HSP40 translocates rapidly from the cytoplasm to the nucleus, and the intracellular pattern is similar to HSP70 [33]. In this study, we found that the expression of HSP40 as well as HSP70 was markedly up-regulated in MIF$^{-/-}$ mice in response to DSS. However, the up-regulation of HSP40 as well as HSP70 was lesser in the colon of WT mice than that of MIF$^{-/-}$ mice. These data provide the idea that MIF negatively interacts with HSP40 and HSP70 in the process of intestinal inflammation. Furthermore, we demonstrated that quercetin, which specifically inhibits the HSP synthesis via blocking the activation of heat shock factor-1 [34], inhibited the up-regulation of the HSP40 and HSP70 expressions and developed severe DSS-induced colitis in MIF$^{-/-}$ mice, suggesting that these HSPs are important molecules in the protection against DSS-induced colitis, especially in MIF$^{-/-}$ mice.

Very recently, we have demonstrated that geranylgeranylacetone up-regulates the expression of HSP70 and protect mice from DSS-induced colitis [35]. Moreover, we have shown that HSP70 induced by geranylgeranylacetone protects rat intestinal cells against oxidative injury [36]. Therefore, overexpression of HSP in colon is thought to reduce the severity of colitis. Since the induction of some HSPs was much greater in MIF$^{-/-}$ mice than in WT mice following DSS treatment, it is suggested that one of the mechanisms underlying the proinflammatory/protoxicant properties of MIF may be its inhibitory effects on induction of HSP in the event of inflammation and immune responses. Our results in this study provide the evidence that MIF affects the function of HSP in colon, however, further study is needed for full elucidation of interaction of MIF with HSP in colon.

In conclusion, we discovered that disruption of the MIF gene completely prevented mice from experimental colitis. More importantly, the diverse proinflammatory effects of MIF, including its novel inhibitory effects on HSP-responsiveness lend further support to the use of strategies which counter the biological activities of MIF.

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RAPID COMMUNICATION

Polaprezinc protects human colon cells from oxidative injury induced by hydrogen peroxide: Relevant to cytoprotective heat shock proteins

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Abstract

AIM: To investigate the effect of polaprezinc on cellular damage induced by hydrogen peroxide (H_2O_2) in human colon CaCo2 cells.

METHODS: CaCo2 cells were treated with polaprezinc (10-100 μmol/L) for 6 h. After polaprezinc treatment, the cells were incubated with H_2O_2 (20 μmol/L) for 1 h. Cell viability was measured by MTT assay. Western blot analysis for heat shock protein (HSP) 27 and HSP72 in the cells was performed. Moreover, cells were pretreated with quercetin (200 μmol/L), an inhibitor of HSP synthesis, 2 h before polaprezinc treatment, and cell viability and the expression of HSP27 and 72 were assessed in these cells.

RESULTS: Polaprezinc significantly protected CaCo2 cells from cell damage induced by H_2O_2, and up-regulated the expressions of HSP27 and HSP72 in the cells (10, 30 and 100 μmol/L of polaprezinc; 35.0% ± 7.7%, 58.3% ± 14.6% and 64.2% ± 8.2%, respectively. $P < 0.01$ *versus* polaprezinc-nontreated cells; 6.0% ± 4.4%). Quercetin inhibited the up-regulation of HSP27 and HSP72 by polaprezinc and diminished the protective effect of polaprezinc against H_2O_2-caused injury in the cells.

CONCLUSION: Polaprezinc is a useful therapeutic agent for treatment of colitis and its effects depend on the function of cytoprotective HSP in colon.

Key words: Heat shock protein; Oxidative injury; Polaprezinc

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INTRODUCTION

Polaprezinc [N-(3-aminopropionyl)-L-histidinato zinc], an antiulcer drug, is a chelate compound consisting of zinc ion, L-carnosine, dipeptide of β-alanine, and L-histidine and has an antioxidant effect and anti-*H pylori* activity[1-4]. It has been reported that administration of polaprezinc prevents gastric mucosa from tissue injury in experimental models[5-9]. Additionally, recent works indicate that polaprezinc has a therapeutic effect in two models of experimental colitis[10,11]. On the other hand, some studies have shown that polaprezinc up-regulates the expression of heat shock protein (HSP) in stomach and colon[11,12].

HSP, a highly conserved and ubiquitous protein, is up-regulated to protect against various physiological stress conditions such as infection and ishcemia[13]. Some HSPs are now accepted to be key anti-inflammatory molecules and play an important role in the protection against physiologic and environmental stressors[14]. Overexpression of these HSPs are thought to prevent apoptosis by regulating intracellular intermediates intimately involved in apoptotic signaling. In intestine, up-regulation of these HSPs by chemicals or non-lethal thermal stress has been shown to protect intestinal epithelial cells and colon tissues against injurious stimulants *in vitro* and *in vivo*[15-20]. In particular, HSP27 and HSP72 protect cells and tissues from chemical, infectious and ischemic injury[13]. On the other hand, our previous study demonstrated that polaprezinc up-regulates the expression of HSP27 and HSP72 in the mouse colon[11], but there are no investigations of the effect of polaprezinc on HSP expression in intestinal epithelial cells *in vitro*. Thus, we herein assessed the effect of polaprezinc on cell damage induced by oxidative stress, which often occurs in intestine

and injured colonic epithelial cells *in vitro*. Moreover, we investigated whether the protective effect of polaprezinc depends on the function of HSPs in human colon cells.

MATERIALS AND METHODS

Materials

Polaprezinc was a gift from Zeria Pharmaceuticals Co. (Saitama, Japan). Antibodies against HSP25 and HSP72 were obtained from Stressgen (Victoria, BC, Canada). Nitrocellulose membrane filters were from Millipore (Bedford, MA). An ECL Western blotting detection system kit was from Amersham Bioscience (Piscataway, NJ, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody and a Micro BCA protein assay kit were from Piece (Rockford, IL, USA). Quercetin was from Wako Pure Chemical Industries (Osaka, Japan). A CellTiter 96 aqueous one solution cell proliferation assay kit was from Promega (Madison, WI, USA) and an anti-actin antibody was from Sigma-Aldrich Co. (Temecula CA, USA). All other chemicals were of reagent grade.

