVacA and rVacA was unlikely to change with anti-VacA antibody. The level of villin, however, was significantly reduced under the presence of anti-VacA antibody (Fig. 7, A and B). Cell viability depending on incubation time showed that upon stimulation with 0.2 μg of VacA cell viability decreased up to 5 h, reincreased up to 10 h, and then decreased thereafter. VacA may induce cell apoptosis followed by cell proliferation to compensate for cell loss wherein SOD-1 and villin might be involved via ERK1/2 activation. Whereas NPPB did not show significant effects on cell viability, anti-VacA antibody protected cells from VacA intoxication and lasted by 20 h. rVacA, however, decreased net cell viability in a time-dependent manner and, contrary to Fig. 7C, NPPB protected moderately but not significantly cell from rVacA intoxication. Anti-VacA antibody protected cell from rVacA-induced cell death by 10 h but could not at 20 h. The differences between nVacA and rVacA may be caused by the crudity of nVacA, which might have a proliferating factor.

**rVacA-induced protein expression in AGS cells in a time-dependent manner.** AGS cells were exposed to 10 μg/ml of rVacA for the indicated times over a 16-h period in serum-free medium, and cells were treated without rVacA for 0 min. A: AGS cells were incubated in the above conditions and fixed with 2% paraformaldehyde in PBS, followed by staining with DAPI. The changes in nuclear morphology and DNA fragmentation were revealed in a time-dependent manner by DAPI staining. B: histograms represent the percentage of apoptotic cells in AGS cells exposed to rVacA in a time-course experiment. The values represent means and SD determined from 3 areas per specimen. C: identification of the expression of proteins at the indicated time point was by an immunoblot using antibodies for the corresponding proteins. Data are representative of at least 2 experiments.
effect of rVacA was measured after 16 h of incubation with 0–4 μg of rVacA (Fig. 8B). Cell viability was decreased in a dose-dependent manner when AGS cells were treated with rVacA in the presence or absence of anti-VacA antibody, with only a slight decrease in cell viability at higher doses (4 μg of rVacA). PD-98059, which totally abrogated ERK1/2 activation in the AGS cells, significantly decreased cell viability compared with control, but the rate of cell death at the indicated concentrations of rVacA was similar to that induced by rVacA only.

Inhibition of the p38 pathway protects the AGS cells partially from rVacA-induced apoptosis and activates ERK1/2. The involvement of p38 in the VacA-induced apoptosis in AGS cells was confirmed by use of SB-203580, a specific inhibitor of p38. Although SB-203580 did not completely inhibit the cytotoxic activity of rVacA, it did protect the cells partially from rVacA-induced apoptosis, which may be at least partially associated with ERK1/2 activation in AGS cells treated with the p38 inhibitor (Fig. 9, A and B). These results imply that there may be a mutual induction between the two pathways: one leading to inhibit and the other to promote apoptosis, especially against rVacA cytotoxicity in AGS cells. Increased SOD-1 expression was likely to be associated with increased ERK phosphorylation and, in part, decreased p38 phosphorylation in the presence of anti-VacA antibody or SB-203580 (Fig. 9A).

Inhibition of p38 MAP kinase with SB-203580 suppresses villin and Bcl2 expression. Despite cytoprotective effect, SB-203580 was unlikely to prevent VacA-induced Bax dimerization (Fig. 10). But a higher level of SB-203580 (60 μM), partially inhibited rVacA-induced Bax dimerization, implying that inhibition of p38 MAP kinase activity may not directly lead to the inhibition of VacA-induced Bax activation. Interestingly, SB-203580 inhibited the expression of Bcl2 and villin expression, indicating p38 may play a role in cell proliferation or differentiation for survival upon VacA insult. In accordance with Fig. 9A, VacA-induced SOD-1 expression was not inhibited by SB-203580 and furthermore prevented SOD-1 reduction from the higher dose of rVacA. The results indicate that VacA-induced p38 MAPK activation is not only involved in apoptosis but also involved in survival mechanism.

