The effect of bortezomib on expression of inflammatory cytokines and survival in an experimental sepsis model induced by cecal ligation and puncture

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Directed by Professor June Myung Kim

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Sang Hoon Han

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This certifies that Doctoral Dissertation of Sang Hoon Han is approved.

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ACKNOWLEDGEMENTS

I have been extremely interested in how we can improve the survival rate in severe sepsis patients with multiorgan failure from when I major in infectious disease. I really believe that the new targeted therapy must be introduced in severe sepsis patients. Although many previous clinical trials for immunomodulating drugs have been not ameliorate the survival rate in severe sepsis patients, we continuously must try to understand the complex pathogenesis of sepsis and find the new therapeutic immunomodulators. I focused that the proteasome inhibitors can serve as the immnuomodulator, and then performed the study that whether the bortezomib, approval proteasome inhibitor, can modulate the hyperimmune status *in vitro* lipopolysaccharide stimulated murine macrophage cell line, and improve the survival rate and decrease the inflammation in cecal ligation and puncture induced murine peritonitis sepsis model.

I owe every piece of my work to my mentor, Professor June Myung Kim, and it may not be possible that this work has been performed without his guidance. Professor Young Soo Ahn, Jun Hee Woo, Jeon-Soo Shin, and Jin Seok Kim, have served as committee member and given me important advices to make this article better.

Finally, I would like to express my gratitude to my lovely wife and daughter for their understanding and encouragement.

Sang Hoon Han

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ABSTRACT

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Bortezomib can modulate the inflammatory process through the nuclear factor-kappa B (NF-κB) signaling pathway. Although only a clinically used proteasome inhibitor, the immunomodulatory effect of pre-incubated bortezomib is not fully evaluated in inflammation caused by infectious agents. The effect of bortezomib on the expression of inflammatory cytokines in lipopolysaccharide (LPS)-stimulated cells was evaluated using an *in vitro* cell line experiment. Bortezomib was pre-applied 1 h before LPS stimulation in RAW 264.7 cells. Subsequently, cellular viability as well as various inflammatory cytokines or cellular adhesion molecule levels were measured by reverse transcription-polymerase chain reaction. In addition, nitric oxide (NO) concentration was measured. C57BL/6J mice were used for *in vivo* cecal ligation and puncture (CLP)-induced murine peritonitis sepsis model experiments. For the negative control, mice received neither surgery nor treatment, and were administered 1 mL of normal saline 1 h before sham surgery. The positive control mice received 1 mL of normal saline 1 h before CLP surgery. To evaluate the impact of bortezomib dose on survival, each group that received bortezomib 0.01 mg/kg or 0.1 mg/kg 1 h before CLP

surgery was compared with the positive control. To evaluate the effect of delayed administration of bortezomib on survival, the mice that were administered bortezomib 0.01 mg/kg at 24 h after CLP surgery were compared with the positive control. The mice were assessed for survival up to seven days following surgery, and then mortality rates were compared between groups. All mice that were alive at day seven after surgery in each group were anesthetized to observe the histopathologic findings and measure pulmonary inflammatory score. Pre-incubation with bortezomib (25 or 50 nM) before LPS (50 or 100 ng/mL) stimulation significantly recovered the number of viable RAW 264.7 cells compared with no pre-incubation. The bortezomib decreased the inflammatory cytokines of TNF-α, IL-1β, IL-6, and IL-10, ICAM-1, as well as NO production in LPS-stimulated cells. The seven-day survival rate in mice administered bortezomib 0.01 mg/kg at 1 h before CLP surgery was significantly higher than in the mice treated with normal saline 1 h before CLP surgery. The administration of bortezomib 0.01 mg/kg 1 h before CLP surgery resulted in the significant decrease of lung parenchymal inflammation. In conclusion, pretreatment with bortezomib showed an increased survival rate and a change of inflammatory mediators. This study suggests the possibility of the pretreatment of bortezomib as the new therapeutic target for overwhelming inflammation characterized in severe sepsis.

Key words**:** Bortezomib; Proteasome inhibitor; Macrophage; Lipopolysaccharide; Inflammatory cytokines, Cecal ligation and puncture

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I. INTRODUCTION

Proteasome inhibitors, which restrain the non-lysosomal degradation of cellular proteins through the ubiquitin-proteasome system (UPS), can modulate excessive inflammations and immune responses as well as regulate the cytokine expression produced by various stimuli[1-4]. The potential role of proteasome inhibitors as a therapeutic modality has been evaluated in chronic inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease[5-7]. The potent anti-inflammatory effects of proteasome inhibitors have been chiefly attributed to reductions in the activation of nuclear factor-kappa B (NF-κB)[8, 9].

Bortezomib is a synthetic peptide boronate and inhibits the chymotrypsin-like activity of the 26S proteasome complex in UPS by a highly selective and reversible covalent bond with threonine in mammalian cells[9, 10]. It was the first proteasome inhibitor approved in 2003 by the US Food and Drug Administration (FDA) for multiple myeloma[9]. Bortezomib has been successfully used as a cytostatic anti-cancer drug for the treatment of mainly refractory or relapsed multiple myeloma[11].

Severe sepsis including septic shock demonstrates an exaggerated and uncontrolled inflammatory process, which results from the activation of NF-

κB through intracellular signaling via Toll-like receptors[12]. Many clinical trials to modulate the excessive inflammatory responses in Gram-negative sepsis mediated by lipopolysaccharide (LPS) have been performed, but immunomodulatory drugs did not show a mortality improvement in severe sepsis until now[13]. However, the development of new therapeutic modalities as the target or tailored-therapy is essential to further ameliorate the high mortality of severe sepsis. Also critical is the adaptation of current evidence-based therapeutic approaches, including early intensive resuscitation through early-goal directed therapy (EGDT) for the restoration of organ hypo-perfusion, as well as the Surviving Sepsis Campaign Program[14].

Proteasome inhibitors as an immune modulator may be a candidate of new therapeutic targets in severe sepsis[15]. The previous two studies using the mouse cecal and ligation puncture (CLP) model revealed that MG-132 and lactacystin (quercetin and mevinolin) in combination with antibiotics as the proteasome inhibitor decreased inflammatory response and prolonged survival[16, 17]. However, there are no known reports whether bortezomib with only clinical approval among the generated proteasome inhibitors can offer beneficial effects in severe sepsis. Therefore, this study evaluated the effect of bortezomib on the expression of inflammatory cytokines and mediators within an *in vitro* LPS-induced macrophage cell line and on survival in a murine peritonitis sepsis model induced by CLP to reveal the possibility of bortezomib as a new drug for severe sepsis.

II. MATERIALS AND METHODS

1. Cell culture

The murine-macrophage-like cell line, RAW 264.7, which is most commonly used in LPS-treated sepsis *in vitro* experiments, was prepared[17]. The RAW 264.7 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). The cells were maintained at 37°C in liquid growth media composed of Dulbecco's Modified Eagle Medium (DMEM), 10% fetal bovine serum (FBS), and penicillin (100 unit/mL)/streptomycin (100 μg/ml) (WelGENE Inc., Korea) in all experiments. The RAW 264.7 cells were cultured in a 37°C incubator with 5% CO_2 and 95% ambient air. Culture media was replaced twice a week.

2. Design and reagents of the *in vitro* experiment

In all *in vitro* experiments, the RAW 264.7 cells were seeded in the plate on day one and the growth media was changed from 10% FBS DMEM to 1% FBS DMEM on day two. On day three, LPS at various concentrations was applied to the RAW 264.7 cells 1 h after the application of bortezomib at various concentrations. All experimental processes were repeated in triplicate with identical protocols.