Cell culture and treatment with polaprezinc

CaCo2 cells (between passages 8 and 14) were grown in high glucose Dulbeco's Vogt modified Eagle's media (DMEM, Sigma, St Louis, MO, USA) supplemented with 100 mL/L fetal calf serum. Cells were incubated at 37℃ in 50 mL/L CO_2 and 90% humidity. Each experiment was performed with an 80%-90% confluent monolayer. Polaprezinc was diluted in DMEM and added to the cultured cells at a final concentration of 10-100 μmol/L for 6 h.

Examination of cell viability

To examine the protective effect of polaprezinc on CaCo2 cells against oxidant-induced injury, we assessed cell viability by MTT assay as described previously[20]. Briefly, cells were grown to an approximate cell concentration of 10^4 cells/well in 96-well plates. Cells in each well were incubated at 37℃ with 150 μL of DMEM and 50 μL of CellTiter 96 aqueous, one solution cell proliferation reagent. Cell viability was determined by the generation of a formazan dye from the substrate. Absorbance (A) at 490 nm was measured with a spectrometer 0 and 90 min after addition of CellTiter 96 aqueous, one solution cell proliferation reagent. Difference in A between 0 and 90 min after incubation was calculated for evaluation of cell viability. The assay was run on cells without treatment as a negative control. The results were compared to those in a negative control and expressed as percentage of cell viability. All experiments were repeated more than three times to confirm reproducibility.

Western blot analysis

Cells were collected with 1 g/L trypsin and homogenated with a Polytron homogenizer (Kinematica, Lucerne, Switzerland). Equal amounts of homogenates were dissolved in 20 μL of Tris-HCL, 50 mmol/L (pH 6.8), containing 10 g/L 2-mercaptoethanol, 20 g/L SDS, 200 mL/L glycerol and 0.4 g/L bromophenol blue. The samples were heated at 100℃ for 5 min, then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically onto a nitrocellulose membrane. The membranes were blocked with 10 mL/L nonfat dry milk in PBS, probed with HSP 27 and 72 Ab, and reacted with goat anti-rabbit IgG Ab coupled with horseradish peroxidase (HRP). The resultant complexes were processed for the ECL detection system according to the manufacturer's protocol. Protein concentration in the homogenate was quantified using a Micro BCA protein assay reagent kit (Pierce, Rockford, IL, USA).

Quercetin treatment

Quercetin is known to strongly inhibit the HSP synthesis[15,20,21]. To investigate the effect of quercetin, cells were pretreated with quercetin at a final concentration of 200 μmol/L for 2 h before polaprezinc treatment. Cell viability and expression of HSP27 and HSP72 were assessed in the cells treated with quercetin.

Statistical analysis

Data obtained by MTT assay were presented as mean ± SD and statistically analyzed using an analysis of variance (ANOVA), followed by Turkey's comparison test (Stat View, SAS Institute, Cary, NC). $P < 0.05$ was considered statistically significant.

RESULTS

Polaprezinc protected CaCo2 cells against hydrogen peroxide

We assessed the effect of polaprezinc on oxidative injury in the CaCo2 cells. Morphological alteration and growth inhibition were not observed in the CaCo2 cells after exposure to polaprezinc (data not shown). To evaluate the effect of polaprezinc on oxidative stress, cell viability in CaCo2 cells treated with NH_2Cl was analyzed by MTT assay. MTT assay showed that, the difference in A at 490 nm, as a parameter of cell viability, was significantly decreased in the cells treated with hydrogen peroxide at a final concentration of 20 mol/L (6.0% ± 4.4%). In contrast, we found that 10, 30 and 100 μmol/L of polaprezinc (35.0% ± 7.7%, 58.3% ± 14.6% and 64.2% ± 8.2%, respectively) significantly improved viability in the cells at 6 h after polaprezinc treatment compared with the cells without polaprezinc treatment ($P < 0.01$).

Enhancement of HSP27 and HSP72 expression in CaCo2 cells treated with polaprezinc

For assessment of the HSP expression in the CaCo2 cells treated with polaprezinc, Western blot analysis for HSPs in the cells was carried out. It was found that HSP27 and HSP72 were constitutively expressed in the CaCo2 cells without treatment. The expressions of HSP25 and HSP72 was greatly up-regulated in the CaCo2 cells treated with 10, 30 and 100 μmol/L of polaprezinc for 6 h compared with those in the non-treated CaCo2 cells (Figure 1).

Quercetin inhibited up-regulation of HSP27 and 72 and diminished the protective effect of polaprezinc on oxidative injury in Caco2 cells

To clarify the effect of HSPs induced by polaprezinc

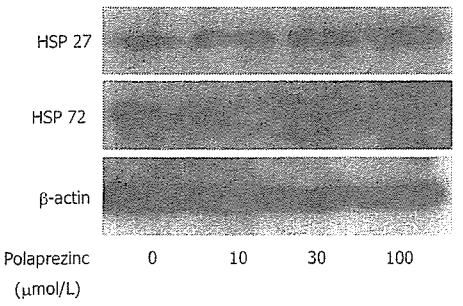

Figure 1 Effect of polaprezinc on up-regulation of heat shock protein HSP27 and HSP70 in CaCo2 cells. The expression of HSP27 and HSP72 was markedly up-regulated in the CaCo2 cells treated with polaprezinc. Representative results of three complete experiments are shown.

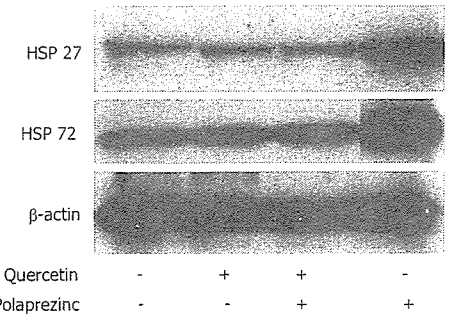

Figure 2 Effect of quercetin on induction of HSP27 and HSP72 in CaCo-2 cells treated with polaprezinc. Quercetin did not up-regulate the expression of HSP27 and HSP72 in the cells. Representative results of three complete experiments are shown.

on cell injury, we investigated the effect of quercetin, an inhibitor of HSP synthesis, and polaprezinc on Caco2 cells injured by H_2O_2. Pretreatment with 200 μmol/L of quercetin for 2 h considerably reduced the cell viability in the CaCo2 cells treated with 30 μmol/L of polaprezinc compared with the cells without quercetin treatment when cells were exposed to H_2O_2 ($P < 0.01$; 12.0% ± 0.3% and 53.6% ± 1.9%, respectively), whereas the cell viability in the quercetin-treated CaCo2 cells without H_2O_2 was minimal compared with non-treated cells (94.3% ± 0.1% and 98.8 % ± 0.1%, respectively). In HSP expression, 200 μmol/L of quercetin completely inhibited the up-regulation of HSP27 and HSP72 by polaprezinc in the CaCo2 cells (Figure 2). On the other hand, quercetin did not alter the baseline level of HSP27 and HSP72 in the cells without polaprezinc treatment (Figure 2).