Inhibition of p38 MAP kinase accelerates rVacA-induced apoptosis after exposure to H2O2. To further confirm the role of MAP kinases in response to ROS induced by rVacA, we investigated the effects of H2O2, product of SOD enzymatic reaction on rVacA-induced apoptosis in the absence or presence of MAP kinase inhibitor or anti-VacA antibody. SB-203580 protected AGS cells partially from rVacA-induced apoptosis as shown in Fig. 9 which appeared to be associated with ERK1/2 activation and SOD-1 expression after 16 h incubation. However, it accelerated cell death of AGS cells treated with rVacA for 4 h after exposure to both 50 and 100 μM H2O2 for 1 h (Fig. 11, A and B). On the contrary, PD-98059 slightly protected cell from rVacA and 100 μM of H2O2 intoxication (Fig. 11C). Therefore, p38 MAP activation may play a critical role in cellular protection in the early stage of response to oxidative stress and conversely, ERK activation promotes oxidative stress- or H2O2-induced apoptosis in the early stage which may trigger cell proliferation thereafter.

Anti-VacA antibody blocks the localization of villin to contractile actin ring. Villin expression in response to VacA or rVacA was very versatile but anti-VacA antibody strongly inhibited villin expression, so we identified the villin expression and localization using an immunofluorescent staining. Villin was mainly observed in contractile actin ring of dividing cells (Fig. 12A). Contractile actin ring, comprised of non-muscle myosin II and actin filaments, assembles equatorially at the cell cortex, driving plasma membrane invagination from the cortex (2). Villin was identified in microvilli, microtubules, and near the perinuclear compartment, but the contractile ring was not seen nearly in AGS cells treated with anti-VacA antibody with or without rVacA (Fig. 12B).

DISCUSSION

Aptosis of gastric epithelial cells induced by H. pylori infection is strongly associated with the development of glandular atrophy and intestinal metaplasia (57, 58). VacA has been considered as an apoptosis-inducing factor both in vivo and in vitro (66). Although the activation of MAP kinases induced by VacA has been demonstrated (14, 18, 45), the involvement in apoptotic event is not clear. Our results showed that the activation of ERK1/2 and p38 by rVacA was link to cell survival and apoptosis, differentially (67, 68).

VacA induces clustering and perinuclear redistribution of late endosomes, which requires a functional microtubule cytoskeleton (41). rVacA also reached late endosomes but could
not induce extensive vacuolar structure and fill the entire cytoplasm. After reaching the compartment, rVacA may attack cytoskeleton structure, which may be causes of defective and localizing vacuolation in perinuclear compartment wherein structure collapsed. It could be a cause of reduced villin levels after high-dose (≥2 μg/ml) and/or prolonged exposure to rVacA, leading to induction of cell cycle arrest and apoptosis. However, rVacA stimulated villin expression at early stages, implying that it may be necessary for inducing vacuolation and morphological change. Villin, an actin-binding protein, is associated with the actin bundles that support microvilli, bundles, caps, and nucleates and sever actin (7, 15, 55). As shown in Fig. 12, we have shown that villin is localized in contractile actin ring during late anaphase and disappeared gradually according to completion of cell division wherein it may sever actin ring. Anti-VacA antibody might suppress cell division by inhibiting villin expression, which may be a cause of the abrupt cell death in AGS cells treated with rVacA plus anti-VacA antibody at 20 h (Fig. 7B). NPPB, an chloride channel blocker, inhibits VacA-induced vacuolation and its internalization (30), and its inhibition of villin expression induced by VacA or rVacA may be associated with the decrease in VacA-induced polymerization of actin due to blocking the membrane trafficking (29, 30). Villin is known...
to an important marker of the preneoplastic cell type; therefore the increase or decrease in the expression of it by VacA suggests VacA may play a role in gastric cancer development. Thus more verification on the role of VacA in regulating of villin is required.