Lipopolysaccharide from *Escherichia coli*, serotype 055:B5 was purchased from Sigma Chemical (St. Louis, MO, USA). Bortezomib (Velcade® inj, 3.5 mg/V composed of 3.5 mg bortezomib and 35 mg mannitol) was obtained from Millennium Pharmaceuticals, Inc. (Cambridge, MA, USA). Bortezomib (1 mg) was dissolved in sterile 0.9% normal saline at a concentration of 1 mg/mL, and was then stored at −20°C until use. For the studies, a 100 μM stock solution was resuspended in 0.9% saline.

3. Proteasome activity assay

The Proteasome-Glo™ Chymotrypsin-like Cell–Based Assay Kit (Promega Corporation, Madison, WI, USA) was used for the measurement of proteasome inhibition activity of bortezomib. This assay uses a luminogenic proteasome substrate containing succinyl-leucine-leucine-valine-tyrosine-aminoluciferin in a buffer optimized for cell permeabilization, proteasome activity, and luciferase activity[18, 19]. Adding Proteasome-Glo™ Cell-Based Reagent in an "add-mix-measure" format results in proteasome cleavage of the substrate and in the generation of a luminescent signal produced by the luciferase reaction. Following cleavage by the proteasome, the substrate for luciferase (aminoluciferin) is released, allowing the luciferase reaction to proceed and produce light. Ten minutes after adding various substrates, the luminescence was measured by luminometer and expressed in relative luminescent units (RLU)[18, 19].

4. Cellular proliferation (viability) assay

The quantitative index of RAW 264.7 cell viability treated with LPS and/or bortezomib of various concentrations was determined by the Cell Counting Kit-8 (CCK-8) colorimetric assay. The CCK-8 assay uses a highly water-soluble tetrazolium monosodium salt, WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium. The sensitivity of CCK-8 is higher than other tetrazolium salts such as MTT, XTT, MTS or WST-1[20, 21]. RAW 264.7 cells were plated in 96-well microtiter plates at a density of 8 X 10^3 cells per well, then the live cell count was assayed using CCK-8 (Dojindo Molecular Technologies, Inc., Rockville, MD, USA) according to the manufacturer's protocol. In brief, 10 μL of CCK-8 solution was added to each well, and the plates were incubated in a CO_2 incubator for 4 h. Thereafter, 10 μL of 1% w/v sodium dodecyl sulfate (SDS) solution was added to each well, stored at room temperature and protected from light before the absorbance (optical density; OD) was measured at 450 nm.

5. Extraction of total cellular RNA and reverse transcription-polymerase chain reaction

The total cellular RNA was extracted using a commercial easy-spin^{TM} (DNA free) total RNA extraction kit, which minimizes the contamination of genomic DNA through the combination of solution formulation and column type (iNtRON BIOTECHNOLOGY Inc., Seoul, Korea)[22]. For RT-PCR study, 6 X 10^5 RAW 264.7 cells were seeded in a 60-mm dish on day one. On day three, the RAW 264.7 cells were harvested after 4 h of LPS treatment. Subsequently, RNA preparation and RT-PCR were performed for various inflammatory cytokines [tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin (IL)-1β, IL-6, and IL-10], and adhesion molecule [intercellular adhesion molecule-1 (ICAM-1)]. The first-strand cDNA was synthesized with Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase[23], oligo dT, and specific primers (50 pmole) from 2 μg of total RNA (BIONEER Inc., Seoul, Korea). Specific murine primers, as well as the amplification cycles and annealing temperatures for semi-quantitative RT-PCR used in this study are summarized in Table 1. Expression of mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was monitored in all semi-quantitative RT-PCR experiments as an internal control. The semi-quantitation for the RT-PCR product was performed using the Multi Gauge Version 3.0 program (Fujifilm, Tokyo, Japan).

Table 1. Primers and methods for reverse-transcription polymerase chain reaction (RT-PCR) used in this study.

Molecule	Primer	Amplification cycle	Annealing temperature	Reference
TNF-α	F:5'-CCTGTAGCCCACGTCGTAGC-3' R:5'-TTGACCTCAGCGCTGAGTTG-3'	30	56°C	24
IFN-γ	F:5'-CTTCTTCAGCAACAGCAAGGCGAAAA-3' R:5'-CCCCCAGATACAACCCCGCAATCA-3'	45	53°C	25
IL-1β	F:5'-ATGGAAATCTGCAGAGGCCTCC-3' R:5'-CTTTAGGAAGACACAAATTGCATGG-3'	30	56°C	26
IL-6	F:5'-TCCAGTTGCCTTCTTGGGAC-3' R:5'-GTGTAATTAAGCCTCCGACTTG-3'	40	53°C	24
IL-10	F:5'-CTGGACAACATACTGCTAACCGAC-3' R:5'-ATTCATTCATGGCCTTGTAGACACC-3'	40	53°C	27
ICAM-1	F:5'-TGCGTTTTGGAGCTAGCGGACCA-3' R:5'-CGAGGACCATACAGCACGTGCAG-3'	30	60°C	28
GAPDH	F:5'-TGAACGGGAAGCTCACTGG-3' R: 5'-TACAGCAACAGGGTGGTGGA-3'	30	56°C	29

Aberrations used: F, forward; R, reverse; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon-gamma; IL, interleukin; ICAM, intercellular adhesion molecule; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase

6. Nitric oxide assay

A nitric oxide (NO) detection kit (iNtRON BIOTECHNOLOGY, Seoul, Korea) utilizing diazotization assay (Griess method)[30] was used to analyze the effect of bortezomib on NO production after LPS stimulation. This kit can accurately detect NO^- concentration by indirectly measuring nitrite (NO_2^-), which is a by-product of NO transformation in living cells, and is based on the colorimetric change that occurs when naphthyl ethylenediamine is added to the by-product of the reaction between sulfanilamide and nitrite. The limit of detection is 2.0 μM nitrite. In advance, the reference curve with the nitrite standard solution was obtained to ensure an accurate nitrite amount. The absorbance was measured using a plate reader with a filter between 520-560 nm within 20-30 mins. On the third day of RAW 264.7 cell growth, the media supernatant, which applied by the bortezomib (0 or 50 nM) and then LPS (0 or 100 ng/mL) with 1 hr interval, was used for NO detection assay 16 and 24 h after bortezomib and/or LPS treatment.

7. Animal preparation and treatment

C57BL/6J mice were given a standard laboratory diet and water *ad libitum* and treated in accordance with the guidelines and regulations for the Care and Use of Laboratory Animals of Yonsei University, Seoul, Korea, and Institute of Laboratory Animal Resources Commission on Life Science National Research Council, USA. The mice were 7–8 weeks of age, and weighed 25–30 g, at the start of the experiments. All bortezomib and normal saline in this study were administrated intraperitoneally. This animal study was approved by Institutional Animal Care and Use Committee in Yonsei University Health System.

8. *In vivo* study design

For the negative control, both mice that received neither surgery nor

treatment (N=5) and were administrated normal saline (1 mL) 1 h before sham surgery (N=5) were used in this study. The mice that received normal saline (1 mL) 1 h before CLP surgery (N=8) were the positive control. To evaluate the impact of bortezomib dose on survival, each group that received bortezomib 0.01 mg/kg (N=8) or 0.1 mg/kg (N=8) 1 h before CLP surgery was compared with the positive control. To evaluate the effect of delayed administration of bortezomib on survival, mice (N=8) that received bortezomib 0.01 mg/kg 24 h after CLP surgery were compared with the positive control. The mice were assessed for survival up to seven days following surgery, and then mortality rates were compared between groups by survival analysis.

