Anti-septic effects of dabrafenib on HMGB1-mediated inflammatory responses

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Supplementary Materials and Methods

Reagents

Dabrafenib was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Bacterial lipopolysaccharide (LPS, serotype: 0111:B4, L5293), Evans blue, crystal violet, 2-mercaptoethanol, and antibiotics (penicillin G and streptomycin) were purchased from Sigma (St. Louis, MO). Human recombinant HMGB1 was purchased from Abnova (Taipei City, Taiwan). Fetal bovine serum and Vybrant DiD were purchased from Invitrogen (Carlsbad, CA).

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex Bio Science (Charles City, IA) and maintained using a previously described method (1-3). HUVECs were used in cell culture at passages 3-5. Human neutrophils were freshly isolated from whole blood (15 mL) obtained by venipuncture from five healthy volunteers, and maintained as previously described (4-6).

Animals and husbandry

Male C57BL/6 mice (6-7 weeks old, 27 g) purchased from Orient Bio Co. (Sungnam, Republic of Korea) were used in this study after a 12 d acclimatization period. Animals (five per polycarbonate cage) were housed under controlled temperature (20-25°C) and humidity (40% - 45% RH), with a 12:12 h light/dark cycle. Animals received a normal rodent pellet diet and water ad libitum during acclimatization. All animals were treated in accordance with the ‘Guidelines for the Care and Use of Laboratory Animals’ issued by Kyungpook National
Cecal ligation and puncture (CLP)

To induce sepsis, male mice were anesthetized with Zoletil (tiletamine and zolazepam, 1:1 mixture, 30 mg/kg) and Rompum (xylazine, 10 mg/kg). The CLP-induced sepsis model was prepared as previously described (1, 7). In brief, a 2 cm midline incision was made to expose the cecum and adjoining intestine. The cecum was then tightly ligated with a 3.0 silk suture 5.0 mm from the cecal tip and punctured once using a 22-gauge needle to induce high grade sepsis (8). The cecum was then gently squeezed to extrude a small amount of feces from the perforation site and returned to the peritoneal cavity. The laparotomy site was then sutured with 4.0 silk. In sham control animals, the cecum was exposed, but not ligated or punctured, and then returned to the abdominal cavity. This protocol was approved by the Animal Care Committee at Kyungpook National University prior to conducting the study (IRB No. KNU 2012-13).

Competitive enzyme-linked immunosorbent assay (ELISA) for HMGB1

A competitive ELISA was performed as previously described to determine the HMGB1 concentrations in cell culture media or mice serum (9). HUVEC monolayers were treated with LPS (100 ng/mL) for 16 h, followed by DAB for 6 h. Cell culture media was collected for the determination of HMGB1. To perform the ELISA, 96 well flat plastic microtiter plates (Corning, NY) were coated with HMGB1 protein in 20 mM carbonate-bicarbonate buffer (pH 9.6) containing 0.02% sodium azide overnight at 4°C. Plates were then rinsed three times in PBS-0.05% Tween 20 (PBS–T) and kept at 4°C. Lyophilized culture media was pre-incubated
with an anti-HMGB1 antibody (Abnova, diluted 1:1000 in PBS-T) in 96-well plastic round microtiter plates for 90 min at 37°C, transferred to the pre-coated plates, and incubated for 30 min at room temperature. Plates were then rinsed three times in PBS-T, incubated for 90 min at room temperature with peroxidase-conjugated anti-rabbit IgG antibodies (diluted 1:2000 in PBS-T, Amersham Pharmacia Biotech), rinsed three times with PBS-T, and incubated for 60 min at room temperature in the dark with 200 µL of substrate solution (100 µg/mL o-phenylenediamine and 0.003% H₂O₂). After stopping the reaction with 50 µL of 8 N H₂SO₄, absorbance was measured at 490 nm.