Activation of p38 and accompanying decrease in a phospho-ERK1/2 level coincided with an increase in Bax dimer, a decrease in Bcl2, and apoptosis of AGS cells. rVacA-induced Bax dimerization and its association with apoptosis coincided with the results of Yamasaki et al. (70). Inhibition of ERK1/2 by PD-98059 decreased cell viability but was unlikely to potentiate the cytotoxic effect of rVacA. However, ERK1/2 activation contributed to sustaining cell survival for a limited time. Although anti-VacA antibody inhibited p38 activation and Bax dimerization induced by rVacA, it was unlikely to inhibit ERK1/2 and SOD expression, which may contribute to the ability of the antibody to protect cells from cell death by rVacA intoxication. These results indicated that the effect of VacA on ERK1/2 and p38 activation may be differentially regulated, of which only one is dependent on initial membrane binding of VacA, which is inhibited by anti-VacA antibody but is independent of internalization of VacA. Reportedly, EGF receptor, lipid rafts, and receptor protein tyrosine phosphatase beta have been proposed as VacA receptor, suggesting that VacA may bind to multiple sites on the cell surface (49). Therefore, VacA-induced signal transduction may be different depending on the kind of receptor bound.

ERK inhibition by PD-98059 did not aggravate or abolish the cell death induced by rVacA; however, it did inhibit the induction of SOD-1 by rVacA as shown in Fig. 8. Obst et al. (47) have shown that H. pylori extract directly induces the synthesis of ROS in gastric epithelial cells and causes DNA damage. Induction of SOD-1 by toxic VacA enriched sample or rVacA implies that VacA may stimulate ROS production, probably from the mitochondria or from MAP kinase activation (72). Recent work described how VacA enhances PGE2 production through induction of COX-2 mRNA in a time- and dose-dependent manner via p38 MAP kinase activation in AZ-521 cells (33), which may induce ROS production. Protection of cells against ROS is accomplished through the activation of oxygen-scavenging enzymes such as SOD, catalase, and glutathione peroxidase (61). The superoxide anion-scavenging activity of SOD-1 leads to an increase in H2O2, which may induce catalase expression. AGS cells treated with rVacA with MAP kinase inhibitors showed that rVacA increased catalase expression moderately, ERK inhibitor decreased the expression slightly, and p38 inhibitor (SB-203580) did not affect the VacA-induced catalase expression. However, there were no
significant differences in the expression level of catalase compared with that of SOD-1 (data not shown). Without a concomitant increase in peroxide-scavenging enzymes, excess H$_2$O$_2$ might be involved in the cell damage caused by VacA. Activation of ERK has been shown to promote or block H$_2$O$_2$-dependent apoptosis (69, 73). In the present work, H$_2$O$_2$ accelerated rVacA-induced cell death, especially in p38 inhibitor-pretreated AGS cells, suggesting that p38 MAPK plays a pivotal role in protection against H$_2$O$_2$-induced cell death (11, 27) and, conversely, that ERK1/2 aggravates H$_2$O$_2$-induced cell death.

Van Laethem et al. (64) reported that activation of p38 by UVB required for the translocation of Bax to the mitochondria in human keratinocytes. Association of p38 with rVacA-induced apoptosis in our results was based on the effect of SB-203580 on AGS cells treated with rVacA, resulting in the attenuation of cell death. However, despite the cytoprotective effect of SB-203580, inhibition of p38 MAPK activation was unlikely to reduce Bax dimerization induced by rVacA and furthermore led to decrease in Bcl2 and villin expression. Nakayama et al. (46) reported that VacA activates the p38 signal pathway, which is independent of cytochrome c release from mitochondria caused by VacA in AZ-521 cells. Pleiotropic activity of p38 MAPK such as proliferation, differentiation, and survival upon a stimulus may be explained by the existence of several p38 MAPK isoforms with distinct functions (3, 23, 54). According to some literatures, p38 promotes but p38$\beta$ inhibits apoptosis, and both p38 isoforms are suppressed by SB-203580 (35, 52, 63). From this, we deduce that SB-203580 may not only protect cell from cell death via p38$\alpha$ but also suppress host defense mechanisms via p38$\beta$ in response to VacA intoxication. Among p38 isoforms, p38$\alpha$ has been more abundantly expressed and active than other isoforms (32). According to the paper of Abdollahi et al. (1), mRNA expression of p38$\alpha$ appears to be constant but p38$\beta$ to be induced at 16 h and maintained over a 24-h period without any stress. In the presence of some stress, however, expression of p38$\beta$ was induced at 8 h, maximal at 16 h and then declined thereafter. Therefore differential activation of p38$\alpha$ and p38$\beta$ MAP kinase coupled with ERK activation may result in various phenotypic consequences in response to VacA intoxication. Taken together, these results indicated that there is an antagonistic regulation between ERK1/2 and p38 on VacA-induced apoptosis. These antagonistic regulations on taxol-induced apoptosis and nitric oxide-induced apoptosis have been described (38, 60). Thus the roles of ERK and p38 MAPKs in apoptosis or proliferation might be dependent on cell type and state (19).