9. CLP and sham surgery

Mice were anesthetized with an intraperitoneal injection of 10 mg/kg (0.004 mL/10 g) xylazine (2% Rompun inj®, Bayer Korea. Ltd., Seoul, Korea) and 30 mg/kg (0.006 mL/10 g) of a 1:1 mixture of tiletamine and zolazepam (Zoletil™ 250 mg/5 mL, Virbac Korea, Seoul, Korea). The cecum was exteriorized through a midline abdominal incision of approximately 1 cm. For the induction of mid-grade murine peritonitis sepsis resulting in seven-day survival rate of approximately 40%, the cecum was ligated at half the distance between distal pole and the base of the cecum with 5.0 monofilament[31]. The ante-mesenteric side of the cecum was punctured through and through with a 23-gauge needle. A scant amount of luminal content was expressed through both puncture sites to ensure patency. The cecum was returned to the abdominal cavity, and the fascia and skin incision was closed with a 6.0 monofilament and surgical staples, respectively. Topical 1% lidocaine and bacitracin were applied to the surgical site post-operatively. All animals received a single intramuscular injection of trovafloxacin (Pfizer Inc, New York, NY, USA) at a dose of 20 mg/kg and subcutaneous fluid resuscitation

with 1.0 mL of normal saline immediately postoperatively. The mice were then returned to their individual cages and rewarmed using heat lamps until they regained normal posture and mobility[32]. Sham-operated animals underwent the same procedure without ligation and puncture of the cecum.

10. Histologic findings and pulmonary inflammatory score

All mice that were alive at day seven after surgery in each group were anesthetized with an intraperitoneal injection of a combination of 10 mg/kg (0.004 mL/10 g) of xylazine (2% Rompun inj®, Bayer Korea. Ltd. Seoul, Korea) and 30 mg/kg (0.006 mL/10 g) of a 1:1 mixture of tiletamine and zolazepam (ZoletilTM 250 mg/5 mL, Virbac Korea, Seoul, Korea). Lung tissue was harvested from all mice that were alive at day seven after surgery and immediately frozen in a -70°C LN_2 (liquid nitrogen) container until homogenization. All harvested lung tissues were stained with hematoxylin and eosin, and reviewed by a pathology specialist. Also, the same pathology specialist, who was unaware of the treatment assignment for these histologic samples, evaluated the lung inflammation scale through pulmonary inflammatory score[33]. The pulmonary inflammatory score was graded from 0 to 3 with this definition; 0=normal, 1=mild inflammation involving the peribronchial or perivascular area; 2=moderate inflammation involving less than 50% of parenchyma, and 3=severe inflammation involving more than 50% of the parenchyma [33].

11. Statistical analysis

Each *in vitro* experiment was replicated in triplicate, and the mean values of these results were used for the statistical quantification analysis. Results were expressed as mean ± the standard error of the mean (SEM). The mean values between the two groups were compared using an independent sample student T-test and a non-parametric Mann-Whitney U-test. Survival analysis was

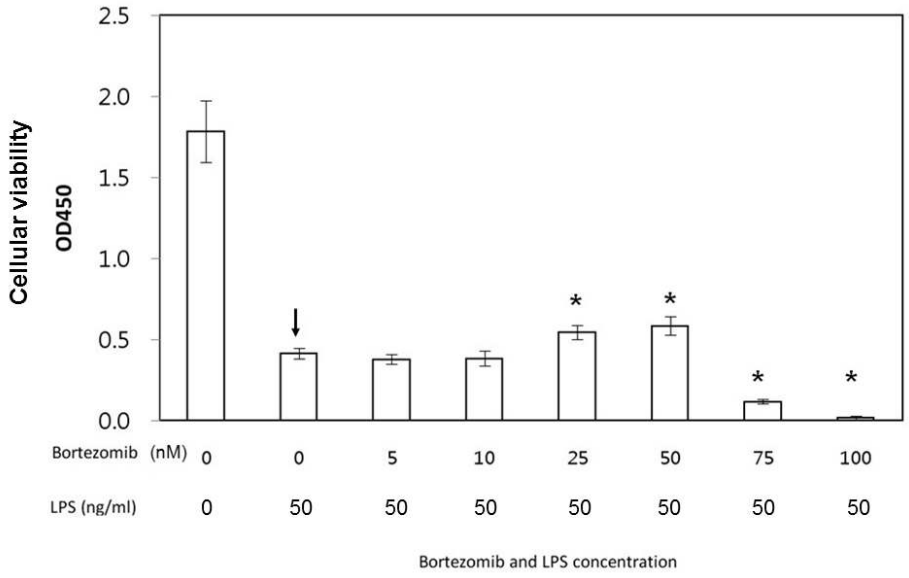
performed using the Kaplan-Meier curves. All statistical analyses were performed using Statistics Package for Social Science (SPSS version 18.0 for Windows; SPSS Inc., Chicago, IL, USA). All *P*-values were two-tailed and $P < 0.05$ was considered statistically significant.

III. RESULTS

1. The effect of pre-incubation with bortezomib 1 h before LPS treatment on cell proliferation

The concentrations of viable RAW 264.7 cells significantly decreased after the treatment of LPS alone at 50 and 100 ng/mL without pre-incubation of bortezomib (arrow in Fig. 1A, B). When bortezomib was pre-incubated 1 h before LPS application of 50 and 100 ng/mL, both 25 and 50 nM bortezomib significantly recovered the concentrations of viable RAW 264.7 cells compared with no pre-incubation. However, pre-incubation of higher bortezomib dose (75 and 100 nM) significantly decreased the concentrations of viable RAW 264.7 cells in both LPS 50 and 100 ng/mL groups (Fig. 1A, B).

(A)



(B)

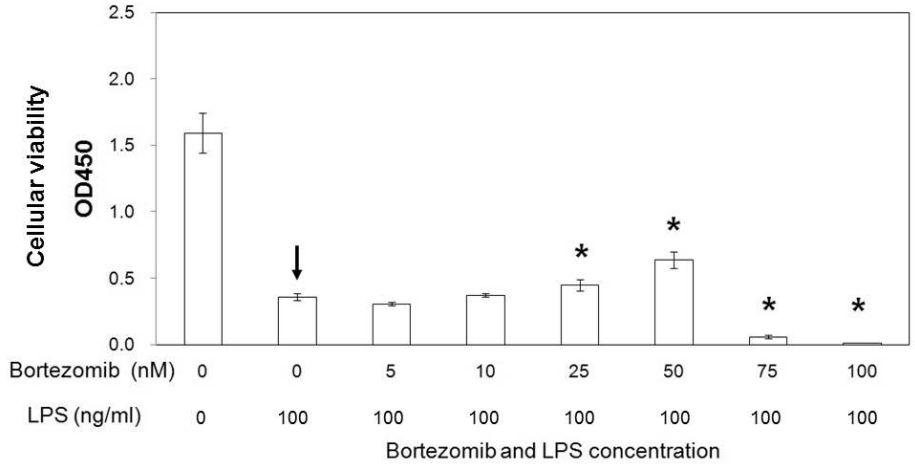


Fig. 1. The measurement of viable RAW 264.7 cell concentration using CCK-8 assay to evaluate the effect of pre-incubation with bortezomib 1 h before LPS treatment on the cell proliferation. On experimental day three, LPS at 50 ng/mL **(A)** and 100 ng/mL **(B)** were applied to growing RAW 264.7 cells of 8 X 10^3 cells/mL 1 h after the application of bortezomib with various concentrations from 0 to 100 nM. Arrows indicate the reference values for LPS application alone at 50 and 100 ng/mL without pre-incubation with bortezomib. Asterisks indicate statistical significance with *P*-value of less than 0.05 when compared with reference values (LPS alone, arrows).