**RNA isolation and real-time PCR**

Total cellular RNA was isolated using TRI-Reagent (Invitrogen, Life Technologies, Carlsbad, CA), according to the manufacturer’s suggested protocol. An aliquot (5 µg) of extracted RNA was reverse transcribed into first-strand cDNA using a PX2 Thermal Cycler (Thermo Scientific) by using 200 U/µL M-MLV reverse-transcriptase (Invitrogen) and 0.5 mg/µL of oligo(dT)-adapter primer (Invitrogen) in a 20-µL reaction mixture. Real-time PCR for moesin and GAPDH was performed using a Mini Opticon Real-Time PCR System (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). The following primers were used to amplify HMGB1: sense 5′-GGA CAA GGC CCG TTA TGA AAG AGA AAT GA-3′ and antisense 5′- GAT GTG CGG AAG GAA AGC -3′; and for GAPDH: sense 5′-TCG GAG TCA ACG GAT TT-3′ and antisense 5′- CCA CGA CGT ACT CAG C -3′. The PCR settings were as follows: the initial denaturation at 95°C was followed by 30 cycles of amplification at 95°C for 15 s and at 58°C for 20 s, with subsequent melting curve analysis by increasing the temperature from 72°C to 98°C. The gene expression levels were quantified relative to GAPDH.
Permeability assay in vitro

For the spectrophotometric quantification of endothelial cell permeability in response to increasing concentrations of each compound, the flux of Evans blue-bound albumin across functional cell monolayers was measured using a modified 2-compartment chamber model, as previously described (10-11). HUVECs were plated (5 × 10^4 cells/well) in transwells with a pore size of 3 µm and a diameter of 12 mm for three days. Confluent monolayers of HUVECs were treated with LPS (100 ng/mL) for 4 h or HMGB1 (1 µg/mL) for 16 h, followed by treatment with DAB. Transwell inserts were then washed with PBS (pH 7.4), and growth medium containing 0.5 mL of Evans blue (0.67 mg/mL) and 4% BSA was added. Fresh growth medium was then added to the lower chamber, and the medium in the upper chamber was replaced with Evans blue/BSA. Ten minutes later, optical density in the lower chamber was measured at 650 nm.

Cell-Cell adhesion assay

Human neutrophils (1.5 × 10^6 cells/mL, 200 µL/well) were labeled with Vybrant DiD dye, and then added to washed and stimulated HUVECs. HUVEC monolayers were treated with HMGB1 (1 µg/mL) for 16 h followed by treatment with DAB for 6 h. Human neutrophils were allowed to adhere, and non-adherent neutrophils were removed by washing. The percentage of adherent neutrophils was calculated using the formula: % adherence = (adherent signal/total signal) × 100.

Migration assay in vitro
Migration assays were performed in transwell plates with a diameter of 6.5 mm containing filters with a pore size of 8 µm. HUVECs ($6 \times 10^4$) were cultured for 3 d to obtain confluent endothelial monolayers. Prior to addition of neutrophils to the upper compartment, cell monolayers were treated with HMGB1 (1 µg/mL) for 16 h, followed by treatment with DAB for 6 h. Transwell plates were then incubated at 37°C in 5% CO₂ for 2 h. Cells in the upper chamber were then aspirated, followed by the removal of non-migrating cells on top of the filter using a cotton swab. Neutrophils on the lower side of the filter were fixed with 8% glutaraldehyde and stained with 0.25% crystal violet in 20% methanol (w/v). Experiments were repeated twice per well on duplicate wells, and nine randomly selected high power microscopic fields (HPF, 200×) were counted. The results are presented as migration indices.