DISCUSSION

In this study, we demonstrated that polaprezinc improved cell viability of CaCo2 cells injured by oxidative chemicals. Moreover, polaprezinc remarkably up-regulated the expression of HSP27 and HSP72, which play an important role in protecting the cells from stresses. Additionally, quercetin, an inhibitor of HSP synthesis diminished the protective effect of polaprezinc on cell injury by H_2O_2 and the up-regulation of HSP27 and HSP72 in CaCo2 cells. These results are consistent with our previous observation that polaprezinc can up-regulate the expressions of HSP27 and HSP70 in murine experimental colitis[11]. Recent studies have demonstrated that the expression of HSP27 and HSP70 is up-regulated by mild physiological stress and irritants[13,14]. Furthermore, HSPs have been suggested to be key anti-inflammatory molecules and play a critical role in protective mechanism against severe physiologic and environmental stressors[13,14]. High expression levels of HSPs are thought to prevent apoptosis by regulating intracellular intermediates intimately involved in apoptotic signaling.

In intestine, induction of HSPs by chemicals or non-lethal thermal stress has been shown to protect intestinal cells and colon tissues against injury and damage[15-19]. Musch *et al*[15] reported that induction of HSP72 by hyperthermia protects rat intestinal epithelial cells from oxidative injury. Hyperthermia rapidly and reproducibly induces HSPs in intestinal epithelial cells. Otani *et al*[16] demonstrated that preinduction of HSPs by non-lethal hyperthermia protects rats from colitis induced by acetic acid. Hyperthermia elevates the expression of HSPs, including HSP72, and remarkably reduces the severity of acetic acid-induced colitis in the colons of rats, suggesting that HSPs are important for the protection against colon tissue damage such as colitis. Although numerous studies have clarified the protecting effect of polaprezinc, there are a few investigations on the up-regulation of HSP by polaprezinc. Very recently, it has been reported that polaprezinc up-regulates the expression of HSP72 in cultured rat gastric mucosal cells (RGM1) and rat gastric mucosa[12]. In colon, our previous study demonstrated that polaprezinc enhances the up-regulation of HSP72 in mouse colon during colitis[11]. However, these studies have not fully clarified the role of HSP induced by polaprezinc. In this study, we demonstrated that quercetin, an inhibitor of HSP synthesis, diminished the protective effect of polaprezinc on cell damage induced by H_2O_2 and up-regulated HSP, suggesting that polaprezinc-induced HSP is essential for protection against oxidative injury in colon cells.

Besides HSP70, HSP27, a member of the small HSP family, have also been found to play an important role in protection of cells against stresses[13]. Ropeleski *et al*[18] demonstrated that IL-11-induced HSP27 has a protectiefve effect against oxidative stress induced by monochrolamine in cultured rat intestinal epithelial cells, as HSP27 plays a potential role in cytoprotection in intestine. Consistent with these findings and our previous findings, the expression of HSP27 is markedly up-regulated in the CaCo2 cells treated with polaprezinc. Howevere, quercetin did not up-regulate the expression of HSP27 in CaCo2 cells treated with polaprezinc in our study. These findings provide the idea that the protective effect of polaprezinc depends not only on the function of HSP72 but also on HSP27 in colon cells.

The mechanism by which polaprezinc up-regulates the expression of HSPs has not been precisely elucidated. Interestingly, it has been reported that zinc or L-carnocine

cannot up-regulate the expression of HSPs in gastric epithelial cells and rat gastric mucosa[12]. Our previous study indicates that zinc supplement does not induce HSPs in mouse colon[11]. Although we did not show the effects of zinc or L-carnocine on induction of HSPs in colon cells, polaprezinc may be a powerful inducer of HSPs compared with zinc or L-carnocine. The reason why polaprezinc but not zinc or L-carnocine markedly up-regulates the expression of HSPs is unclear, thus, further study is needed to clarify this result.

In conclusion, polaprezinc protects colon cells from oxidative injury induced by hydrogen peroxide, and enhances the expression of HSP27 and HSP72 in the CaCo2 cells. The up-regulation of cytoprotective HSPs by polaprezinc is potentially therapeutic for intestinal injuries such as colitis.

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Protective effect of geranylgeranylacetone on trinitrobenzene sulfonic acid-induced colitis in mice

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Abstract. Geranylgeranylacetone (GGA) has recently been reported to have a protective effect against ischemic, injurious and apoptotic stress in several tissues. The aim of this study was to determine the effect of GGA on colitis induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS) in mice. Colitis was induced by intrarectal instillation of TNBS in 50% ethanol in BALB/c mice. Survival, change in body weight and change in wet colon weight were assessed. Histological score in the colon was evaluated 5 days after TNBS treatment. The level of myeloperoxidase (MPO) activity in the colon was also determined. Immunohistochemistry for CD4 in the colon was performed. In addition, the level of heat shock protein (HSP) 70 in the colon was determined by Western blot analysis. Mice were orally treated with GGA (300 mg/kg) 2 h before and every other day after starting TNBS administration. Treatment with GGA markedly improved the survival rate, and reduced the loss of body weight and loss of wet colon weight in mice with TNBS-induced colitis. GGA also suppressed the increase in MPO activity and the number of CD4-positive cells infiltrating the colons of mice with TNBS-induced colitis. Furthermore, treatment with GGA remarkably up-regulated the expression of HSP70 in the colons of mice with TNBS-induced colitis. Our results provide further evidence that GGA has therapeutic potential for intestinal inflammation.