2. The proteasome inhibition activity of bortezomib

The chymotrypsin-like activity of the proteasome in the RAW 264.7 cells, which were treated with only LPS (100 ng/mL) without bortezomib, did not decrease when compared with cells not treated with both bortezomib and LPS (Fig. 2). Whether the bortezomib (25 and 50 nM), which significantly increased the cell viability against LPS effect (Fig. 1A, B**)**, effectively inhibited the proteasome activity or not, was evaluated. Bortezomib treatment (25 and 50 nM) significantly decreased the proteasome activity when compared with the cells that were not treated with both bortezomib and LPS regardless of post-treatment with 100 ng/mL of LPS. Moreover, the proteasome activity exposed to high dose bortezomib at 50 nM was decreased compared to those in the low dose bortezomib at 25 nM (Fig. 2).

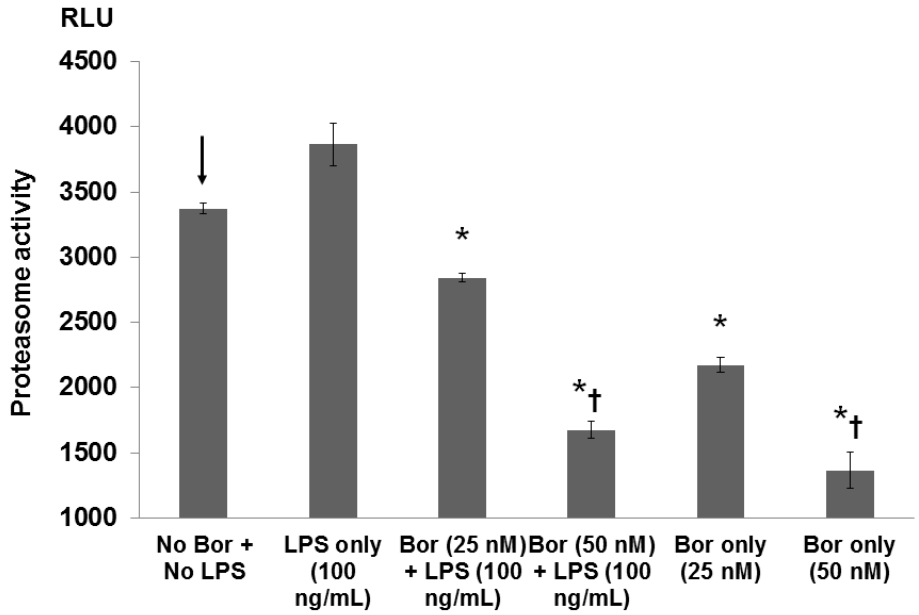


Fig. 2. The chymotrypsin-like activity of proteasome measured by

bioluminescent proteasome assay. The RAW 264.7 cells (arrows), which were not treated with bortezomib nor LPS (100 ng/mL), were used as the reference control for statistical significance (*P<0.01). † denotes statistical significance with a P-value of less than 0.01 when compared with bortezomib 25 nM (asterisks), respectively. Aberrations used: RLU, relative luminescent units. The results are reported as mean ± SEM of three independent experiments in triplicate.

3. The expression of inflammatory cytokines and adhesion molecules

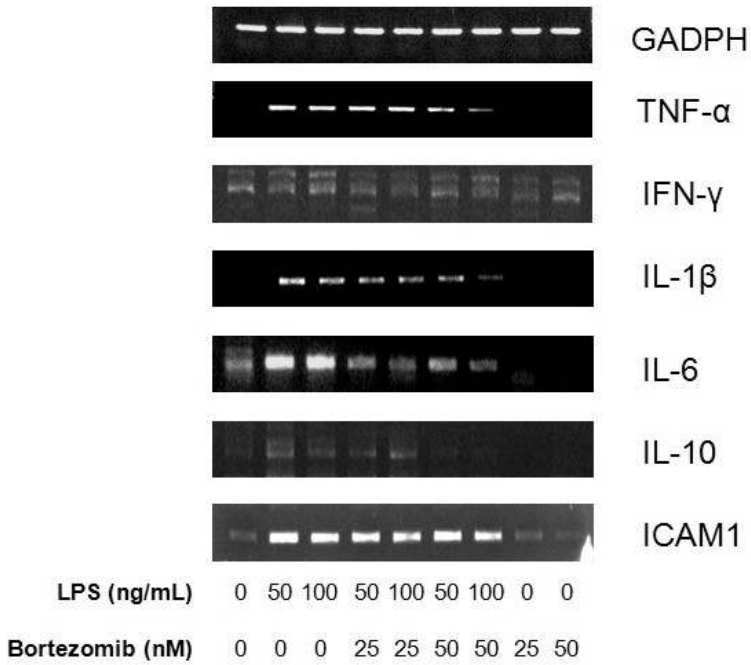
The degree of expression of inflammatory cytokines and adhesion molecules was evaluated through RT-PCR, to reveal the effects of bortezomib in cells stimulated by LPS. Bortezomib at concentrations of 0, 25, and 50 nM were administrated 1 h before the stimulation by LPS at 0, 50, and 100 ng/mL. TNF-α, IL-1β, and ICAM-1 were not expressed in RAW 264.7 cells, which received neither the bortezomib nor LPS treatment. In addition, these three molecules were also not expressed when the cells received only bortezomib (25 or 50 nM) without LPS stimulation. IL-6 and IL-10 were expressed in the RAW 264.7 cells, but not significantly detected in only bortezomib (25 or 50 nM)-treated cells except at very low levels. However, the IFN-γ shown the all expression without the significant difference irrespective of changes of bortezomib and LPS concentration (Fig. 3A). Therefore, we did not perform semi-quantitation for IFN-γ.

The results for the semi-quantitation are expressed in Fig. 4B. The levels of TNF-α significantly decreased in the cells that were pretreated with bortezomib at only 50 nM before the application of LPS at both 50 and 100 ng/mL when compared with the cells not pretreated with bortezomib before LPS stimulation. The lower dose of bortezomib (25 nM) did not show an effect. The expression levels of IL-1β and IL-6 significantly decreased in the cells pretreated bortezomib (25 and 50 nM) before the application of LPS with both 50 and 100 ng/mL when compared with the cells not pretreated bortezomib. The higher dose bortezomib (50 nM) showed a larger decrease effect for IL-1β than 25 nM in LPS-treated (50 and 100 ng/mL) mice. However, the decreased degrees of IL-6 level were similar irrespective of bortezomib and LPS concentrations.

The expression levels of IL-10 significantly decreased in the cells pretreated with bortezomib at both 25 and 50 nM before the application of LPS at 50 ng/mL when compared with the cells not pretreated with bortezomib. In the

cells receiving 100 ng/mL of LPS, pre-incubation with only 50 nM bortezomib significantly decreased the expression of IL-10. In the ICAM-1 results, the cells which were stimulated by the higher dose (100 ng/mL) LPS expressed significantly lower levels upon the pre-incubation with bortezomib at both 25 and 50 nM. However, the cells which were stimulated by the lower dose LPS of 50 ng/mL expressed significantly lower levels upon pre-incubation with only the lower dose (25 nM) bortezomib (Fig. 3B).