In vivo permeability and the leukocyte migration assay

For the in vivo study, male mice were anesthetized with 2% isoflurane (Forane, JW Pharmaceutical, South Korea) in oxygen delivered via a small rodent gas anesthesia machine (RC2, Vetequip, Pleasanton, CA), first in a breathing chamber and then via facemask. Mice were allowed to breath spontaneously during the procedure. CLP-operated mice or mice were treated with HMGB1 (2 µg/mouse, i.v.) for 16 h, followed by treatment with DAB (260 or 520 µg/kg, i.v.). For the in vivo permeability assay, after 6 h, 1% Evans blue dye solution in normal saline was injected intravenously into each mouse. Mice were euthanized after 30 min, and the peritoneal exudates were collected by washing cavities with 5 mL of normal saline and centrifuging at $200 \times g$ for 10 min. Absorbance of the supernatants was read at 650 nm. Vascular permeability is expressed as µg of dye in the peritoneal cavity/mouse, and were determined using a standard curve, as previously described (12-13).
To assess leukocyte migration, mice were euthanized after 6 h, and the peritoneal cavities were washed with 5 mL of normal saline. The obtained samples of peritoneal fluids (20 µL) were mixed with 0.38 mL of Turk’s solution (0.01% crystal violet in 3% acetic acid), and the number of leukocytes was counted under a light microscope.

Expression of cell adhesion molecules (CAMs) and HMGB1 receptors

The expression of VCAM-1, ICAM-1, and E-selectin was determined by whole-cell ELISA, as previously described (14-15). Briefly, confluent monolayers of HUVECs were treated with HMGB1 (1 µg/mL) for 16 h (VCAM-1 and ICAM-1) or 22 h (E-Selectin), treated with DAB, and fixed in 1% paraformaldehyde. After washing three times, mouse anti-human monoclonal antibodies (VCAM-1, ICAM-1, and E-selectin, Temecula, CA, 1:50 each) were added, and samples were incubated for 1 h (37°C, 5% CO₂). Cells were then washed, treated with peroxidase-conjugated anti-mouse IgG antibody (Sigma, St. Louis, MO) for 1 h, washed three times, and treated with o-phenylenediamine substrate (Sigma, St. Louis, MO). The same experimental procedures were used to monitor the cell surface expression of the toll like receptor (TLR)2, TLR4, and receptor for advanced glycation end products (RAGE), using specific antibodies (A-9, H- 80, and A-9, respectively) obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

ELISA for phosphorylated p38 mitogen-activated protein kinase (MAPK), nuclear factor (NF)-kB, TNF-α, extracellular regulated kinases (ERK) 1/2, and IL-6

The activity of phosphorylated p38 MAPK was quantified according to the manufacturer’s instructions.
using a commercially available ELISA kit (Cell Signaling Technology, Danvers, MA). Total and phosphorylated p65 NF-κB (#7174, #7173, Cell Signaling Technology, Danvers, MA) or total and phosphorylated ERK 1/2 (R&D Systems, Minneapolis, MN) activities in nuclear lysates were determined using ELISA kits. The concentrations of IL-6 and TNF-α in cell culture supernatants were determined using ELISA kits (R&D Systems, Minneapolis, MN). Values were measured using an ELISA plate reader (Tecan, Austria GmbH, Austria).

**Hematoxylin & eosin staining and histopathological examination**

Male C57BL/6 mice underwent CLP and were administered DAB (260 or 520 μg/kg, i.v.) at 12 h and 50 h after CLP (n = 5). Mice were euthanized 96 h after CLP. To analyze the phenotypic change in the lungs, samples were removed from each mouse, washed three times in PBS (pH 7.4) to remove the remaining blood, and fixed in 4% formaldehyde solution (Junsei, Tokyo, Japan) in PBS for 20 h at 4°C. After fixation, the samples were dehydrated using an ethanol series, embedded in paraffin, sectioned (4 μm), and placed on a slide. The slides were deparaffinized in a 60°C oven, rehydrated, and stained with hematoxylin (Sigma). To remove over-staining, the slides were quickly dipped in 0.3% acid alcohol three times, and counterstained with eosin (Sigma). Over-staining was then removed by an ethanol series and xylene, and the samples were placed under a coverslip. Light microscopic analysis of the lung specimens was performed by a blinded observer to evaluate pulmonary architecture, tissue edema, and infiltration of the inflammatory cells as previously defined (16). The results were classified into four grades, where Grade 1 represented normal histopathology; Grade 2 indicated minimal neutrophil leukocyte infiltration; Grade 3 represented moderate neutrophil leukocyte infiltration, perivascular edema formation, and partial destruction of pulmonary architecture, and Grade 4 included dense neutrophil
leukocyte infiltration, abscess formation, and complete destruction of pulmonary architecture.