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Abbreviations: HSP, heat shock protein; GGA, geranylgeranylacetone; TNBS, trinitrobenzene sulfonic acid; MPO, myeloperoxidase; IBD, inflammatory bowel disease

Key words: colitis, geranylgeranylacetone, heat shock protein, inflammatory bowel disease, trinitrobenzene sulfonic acid

Introduction

Ulcerative colitis and Crohn's disease are the major inflammatory bowel diseases (IBDs) in humans and are characterized by chronic relapsing intestinal inflammation. Although numerous studies on IBD have been carried out, the etiology of IBD still remains unknown (1). Recent studies have demonstrated that various inflammatory mediators, including tumor necrosis factor (TNF)-α and macrophage migration inhibitory factor (MIF) are involved in the pathogenesis and exacerbation of IBD (2-4).

5-Aminosalicylic acid (ASA), glucocorticoids, and immunosuppressive and immunoregulatory agents are commonly used for the treatment of IBD (1). Unfortunately, some IBD patients are unresponsiveness to treatment with these agents. Recently, infliximab, an anti-TNF-α monoclonal antibody, has been used for the treatment of Crohn's disease (5,6). However, side effects, such as infection and malignancy, have been reported in some IBD patients (7,8).

Geranylgeranylacetone (GGA) is an acyclic polyisoprenoid that can protect the stomach from mucosal injury such as ulceration. This compound is effective for protecting the gastric mucosa from various stresses (9,10). In addition, GGA increases synthesis and secretion of gastric mucin (11) as well as components of high-molecular-weight glycoproteins and surface-active phospholipids (12). Interestingly, it has been reported that GGA enhances the expression of heat shock protein (HSP) in gastric mucosal cells and in several tissues and that it protects cells and tissues from destruction and inflammation (13-16). HSP is considered to be a stress-inducible protein that exhibits protective properties and regulates the immune response (17). HSP plays a pivotal role in operating as an intracellular chaperone for aberrantly folded or mutated proteins, and it provides a protective function to cells under stressful conditions (17). HSP70, named after its molecular weight of approximately 70 kDa, has a strong cytoprotective function against stress in the stomach, liver and heart (18-20). Transgenic overexpression of HSP70 also protects the mouse heart against tissue injury (21,22). In the human bowel, the expression of HSP70 is enhanced in ulcerative colitis compared to non-specific colitis in the colonic mucosa (23). Another study has shown that HSP70 induced

by heat stress contributes to the suppression of acetic acid-induced colitis in rats (24). However, the role of HSP70 in colitis has not been fully elucidated. Therefore, the aim of this study was to investigate the effect of GGA on experimental colitis induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS) in mice and to determine whether GGA enhances the expression of HSP70 in mice with TNBS-induced colitis as well as in other experimental models.

Materials and methods

Animals and study protocol. Specific pathogen-free 8-week-old BALB/c male mice weighing 22-25 g were obtained from Japan Charles River Co. (Shizuoka, Japan). Mice were comfortably housed under standard conditions of temperature, humidity and light/dark cycle. Mice could access food and water *ad libitum*. All experimental protocols adhered to the Declaration of Helsinki and were approved by the Animal Experiment Ethics Committee of the Graduate School of Medicine of Hokkaido University.

Mice were starved for 12 h and colitis was induced in mice by intrarectal administration of 0.5 mg of TNBS (Sigma, St. Louis, MO) in 0.1 ml of 50% ethanol using a nylon catheter as previously described (25). The mice were weighed and visually inspected every day beginning on day 0. The percent weight change for each individual mouse compared to the weight on day 0 was calculated. On the 5th day after TNBS administration, the mice were euthanised by intraperitoneal injection of thiopental and the colon tissue was removed. The wet colon weight was immediately measured. For assessment of histology, colon tissue was longitudinally opened, fixed with 10% neutral buffered formalin, and embedded in paraffin. For analyses of myeloperoxidase (MPO) activity and HSP70, colon tissues were stored at -80°C until use. For determination of the survival rate, each mouse was intrarectally administered 1 mg of TNBS in 0.1 ml of 50% ethanol.

Histologcal evaluation. After deparaffinization of thin tissue sections on glass slides, the colon tissue was stained with hematoxylin and eosin (H&E). Histological evaluation was microscopically evaluated and quantitated by an expert pathologist using the method described previously (25). Briefly, the degree of inflammation was graded from 0 to 4 as follows: 0, no leukocyte infiltration; 1, low level of leukocyte infiltration; 2, moderate level of leukocyte infiltration; 3, high level of leukocytic infiltration, high vascular density, and thickening of the colon wall; 4, transmural leukocyte infiltration, loss of goblet cells, high vascular density, and thickening of the colon wall. All slides were blinded for scoring.

GGA treatment. GGA was provided by Eisai Co. (Tokyo, Japan), as an emulsion with 5% gum arabic and 0.0008% α-tocopherol. GGA was given to mice orally in a volume of 5 ml/kg/time at a dose of 300 mg/kg/time through metal tubing attached to a 1-ml syringe. Mice in the control group were given the same dose of vehicle. Mice in each group were given GGA or vehicle 2 h before and every other day after starting administration of TNBS.

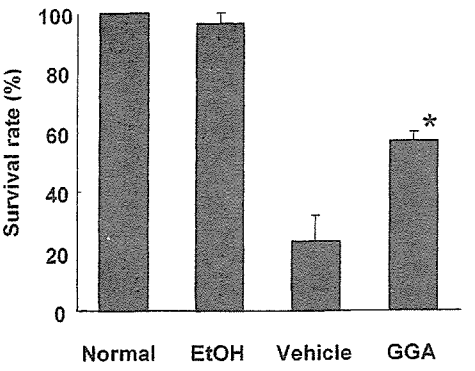


Figure 1. Effect of GGA on survival rate of mice with severe TNBS-induced colitis. Colitis was induced by intrarectal instillation of 1 mg of TNBS with 0.1 ml of 50% ethanol. Mice were repeatedly treated with 300 mg/kg of GGA every other day up to day 5. Values are expressed as mean ± standard error (SE). Three separate experiments were performed (n=10/time). *P<0.01 vs. vehicle-treated mice.