(A) RT-PCR



(B) Semi-quantitation of RT-PCR

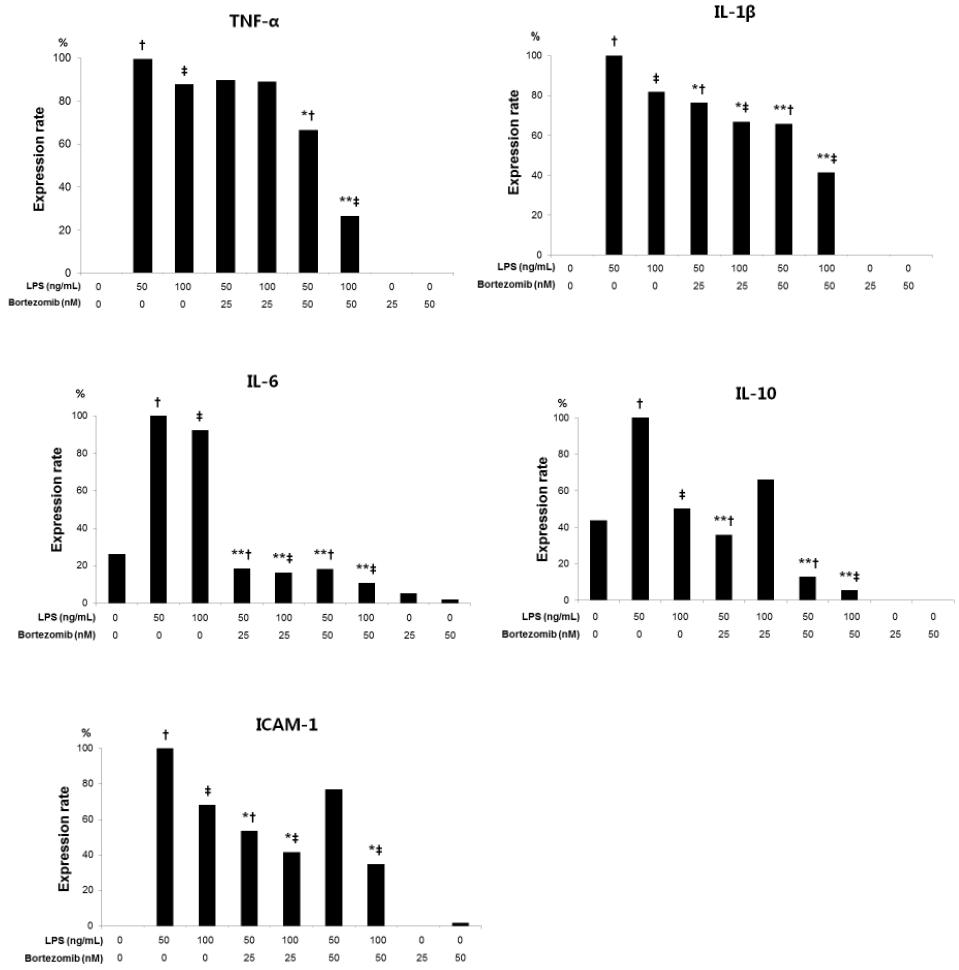


Fig. 3. The expression levels of inflammatory cytokines and intercellular adhesion molecule according to the application of bortezomib and LPS at various concentrations. Asterisks indicate statistical significance with a *P*-value of less than 0.01. Double asterisks indicate statistical significance with a *P*-value of less than 0.001. † and ‡ denote the reference values for the statistical comparison.

4. Measurement of nitric oxide levels after treatment of LPS and bortezomib

We confirmed a six-fold increase of NO production in RAW 264.7 cells after both 16 and 24 h treated only with LPS (100 ng/mL) without bortezomib. When bortezomib (50 nM) was pretreated before the application of LPS (100 ng/mL), NO levels significantly decreased compared with LPS alone, but increased compared with the RAW 264.7 cells that did not receive bortezomib or LPS as the negative control. However, the bortezomib (50 nM) treatment without LPS resulted in a change of NO levels, which was similar with the negative control (Fig. 4).

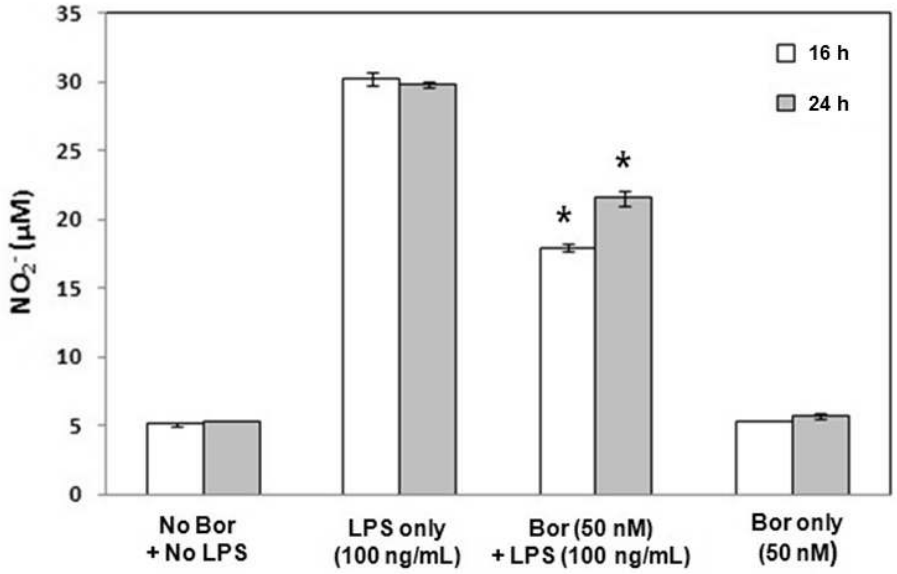


Fig. 4. The measurement of nitric oxide concentrations using diazotization assay (Griess method) to evaluate the effect of bortezomib on nitric oxide production after LPS stimulation. On the third day of RAW 264.7 cell growth, the supernatant of the media, which was applied upon bortezomib (0 or 50 nM) and then LPS (0 or 100 ng/mL) with 1-h interval treatment, was used for nitric oxide detection assay 16 and 24 h after bortezomib and/or LPS treatment. Asterisks denote a *P*-value less than 0.001 compared with the LPS

only treatment group for 16 or 24 h.

5. Impact of bortezomib administration on survival in the CLP-induced mouse sepsis model

In the negative control groups, all mice were living seven days after surgery. The seven-day survival rate in mice receiving bortezomib 0.01 mg/kg 1 h before CLP surgery was significantly higher than in the positive control (the mice receiving 1 mL normal saline 1 h before CLP surgery) (P=0.006) (Fig. 5A). However, the seven-day survival rate between mice administered bortezomib 0.1 mg/kg at 1 h before CLP surgery and the positive control was not significantly different (Fig. 5B).

The delayed administration of bortezomib 0.01 mg/kg at 24 h after CLP surgery had the higher seven-day survival rate compared with the positive control with borderline statistical significance (P=0.096) (Fig. 5C).