**Immunofluorescence staining**

HUVECs were grown to confluence on glass cover slips coated with 0.05% Poly-L-Lysine in complete media containing 10% FBS and maintained for 48 h. Cells were then stimulated with HMGB1 (1 µg/mL) for 16 h with or without 6-h DAB treatment (10 µM). For cytoskeletal staining, the cells were fixed in 4% formaldehyde in PBS (v/v) for 15 min at room temperature, permeabilized in 0.05% Triton X-100 in PBS for 15 min, and blocked in blocking buffer (5% BSA in PBS) overnight at 4°C. Then, the cells were incubated with F-actin labeled fluorescein phalloidin (F 432; Molecular Probes, Invitrogen) or primary rabbit monoclonal NF-κB p65 antibody and anti-rabbit alexa 488 overnight at 4°C. Nuclei were counterstained with 4,6-diamidino-2-phenylindole dihydroChloride (DAPI) and were visualized by confocal microscopy at a 630× magnification (TCS-Sp5, Leica microsystem, Germany).

**Measurements of organ injury markers**

Plasma levels of aspartate transaminase (AST), alanine transaminase (ALT), blood urea nitrogen (BUN), and creatinine were measured using commercial assay kits (Pointe Scientific, Linclon Park, MI).

**Statistical Analysis**

All experiments were performed independently at least three times. Values are expressed as the mean ± SEM. The statistical significance of differences between test groups was evaluated using SPSS for Windows,
version 16.0 (SPSS, Chicago, IL). Kaplan-Meier survival analysis was performed to evaluate the overall survival rates. Statistical significance was accepted for p values < 0.05.

References

6. Bae JS and Rezaie AR (2013) Thrombin inhibits HMGB1-mediated proinflammatory signaling responses when endothelial protein C receptor is occupied by its natural ligand. BMB Rep 46, 544-549
Supplementary Figure 1. Effects of DAB on HMGB1-stimulated production of IL-6/TNF-α and activation of NF-κB/ERK. A-B, HUVECs were stimulated with HMGB1 (1 μg/mL) for 16 h, followed by treatment with DAB for 6 h. HMGB1-mediated production of TNF-α (A) or IL-6 (B) in HUVECs was analyzed after treatment of cells with DAB for 6 h. C-D, Confluent HUVECs were activated with HMGB1 (1 μg/mL, 16 h), followed by incubation with DAB for 6 h, and HMGB1-mediated activation of phospho-NF-κB p65 (white bar) or total NF-κB p65 (black bar) in HUVECs was analyzed (C) or phospho-ERK1/2 (white box) or total ERK1/2 (black box) in HUVECs was analyzed (D). E, Immunofluorescence microscopy analysis of the nuclear translocation of p65 in HUVECs. HUVECs were stimulated (or not) for 1 h with 1 μg/mL HMGB1 and treated or not with 10 μM DAB for 6 h. The subcellular localization of p65 was examined by IF staining. The representative images were from three separate experiments in different days with similar results. Results are expressed as the mean ± SEM of three separate experiments in different days. *p < 0.05 vs. HMGB1.
The figures show the effects of HMGB1 and DAB on various biological markers. The x-axes represent different concentrations of DAB (in μM), while the y-axes in panels A and B represent the levels of TNF-α and IL-6, respectively. In panels C and D, the y-axes represent the NF-κB phosphorylation levels and Egr-1 mRNA expression, respectively. Panel E illustrates the immunofluorescence staining for NF-κB p65 and DAPI. The results indicate a concentration-dependent effect of DAB on these markers in the presence or absence of HMGB1.