Measurement of myeloperoxidase activity. The level of myeloperoxidase (MPO) activity was determined in the colon as described previously (27,28). Briefly, a tissue specimen (~300 mg) was homogenized in 50 mM potassium phosphate buffer (pH 6.0) with 0.5% hexadecyltrimethylammonium bromide using a Polytron-type homogenizer for 3x30 sec on ice. The sample was centrifuged at 20,000 x g for 20 min at 4°C, and the supernatant was collected. Each sample (100 μl) was added to 2.9 ml of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/ml O-dianisidine hydrocholoride and 0.0005% hydrogen peroxide, and the MPO activity in the sample was measured using a spectrometer at 25°C. The protein concentration of the supernatant was determined using a Bradford assay kit (Bio-Rad Laboratories, Hercules, CA) for calibration, and values were standardized using MPO purified from human leukocytes (Sigma). Absorbance at 460 nm was determined for three separate 30-sec intervals. One unit of MPO activity was defined as 1 μmol H_2O_2 broken down to H_2O and O- by MPO. The final value of MPO activity was represented as units per gram of tissue.

Immunohistochemistry. Immunohistochemical analysis for CD4 was performed with a Vectastain ABC kit according to the manufacturer's protocol. A frozen sample of colon tissue was cut to a 5-μm-thick section. The section was pretreated with 3% H_2O_2 for 10 min at 4°C and then treated with 10% normal goat serum for 30 min at room temperature followed by overnight incubation with an anti-CD4 antibody (diluted 1:50, BD Bioscience, San Jose, CA, USA) at 4°C. CD4-positive staining was visualized with diaminobenzidine as a chromogen. The numbers of CD4-positive staining cells in the colon mucosa were counted microscopically in a blind fashion.

Western blot analysis for HSP in colon tissue. Colon tissue was disrupted using a Polytron homogenizer (Kinematica, Lucerne, Switzerland). The homogenate was dissolved in 20 μl of Tris-HCl, 50 mM (pH 6.8), containing 2-mercaptoethanol (1%), SDS (2%), glycerol (20%) and bromophenol blue (0.04%), and heated at 100°C for 5 min. The sample was then subjected to SDS-polyacrylamide gel electrophoresis (SDS-

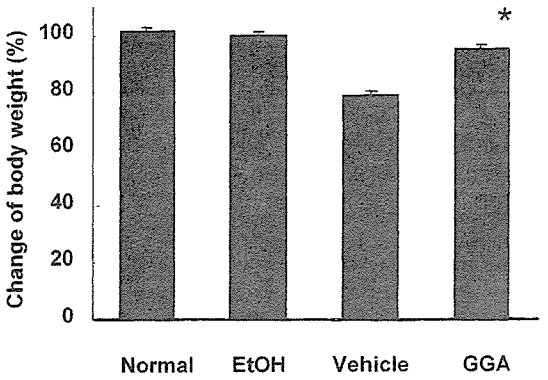


Figure 2. Effect of geranylgeranylacetone (GGA) on body weight in mice with TNBS-induced colitis. Colitis was induced by intrarectal instillation of 0.5 mg of TNBS with 0.1 ml of 50% ethanol. Mice were treated with 300 mg/kg of GGA every day up to day 5. Normal, non-treated mice; EtOH, ethanol-treated mice; vehicle, vehicle-treated mice with TNBS colitis; GGA, 300 mg/kg of GGA-treated mice with TNBS-induced colitis. Values are expressed as mean ± SE. n=5; *P<0.01 vs. vehicle-treated mice.

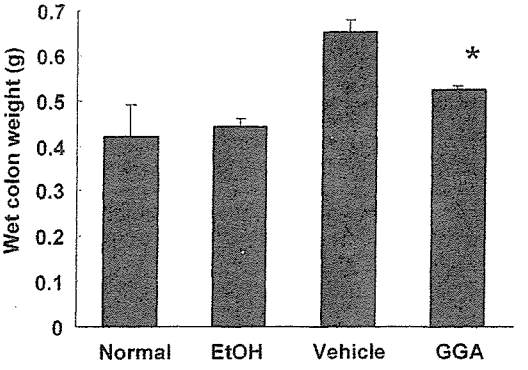


Figure 3. Effect of GGA on wet colon weight of mice with TNBS-induced colitis. Colitis was induced by intrarectal instillation of 0.5 mg of TNBS with 0.1 ml of 50% ethanol. Mice were repeatedly treated with 300 mg/kg of GGA every other day up to day 5. Values are expressed as mean ± SE. n=5; *P<0.05 vs. vehicle-treated cells.

PAGE) and transferred electrophoretically onto a nitrocellulose membrane. The membrane was blocked with 1% non-fat dry milk in phosphate-buffered saline (PBS), probed with an anti-HSP70 antibody (diluted 1:1000, Stressgen, Victoria, Canada) and ß-actin (Sigma), and then reacted with a goat anti-rabbit IgG antibody coupled with horseradish peroxidase (HRP). The resultant complexes were processed for the detection system according to the manufacturer's protocol. The protein concentration of the cell homogenate was quantified using a Micro BCA protein assay reagent kit.

Statistical analysis. All results were expressed as means ± standard error (SE) and statistically analyzed using Student's t-test (StatView, SAS Institute, Cary, NC). $P<0.05$ was considered as significant difference.

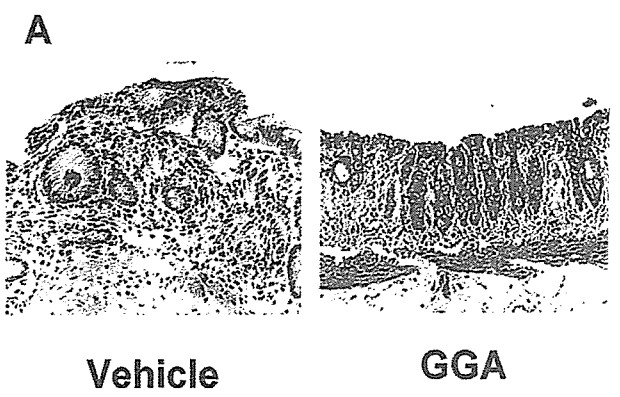
Results

Effects of GGA on survival, body weight and wet colon weight in mice with TNBS-induced colitis. In this model, a high dose of TNBS leads to death within 5 days. In mice treated with ethanol alone, the survival rate slightly decreased to 96.7±3.3% in comparison with non-treated controls (Fig. 1). The survival rate was remarkably decreased to 23.3±8.8% in mice given 1 mg of TNBS. On the other hand, the survival rate was greatly improved in mice treated with 300 mg/kg of GGA (56.7±3.3%, $P<0.01$, vs. vehicle-treated mice). The percent change in body weight was markedly decreased to 77.9±1.7% in vehicle-treated mice 5 days after initial TNBS administration in comparison with that on day 0 (100%) (Fig. 2). In contrast, the percent loss in body weight was significantly smaller in mice treated with 300 mg/kg of GGA than in vehicle-treated mice (94.4±1.3%; $P<0.01$ vs. vehicle-treated mice) (Fig. 2). Wet colon weight correlates with the severity of TNBS colitis and is used as a parameter of colitis (23). Vehicle-treated mice given TNBS showed an increase in wet colon weight compared with non- or ethanol alone-treated mice (0.63±0.04, 0.42±0.07 and 0.44±0.02 g, respectively, $P<0.01$ vs. non- or ethanol-treated mice) (Fig. 3). On the other hand, treatment with 300 mg/kg of GGA for 5 days significantly suppressed the increase in wet colon weight of mice with TNBS-induced colitis compared with treatment with the vehicle (0.51±0.01 g, $P<0.05$) (Fig. 3).