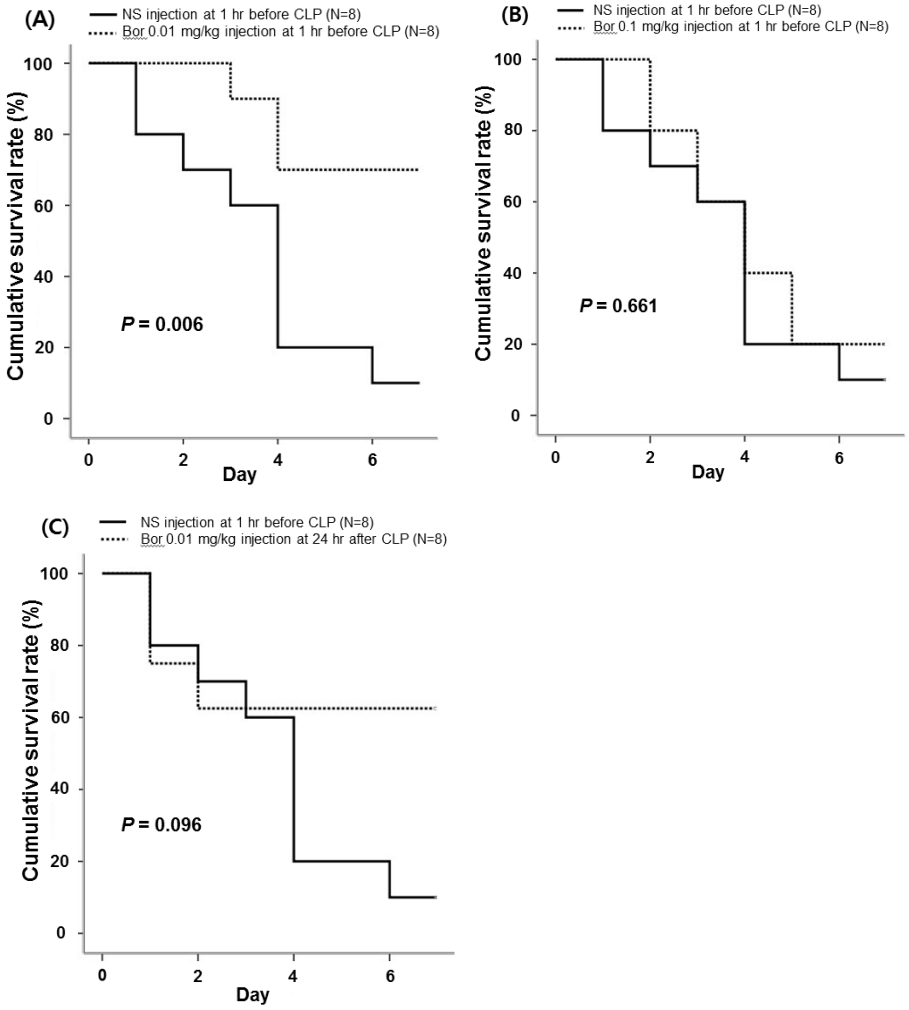


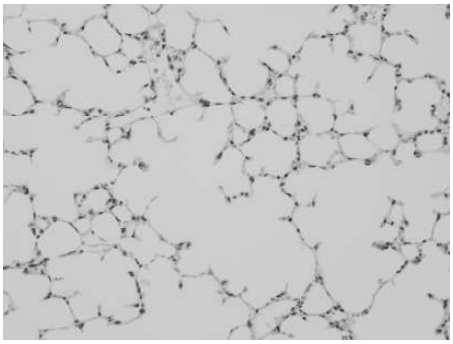
Fig. 5. Comparison of seven-day mortalities after CLP surgery by Kaplan-Meier survival curve. All bortezomib and normal saline treatments were injected intraperitoneally.

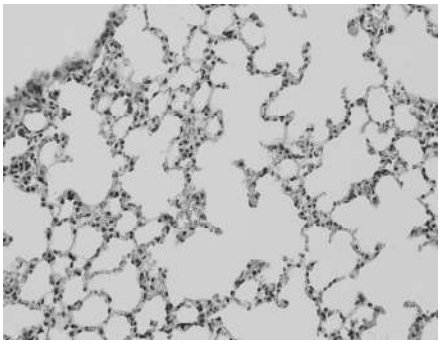
6. Histologic findings and mean pulmonary inflammatory score in the harvested lung tissue

In the H & E stain, the lungs of mice that received the bortezomib 0.01 mg/kg 1 h before CLP surgery showed minimal infiltration of inflammatory cells in the pulmonary parenchyma, smilar with the lungs of mice that received normal saline 1 h before sham surgery (Fig. 6B, D). However, the inflammation in mice that received bortezomib 0.1 mg/kg 1 h before CLP surgery was much higher compared to mice that received bortezomib 0.01 mg/kg 1 h before CLP surgery (Fig. 6D, F). The lung parenchyma in mice that received bortezomib 0.01 mg/kg 24 h after CLP surgery revealed increased inflammatory cell infiltration compared to mice that received bortezomib 0.01 mg/kg 1 h after CLP surgery (Fig. 6D, E), but a lower infiltration compared to mice that received bortezomib 0.1 mg/kg 1 h after CLP surgery (Fig. 6E, F).

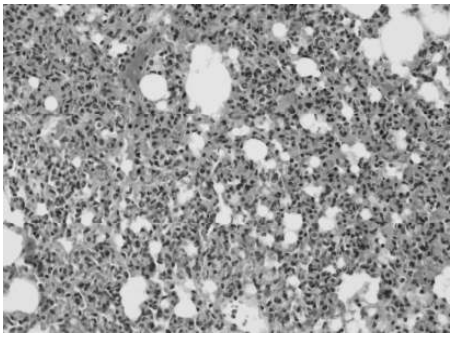
In the objective semi-quantitative analysis, the mice groups that received both bortezomib 0.01 mg/kg 1 h before and 24 h after CLP surgery had a significantly lower pulmonary inflammatory score than that of the CLP positive control group (Fig. 7)

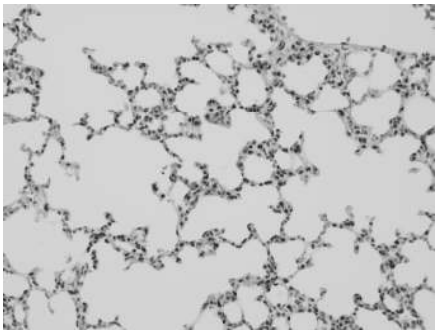
(A) **(B)**



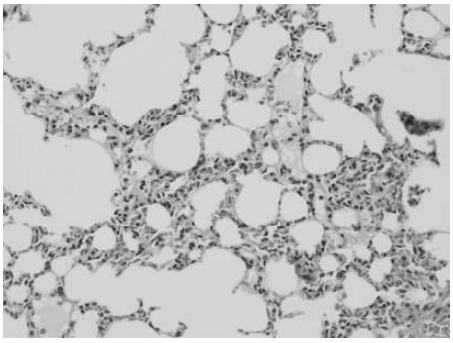


(C) **(D)**





(E) **(F)**



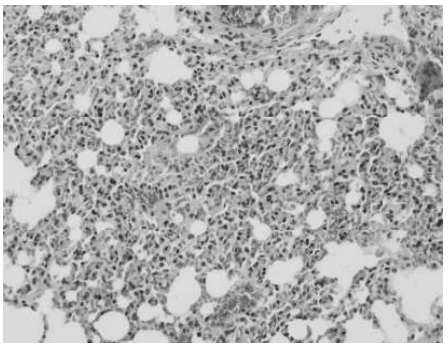


Fig. 6. Histologic findings of lung tissue, H & E staining (X 200). All lung tissues were harvested from the mice that were alive at day seven after surgery. (A) Normal mice (negative control), (B) Normal saline 1 mL injection i.p, 1 h before sham surgery (negative control), (C) Normal saline 1 mL injection i.p, 1 h before CLP surgery (positive control), (D) Bortezomib 0.01 mg/kg injection i.p., 1 h before CLP surgery, (E) Bortezomib 0.01 mg/kg injection, i.p., 24 h after CLP surgery, (F) Bortezomib 0.1 mg/kg injection, i.p., 1 h before CLP surgery.