In addition, Ranganathan et al. (33) demonstrated that Mn-SOD dependent H$_2$O$_2$ production leads to the activation of the ERK1/2 signaling and subsequent downstream transcriptional

Fig. 11. Inhibition of p38 MAP kinase accelerates rVacA-induced apoptosis after exposure to H$_2$O$_2$. Cell viability was assessed by an MTT assay in AGS cells treated with 0–4 $\mu$g/ml of rVacA for 4 h (A) or followed by exposure to 50 (B) or 100 $\mu$M H$_2$O$_2$ (C) for 1 h. The cell viability represents a percentage of the values of cells without stimulation of rVacA and exposure to H$_2$O$_2$ and the means and SD from tripel or quadruplet samples. *P < 0.05, †P < 0.01 vs. nontreated with rVacA; **P < 0.05, ††P < 0.01 vs. 2 $\mu$g/ml rVacA treatment; and ‡P < 0.05, ‡‡P < 0.01 vs. H$_2$O$_2$ negative control, Student’s t-test.

Fig. 12. Immunofluorescent staining for villin in rVacA-treated AGS cells. A: villin is shown mainly in dividing cells, especially contractile ring (left) and cell nuclei was visualized by DAPI (right). Villin is strongly expressed in dividing cells (arrows) and then decreased after completion of cell cleavage (arrowheads). B: villin is localized with actin in contractile ring and microtubules in rVacA treated AGS cells (left), but it is localized with actin in microvilli and in perinuclear compartment (right).
increases in MMP-1 expression, which is involved in extracellular matrix remodeling. Therefore, it is likely that VacA-induced ROS stimulates ERK1/2-dependent SOD-1 expression by which H₂O₂ production might lead to another ERK1/2 activation and subsequent downstream transcriptional increases in MMP expression, which may be involved in actin cytoskeleton degradation, leading to apoptosis. A recent report by Pillinger et al. (50) suggests that H. pylori-induced MMP-1 secretion can be both dependent on and independent of CagA and is regulated positively via ERK and negatively via p38 MAPK. Thus the association of VacA with MMP-1 secretion needs further investigation.

Apoptosis in gastric epithelium by H. pylori infection and ROS production have been associated with the incidence of gastric cancer. Moreover, Lin et al. (42) reported in a case-control study that higher SOD-1 levels may be associated with an increased risk of gastric cancer. H. pylori-associated gastritis is predominant in the antrum, where a significant increase of SOD activity has been detected (24, 25). Malignant cells produce active superoxide, which makes the cancer cell highly dependent on SOD for survival and thus more sensitive to inhibition of SOD; hence Huang et al. (34) suggested that targeting SOD may be a promising approach to the selective killing of cancer cells.

In summary, we first demonstrated that VacA stimulates expression of SOD-1 via ERK1/2 activation and the expression and degradation of villin using recombinant VacA. These results suggest that SOD-1 and villin are expressed differentially upon VacA insult depending on dose and exposure time via ERK and p38 MAP kinases; decrease in SOD-1 and villin expression coupled with Bax dimerization leads to apoptosis of gastric epithelial cells. Therefore, VacA may be a crucial factor of gastric cancer development in subjects with chronic infection by H. pylori. Expression coupled with Bax dimerization leads to apoptosis of gastric epithelial cells. Therefore, VacA may be a crucial factor of gastric cancer development in subjects with chronic H. pylori infection by a repeated stimulation of cellular proliferation and apoptosis mediated by a fine-tuned control between infection by a repeated stimulation of cellular proliferation and apoptosis mediated by a fine-tuned control between ERK and p38 MAP kinase.

GRANTS

This research was supported by a grant (CBM31-B3003-01-01-00) from the Center for Biological Modulators of the 21st Century Frontier R & D Program, the Ministry of Science and Technology, Korea.

REFERENCES


VACA-INDUCED SOD-1 AND VILLIN EXPRESSION

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