Effects of GGA on histological findings in the colons of mice with TNBS-induced colitis. Colon tissue from mice with TNBS-induced colitis was histologically evaluated using H&E staining. In non-treated mice and ethanol-treated mice, there were no findings of colitis (histological scores: 0±0 and 0.2±0.2, respectively) (Fig. 4A). On the other hand, transmural infiltration of leukocytes with crypt loss and destruction of epithelial cells were found in colon tissues from vehicle-treated mice with TNBS-induced colitis (Fig. 4A, left panel). Treatment with 300 mg/kg of GGA for 5 days greatly improved inflammatory infiltration and tissue destruction in the colon tissues (Fig.4A, right panel). The histological score was significantly decreased in GGA-treated mice with TNBS-induced colitis compared with that in vehicle-treated mice with TNBS-induced colitis (1.8±0.5 and 2.8±0.4, respectively, $P<0.05$ vs. vehicle-treated mice) (Fig. 4A).

Effect of GGA on MPO activity in mice with TNBS-induced colitis. To assess the effect of GGA on neutrophil accumulation, we measured the levels of MPO activity in the colon tissue. The levels of MPO activity were low in non-treated mice and ethanol-treated mice (0.30±0.18 and 0.47±0.23 U/g protein, respectively) (Fig. 5). Intrarectal administration of TNBS markedly increased the level of MPO activity in the colon tissue of mice treated with the vehicle (2.40±0.25 U/g protein) (Fig. 5). On the other hand, mice treated with 300 mg/kg of GGA for 5 days showed a remarkable reduction in the level of MPO activity in colon tissue compared with vehicle-treated mice (1.38±0.07, $P<0.01$) (Fig. 5).

Effect of GGA on infiltration of CD4-positive T cells in the colons of mice with TNBS-induced colitis. Immunohistochemistry for CD4 was performed to evaluate the effect of GGA on the infiltration of T cells in mice with TNBS-induced colitis. The number of CD4-positive stained cells was increased in the colon tissue of mice given TNBS and



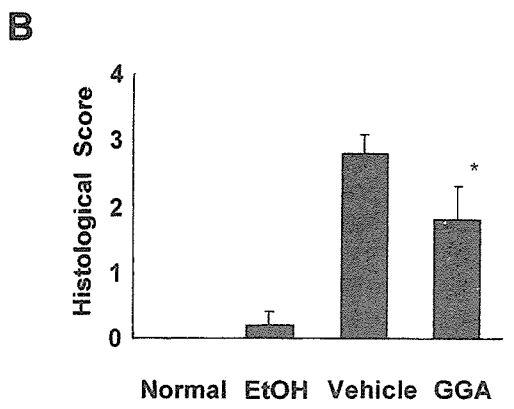


Figure 4. Effects of GGA on histological findings in the colon tissue of mice with TNBS-induced colitis. Colitis was induced by intrarectal instillation of 0.5 mg of TNBS with 0.1 ml of 50% ethanol. Mice were repeatedly treated with 300 mg/kg of GGA every other day up to day 5. (A) The microphotograph of the colon mucosa of mice with TNBS-induced colitis. Left panel, vehicle-treated mice; right panel, 300 mg/kg of GGA-treated mice. Representative pictures are shown. Similar results were obtained from the samples from other mice (original magnification x200). (B) Histological scores in the colon tissue. Values are expressed as mean ± SE. n=5; *P<0.01 vs. vehicle-treated mice.

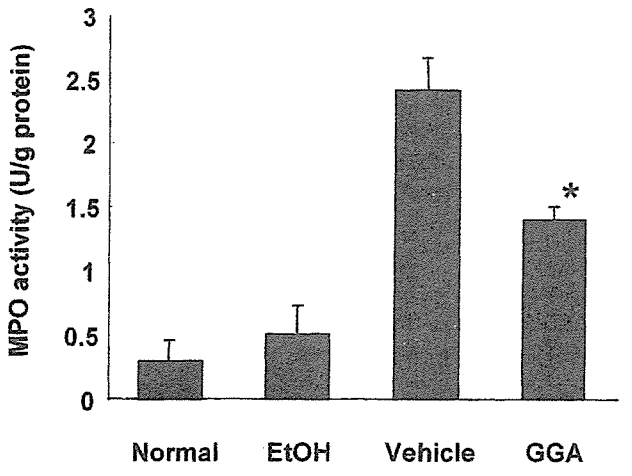
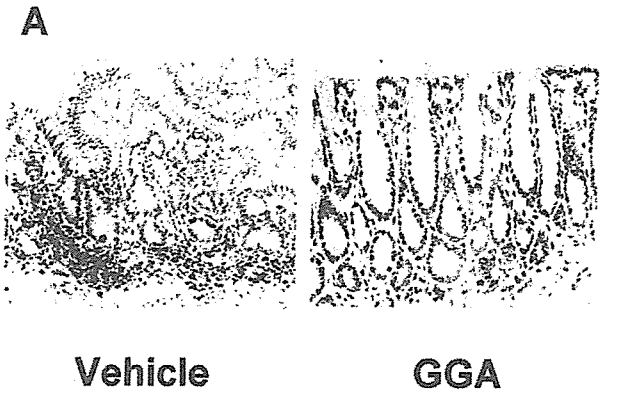


Figure 5. Effect of GGA on MPO activity in mice with TNBS-induced colitis. Colitis was induced by intrarectal instillation of 0.5 mg of TNBS with 0.1 ml of 50% ethanol. Mice were repeatedly treated with 300 mg/kg of GGA every other day up to day 5. Normal, non-treated mice; EtOH, ethanol-treated mice; vehicle, vehicle-treated mice with TNBS colitis; GGA, 300 mg/kg of GGA-treated mice with TNBS colitis. Values are expressed as mean ± SE. n=5; *P<0.01 vs. vehicle-treated mice.