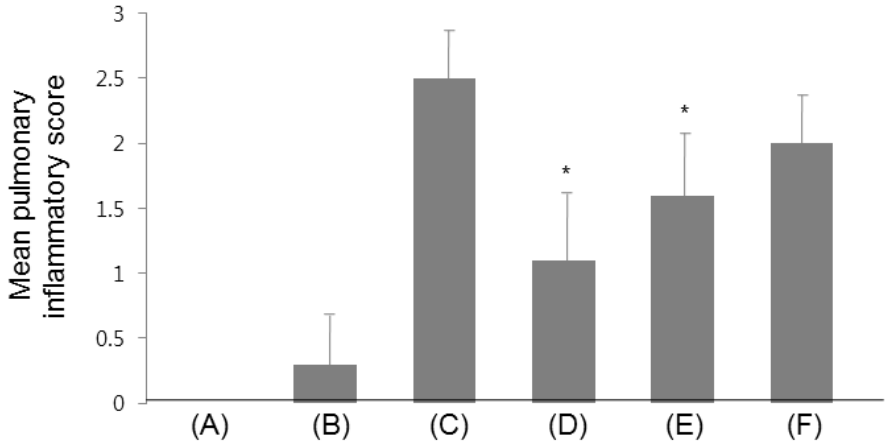


Fig. 7. Mean pulmonary inflammatory score in the harvested lung tissue. $^{*}P<0.05$ when compared to the CLP-positive control group (C). The upper bars represent the standard error of the mean (SEM). (A) Normal mice (negative control), (B) Normal saline 1 mL injection i.p, 1 h before sham surgery (negative control), (C) Normal saline 1 mL injection i.p, 1 h before CLP surgery (positive control), (D) Bortezomib 0.01 mg/kg injection i.p., 1 h before CLP surgery, (E) Bortezomib 0.01 mg/kg injection, i.p., 24 h after CLP surgery, (F) Bortezomib 0.1 mg/kg injection, i.p., 1 h before CLP surgery.

IV. DISCUSSION

Severe sepsis is the leading cause of death in critically ill patients and leads to multiple organ failure with injuries involving the kidneys, lungs and liver. The principal pro-inflammatory mediators in the pathophysiology of sepsis are TNF-α and IL-1β, which are released in response to bacterial toxins by monocytes, macrophages, and other leukocytes[34]. TNF-α and IL-1β activate NF-κB by triggering a signaling pathway that leads to the phosphorylation and consequent degradation of the inhibitor κBα (IκBα)[35]. The degradation of IκBα exposes a nuclear localization signal on the NF-κB protein, which then moves into the nucleus and stimulates the transcription of specific genes. The secretion of various proinflammatory cytokines characterizes the first phase of sepsis. The overproduction of proinflammatory mediators enhances adhesion molecules and leads to the deleterious effects associated with sepsis, including fever, hypotension, multiple organ failure, and shock[36].

The ubiquitin-proteasome system (UPS) is the major proteolytic system for non-lysosomal degradation of cellular proteins[10]. The UPS has significance in numerous cellular processes such as turnover and quality control of proteins[37], cell cycle and apoptosis[38, 39], transcription and cell signaling[40-43], immune response and antigen presentation[44], and inflammation and development[45, 46]. Bortezomib, a peptide-boronic acid analog, has been shown to have potent inhibitory activity of the chymotrypsin-like subunit in the 20S core of the proteasome[47]. It is the first drug of its kind that has received accelerated approval for the treatment of relapsed or refractory multiple myeloma[47]. A significant decrease in the levels of NF-κB, resulting in decreased production of inflammatory cytokines by the cell, after bortezomib treatment has been noted by several studies[48, 49]. This effect is a result of the lack of degradation of the inhibitor of kappa-B light polypeptide gene enhancer (IκB) protein[47]. In addition, protein processing and degradation is halted by the proteasome inhibitor, resulting in decreased cell adhesion molecule expression and anti-

apoptotic protein expression resulting in increased apoptosis[47]. It was reported that bortezomib significantly inhibited acute lethal graft-versus-host disease (GVHD) induction, associated with a significant decrease in TNF-α, IL-1, and IL-6[50]. Bortezomib also induced selective depletion of human alloreactive T lymphocytes, while decreasing the production of Th1 cytokines (IFN-γ and IL-2) *in vitro*[51].

The individual response for infection is determined by many factors, including the virulence of the organism, the size of the inoculum, coexisting conditions, and polymorphisms in genes for cytokines. The initial immune response is hyperinflammatory, but the response rapidly progresses to a hypoinflammatory reaction. A secondary bump in the hyperimmune state can occur during the hospital course with secondary infections. Otherwise, there may be an initial robust hyperinflammatory response. This patient would have extremely high plasma concentrations of TNF-α and other inflammatory cytokines. Death may occur due to a hyperinflammatory state, and anti-inflammatory treatments may improve the likelihood of survival. In the hypothetical patient with diabetes, chronic renal failure, and pneumonia, the initial response is blunted, and there is prolonged depression of immune function, culminating in death[34].

Numerous therapies that block pro-inflammatory cytokines (anti-TNF-α, TNF-α receptor, and IL-I receptor antagonist *et al.*) have failed, perhaps because the approach was narrowly focused, pathways are redundant, or cytokines are critical to host defense and their blockade is excessively immunosuppressive. Ibuprofen, platelet-activating factor acetyl hydrolase, bradykinin antagonists, and other therapies (elastase inhibitor, nitric oxide synthase inhibitor) have not improved survival among patients with sepsis[52].

Anti-cytokine or anti-inflammatory treatment in sepsis should be tailored to a patient's immune status. Anti-inflammatory strategies applied early in patients with a hyperinflammatory immune response may be life-saving. In

addition to TNF-α and IL-1β, other inflammatory mediators may have critical roles in mediating cell injury in sepsis. Recently, high mobility group 1 protein (HMGB1) was identified as a late mediator of the lethality of endotoxin in mice, and is correlated with sepsis outcome. Measurement of circulating concentrations of inflammatory mediators may prove to be useful in evaluating the stage of sepsis and in tailoring the administration of anti-inflammatory agents. Alternatively, anti-inflammatory agents used during the hypoimmune phase may worsen outcomes. When patients are determined to be in a hypoimmune state, inflammatory strategies that enhance the function of the innate or adaptive immune system may be efficacious[34].

Because the proteasome complex regulates the LPS-induced signal transduction and the proteasome inhibitor blocks the LPS-induced proteasome's chymotrypsin activity, as well as macrophage TNF-α secretion and the expression of multiple inflammatory mediator genes, the proteasome inhibitor may be an important therapeutic target in Gram-negative sepsis and septic shock[15, 53]. Safranek *et al.* reported that CLP induced a significant increase in plasma levels of IL-1, TNF-α, IL-6, and IL-10, but proteasome inhibitor MG-132 treatment resulted in a lower increase in IL-1, TNF-α, and IL-10 levels, and prolonged survival[16]. However, in this study, proteasome inhibitor was administrated 3 h before or after the induction of sepsis (CLP surgery)[16]. A few studies revealed that proteasome inhibition resulted in a decrease of the LPS-induced pro-inflammatory cytokines and adhesion molecules *in vitro,* and prevented LPS-induced shock in mice, but in most of these studies, the proteasome inhibitors were pretreated in macrophages or in mice before LPS challenge[15, 16, 53]. However, the protective effect of proteasome inhibitors in sepsis may not be uniformly applied to various immune states or various time differences after the development of sepsis or multiple organ dysfunctions in human beings. The principal aim of this study was to determine whether the administration of a proteasome inhibitor,

bortezomib, can modulate the expression of pro-inflammatory cytokines and mortality in a murine sepsis model induced by CLP surgery.

Although many animal sepsis models have been described, the best described and most commonly used model is the CLP model. A major advantage of the CLP model is that the animals develop an initial hyperdynamic cardiovascular response similar to what is observed in human sepsis, where cardiac output increase and systemic vascular resistance decrease. Additionally, animals develop bacteremia and signs of systemic sepsis, as well as a cytokine response that is similar to that found in patients with sepsis[54]. Therefore, the CLP mouse model was used in this study.

This study revealed that bortezomib decreases the inflammatory cytokines of TNF-α, IL-1β, IL-6, and IL-10 and ICAM-1 in LPS-stimulated cells of *in vitro* Gram-negative sepsis models. In addition, it decreases NO production. Our results suggest that bortezomib can reduce the uncontrolled exaggerated inflammatory process induced by LPS through the NF-κB pathway *in vitro*. The above data were supported by the cell viability assay and the measurement of proteasome activity. Bortezomib treatment of 25 and 50 nM significantly recovered the counts of viable cells in which the inflammatory process occurred by LPS. Moreover, we confirmed that bortezomib definitely plays a role as a proteasome inhibitor in our study via measurement of the chymotrypsin-like activity of the proteasome. To our knowledge, this study is the first *in vitro* experiment that evaluates the preincubation effect of the uniquely clinically approved bortezomib among proteasome inhibitors on inflammation developed by LPS.

However, bortezomib concentrations of 75 and 100 nM decreased cell viability compared to those LPS-stimulated cells without pre-incubation of bortezomib. This may be caused by the direct cellular toxicity of bortezomib. Proteasome inhibitors, including bortezomib, act as a direct inducer of apoptosis as well as inhibitors of cell proliferation in both tumor and normal

cells [10]. In addition, bortezomib can cause different cellular effects through the inhibition of the proteasome in a dose-dependent manner[55]. Given that peripheral neuropathy[56] is the most common adverse event of using bortezomib, and that accelerated cellular and organ damage can develope in patients with severe sepsis, the optimal dose of bortezomib for the treatment of severe sepsis must be carefully determined to minimize the direct toxicity of bortezomib.

In the bioluminescent proteasome assay, bortezomib treatment of both 25 and 50 nM did not completely inhibit of chymotrypsin-like activity. Therefore, bortezomib treatment of 25 and 50 nM could not sufficiently recover the viable cell count up to similar levels with those in cells without LPS stimulation. However, because of toxicity in higher bortezomib dose, we evaluated the effect of bortezomib on the expression of inflammatory cytokine using lower doses of 25 and 50 nM.

Although delayed administration of bortezomib after the initiation of sepsis via CLP surgery procedure did not have a significant advantage for improving survival, further study is warranted. Moreover, using a combination treatment of bortezomib and other immunomodulating drugs, including statin and/or macrolide or antibiotics, as well as different dose and administration times might reveal that the delayed administration of bortezomib after sepsis pathogenesis has significant therapeutic possibilities in clinical settings. Maninly, this is because most severe sepsis cases present after an initiated or advanced hyperimmune process. The new immunomodulating drug will have a clinically beneficial effect in delayed administration cases, after the development of sepsis. Further experiments of delayed administration with various concentrations of bortezomib and/or time intervals between bortezomib treatment and the development of sepsis will be necessary to determine the detailed clinical usefulness of bortezomib for severe sepsis treatment.

This study has several limitations. First, whether higher concentrations of bortezomib have cellular toxicity was not clearly examined. Second, histopathologic examination to compare the degree of pulmonary inflammation was performed in the delayed harvested lung tissue seven days after sepsis development. Therefore, further study is warranted to confirm the degree of organ failure in liver, kidney, lung, and spleen tissues immediately and shortly after the development of sepsis in all live mice. Third, the immunologic difference of individual mice was not evaluated within *in vivo* experiments, and we hypothesized that all mice have the same immunologic function and response in CLP sepsis model.

We revealed that the pre-incubation of bortezomib 1 h before LPS application reduces important pro-inflammatory cytokines and the pretreatment of bortezomib 1 h before CLP surgery ameliorates survival in a mouse peritonitis sepsis model. In conclusion, our study suggests the possibility of pre-treatment with bortezomib as a new therapeutic target in severe sepsis.

V. CONCLUSION

Proteasome inhibitors, as the immune modulator, may be a candidate for new therapeutic targets in severe sepsis cases. In this work, the effect of bortezomib, with only clinical approval among the proteasome inhibitors, was examined with regard to the expression of inflammatory cytokines and mediators via an *in vitro* LPS-induced macrophage cell line and on survival in a murine peritonitis sepsis model induced by CLP.

1. Preincubation with bortezomib (25 or 50 nM) before LPS (50 or 100 ng/mL) stimulation significantly increased the number of viable RAW 264.7 cells compared with no preincubation.
2. Bortezomib decreased inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-10 and ICAM-1, as well as NO production in LPS-stimulated cells.
3. The seven-day survival rate in mice administered bortezomib 0.01 mg/kg 1 h before CLP surgery was significantly higher than mice receiving normal saline 1 h before CLP surgery.
4. The administration of bortezomib 0.01 mg/kg 1 h before CLP surgery resulted in a significant decrease of lung parenchymal inflammation.

In conclusion, pretreatment with bortezomib showed a change of inflammatory mediators and increased survival rate within *in vitro* and *in vivo* mouse sepsis experiments.

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ABSTRACT (IN KOREAN)

Cecal ligation and puncture에 의하여 유도된 패혈증의 실험실적 모델에서 bortezomib의 투여가 inflammatory cytokine의 발현과 생존률에 미치는 영향

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한 상 훈

Bortezomib은 NF-κB 신호전달체계를 통하여 염증 반응을 조절할 수 있는 프로테아좀 억제제 중의 하나이다. Bortezomib이 임상적으로 사용되고 있는 유일한 프로테아좀 억제제이지만, 감염성 병원체에 의하여 유발되는 염증반응에서 bortezomib을 전처치하였을 경우 발생하는 면역조절 효과는 연구되어 있지 않다. 따라서, lipopolysaccharide (LPS)로 자극한 세포에서 bortezomib이 염증성 사이토카인의 분비에 미치는 영향을 분석하였다. RAW 264.7 세포에 다양한 농도의 bortezomib으로 전처치를 시행하고 1시간 후에 LPS로 세포를 자극하였다. 또한, C57BL/6J 마우스를 사용하여 결장 결찰 및 천공 (cecal ligation and puncture, CLP)을 통한 복막염 패혈증 실험을 시행하였다. Bortezomib 0.01 mg/kg와 0.1 mg/kg를 각각 8마리의 마우스에 복강 내로 투여하고 1시간이 경과하여 CLP 수술을 시행하였으며, bortezomib을 투여하지 않고 CLP 수술만 시행한 양성 대조군과 7일 째 생존률을 비교하였다. 7일까지 생존해 있는 마우스에서 폐 조직을 적출하여 폐 염증 세포의 침윤 정도를 조직병리 소견 및 pulmonary inflammatory

score를 사용하여 비교하였다. Bortezomib 25 또는 50 nM을 세포에 처리하고 1시간 후에 LPS 50 또는 100 ng/mL을 처리하였을 때 살아있는 RAW 264.7 세포의 수가 bortezomib을 전처치하지 않은 경우에 비하여 통계적으로 의미 있게 증가되었다. Bortezomib은 산화 질소 (nitric oxide, NO)의 생성 뿐만 아니라 TNF-α, IL-1β, IL-6, IL-10, ICAM-1의 염증 매게 물질의 생성을 감소시켰다. 마우스 패혈증 실험에서는 CLP 수술을 시행 하기 1시간 전에 bortezomib 0.01 mg/kg를 복강 내로 투여하였을 때 7일 생존률이 증가되고 폐 실질의 염증 세포 침윤 정도가 의미 있게 감소되는 것을 확인할 수 있었다. 결론적으로 본 세포 내 실험과 마우스 패혈증 모델 연구를 통하여 bortezomib의 사전 투여가 패혈증의 생존률을 향상시키고 염증 매게 물질의 발현 정도를 조절하는 것을 확인하였다. 이러한 결과들은 bortezomib이 과도한 염증성 반응을 특징으로 하는 중증 패혈증에서 새로운 염증조절물질제제의 치료제로서 사용될 수 있을 가능성을 제시하고 있다.

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