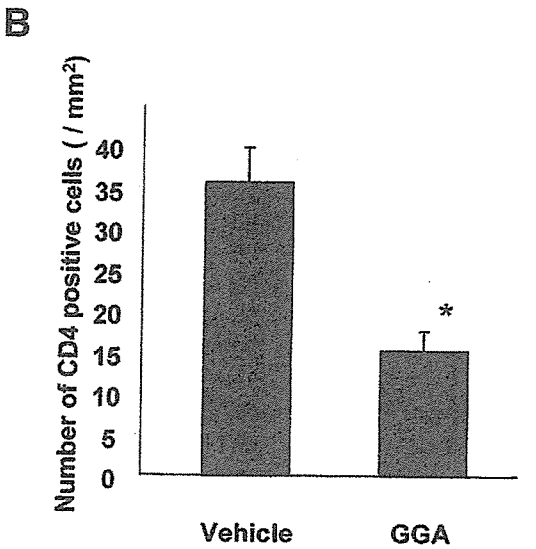


Figure 6. Immunohistochemistry for CD4 in the colon tissue of mice with TNBS-induced colitis. (A) Left panel, the microphotograph in the specimen of colon from vehicle-treated mice with TNBS colitis. Right panel, colon from GGA-treated mice with TNBS-induced colitis (original magnification x200). (B) Number of infiltrating CD4-positive cells. The number of CD4-positive staining cells was counted in the areas of 5 mice with a microscope. Average of cell counts per mm² was calculated. Values are expressed as mean ± SE. n=5; *P<0.05 vs. vehicle-treated mice.

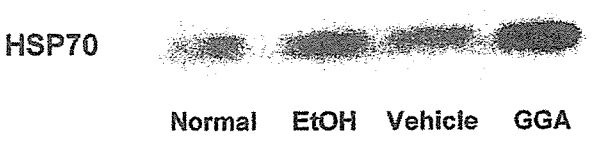


Figure 7. Effect of GGA on induction of HSP70 in the colon tissue of mice. Mice were orally and repeatedly given 300 mg/kg of GGA or vehicle every other day for 5 days. Colon tissue was removed from mice 5 days post-initial treatment with GGA or vehicle. Western blot analysis for HSP70 in the sample of colon was performed. Normal, non-treated mice; EtOH, ethanol-treated mice; vehicle, vehicle-treated mice with TNBS colitis; GGA, 300 mg/kg of GGA-treated mice with TNBS colitis. Representative results are shown. Similar results were obtained from three complete experiments.

vehicle (35.6±4.3/mm²) (Fig. 6A, left panel and B). In mice given TNBS and treated with 300 mg/kg of GGA for 5 days, the increase in numbers of CD4-positive stained cells in the colon tissue was significantly suppressed (15.2±2.5/mm², P<0.01, vs. vehicle-treated mice) (Fig. 6A, right panel and B).

Effect of GGA on enhancement of HSP70 expression in the colons of mice with TNBS-induced colitis. The expression of HSP70 in the colons of mice with TNBS-induced colitis was determined by Western blot analysis with an antibody specific for HSP70. The expression of HSP70 was weakly detected in colon tissue from non-treated, ethanol-treated and vehicle-treated mice on day 5 (Fig. 7). The HSP70 expression level was markedly increased in colon tissue from mice treated with 300 mg/kg of GGA for 5 days (Fig. 7).

Discussion

Several murine models of colitis resembling human IBD have been developed to analyze the pathogenesis and the therapeutic effects of drugs. TNBS-induced colitis, which is induced by intrarectal instillation of TNBS, is one of the useful experimental colitis models in mice (25,28,29). This model is characterized by marked transmural infiltration of leukocytes, mainly T cells. This model of inflammation is considered to be associated with a T-helper 1 cell response and is immunologically similar to Crohn's disease, in which Th1 cells are observed predominantly in the inflammatory lesion (29,30). In this study, we investigated the effect of GGA using a model of colitis induced by TNBS in mice, and we demonstrated that GGA protected mice from TNBS-induced colitis.

GGA has been reported to prevent gastric mucosa from inflammation and ulceration in several experimental models induced by chemicals (9,10,13,31,32). GGA has a protective effect against damage caused by non-steroidal anti-inflammatory drugs (NSAIDs), including indomethacin, in gastric mucosal cells (10,32). In other organs, GGA protects tissues such as the heart and liver from ischemic and surgical damage (14,15). In this study, we demonstrated that GGA clinically and histologically protected mice from TNBS-induced colitis. Our results reveal that GGA potentially plays an anti-inflammatory role in the development of TNBS-induced colitis.

In addition to the clinical and histological findings, it was found that the level of MPO activity, as a critical acute inflammatory mediator, in the colon was suppressed in GGA-treated mice with TNBS-induced colitis compared with that in vehicle-treated mice. MPO activity has been shown to be positively correlated with accumulation of neutrophils in local lesions in colons of mice with colitis (27,33). It is thought that treatment with GGA suppresses the increase in neutrophil accumulation in the colons of mice with TNBS-induced colitis. Recently, it has been reported that GGA regulates the expression of interleukin-8 in the gastric mucosa (34,35). Although the mechanism by which GGA suppressed neutrophil accumulation in colon tissue from mice with TNBS-induced colitis was not clarified in the present study, previous studies and the present study suggest that GGA inhibits neutrophil accumulation via mediating the expressions of chemokines in the colon.

An increase in T cells infiltrating the colon mucosa is commonly observed in mice with TNBS-induced colitis (29,30). We evaluated the infiltration of T cells into the colon mucosa to investigate the effect of GGA in mice with TNBS-induced colitis. In immunohistochemistry for CD4, treatment with 300 mg/kg of GGA for 5 days inhibited the increase in number of T cells infiltrating the colon tissue compared with that in mice treated with the vehicle. There has been no study in which effects of GGA on infiltration of T cells and activation of T cell-related mediators were investigated. Our results suggest that GGA may, at least partly or indirectly, suppress the activation of T cells in mice with TNBS-induced colitis.

Recent studies have partially clarified one of the defense mechanisms of GGA in the protection of cells and tissues. GGA markedly and immediately up-regulates the expression of endogenous HSP70, which plays an important role in the protection of cells from stress (13). An *in vitro* study has shown that up-regulation of HSP70 expression induced by GGA inhibits the process of apoptosis caused by hydrogen peroxide and ethanol in cultured rat hepatocyte (36). Moreover, it has been reported that GGA immediately induces the expression of HSP70 in the rat gastric mucosa (13). These findings suggest that the mechanism of tissue protection by GGA mainly depends on the enhancement of HSP70 expression by GGA. Transgenic overexpression of HSP70 also protects tissues against oxidative and ischemic stresses (21,22). On the other hand, lack of HSP70 severely aggravates cerebral ischemia and infarction in the mouse brain (37). Taken together, the findings suggest that HSP70 plays an essential role in protection against various types of tissue damage. Our previous study also demonstrated a protective effect of GGA on DSS-induced colitis in mice (38). Treatment with GGA significantly improved the clinical and histological findings in the colons of mice with DSS-induced colitis. In addition, administration of GGA enhanced the expression of HSP70 in the colon tissue of mice under normal conditions. Moreover, repeated administration of GGA for 7 days up-regulated the expression of HSP70 in surface epithelial cells in the colon mucosa: In this study, we found that treatment with 300 mg/kg of GGA for 5 days remarkably up-regulated the expression of HSP70 in the colon tissue of mice given TNBS. Although further study is needed to elucidate the effect and mechanism of induction of HSP70 by GGA in the colon, HSP70 enhanced by GGA may partly contribute to the protection of mice against TNBS-induced colitis.

In conclusion, we demonstrated in this study that GGA protects mice against experimental colitis induced by TNBS. In this model, GGA suppressed the increase of neutrophillic accumulation and strongly enhanced HSP70 expression in the colon. These results provide further evidence that GGA has an anti-inflammatory property in intestinal inflammation and that GGA is a useful drug for the treatment of colitis, including IBD.

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An inhibitor of c-*Jun* NH2-terminal kinase, SP600125, protects mice from D-galactosamine/lipopolysaccharide-induced hepatic failure by modulating BH3-only proteins

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Abstract

Fulminant hepatic failure (FHF) is a dramatic clinical syndrome characterized by massive hepatocyte apoptosis and very high mortality. The c-*Jun*-N-terminal kinase (JNK) pathway is an important stress-responsive kinase activated by several forms of liver injury. The aim of this study is to assess the role of JNK during D-galactosamine (GalN)/lipopolysaccharide (LPS)-induced liver injury, an experimental model of FHF, using SP600125, a small molecule JNK-specific inhibitor. Mice were given an intraperitoneal dose of GalN (800 μg/g body weight)/LPS (100 ng/g body weight) with and without subcutaneous SP600125 (50 mg/kg body weight) treatment (at 6 and 2 h before and 2 h after GalN/LPS administration). GalN/LPS treatment induced sustained JNK activation. Administration of SP600125 diminished JNK activity, suppressed lethality and the elevation of both serum alanine aminotransferase and aspartate aminotransferase, but had no effect on serum tumor necrosis factor-α, and reduced hepatocyte apoptosis after GalN/LPS administration. In support of the role of JNK in promoting the mitochondria-mediated apoptosis pathway, SP600125 prevented cytochrome *c* release, caspase-9 and caspase-3 activity. Moreover, SP600125 downregulated the mRNA and protein expression of Bad in the early periods following GalN/LPS injection and prevented Bid cleavage in the late periods. These results confirm the role of JNK as a critical apoptotic mediator in GalN/LPS-induced FHF. SP600125 has the potential to protect FHF by downregulating Bad and inhibiting Bid cleavage.

Keywords: SP600125; JNK; Hepatic failure; Hepatocyte apoptosis; Cytochrome *c*; Bad; Bid

Introduction

Apoptosis, or programmed cell death, is essential to many biological processes in multicellular organisms, including embryonic development, immune responses, tissue homeostasis, and normal cell turnover (Thompson, 1995; Jacobson et al., 1997). Dysregulated apoptosis contributes to many pathogeneses, including tumor promotion, autoimmune and immunodeficiency diseases, neurodegenerative disorders, and fulminant hepatic failure (FHF) (Lee, 1993; Evan and Littlewood, 1998). FHF is a life-threatening illness, which is induced by viruses, alcohol or hepatotoxic drugs, and is marked by a massive degree of hepatocyte apoptosis. Mortality due to FHF remains high despite progress in medical therapy. Recently, liver transplantation has reduced mortality to some degree, but the chronic shortage of donor livers has limited its application. In fact, of 308 patients with FHF in the United States, 28% died while awaiting liver transplantation (Lee, 2003).

Abbreviations: FHF, fulminant hepatic failure; MAPK, mitogen-activated protein kinase; JNK, c-*Jun*-N-terminal kinase; GalN, D-galactosamine; LPS, lipopolysaccharide; TNF, tumor necrosis factor; I/R, ischemia/reperfusion; TBS, Tris-buffered saline; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TUNEL, *in situ* terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; PBS, phosphate-buffered saline; PCR, polymerase chain reaction.

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The mitogen-activated protein kinase (MAPK) family represents a group of proteins involved in the signal transduction of a variety of cellular stimuli. The c-*Jun* NH_2-terminal kinase (JNK) is a member of the MAPK family, and binds to the NH_2-terminal activation domain of c-*Jun* and phosphorylates c-*Jun* on Ser-63 and -73, causing increased transcriptional activity (Pulverer et al., 1991; Adler et al., 1992). JNK activation is known to trigger apoptosis in response to environmental stresses as well as inflammatory cytokines such as tumor necrosis factor (TNF)-α (Ip and Davis, 1998). The JNK signaling pathway is activated in various forms of liver injury (Trautwein et al., 1998; Bendinelli et al., 1996; Bradham et al., 1997; Schattenberg et al., 2006; Chang et al., 2006). Recently, several studies, based on the gene-knockout approach, have convincingly demonstrated the critical role of JNK in hepatocyte apoptosis, which was induced by concanavalin A, a methionine- and choline-deficient diet, or D-galactosamine (GalN)/lipopolysaccharide (LPS) (Schattenberg et al., 2006; Chang et al., 2006; Maeda et al., 2003; Wang et al., 2006). The recent development of JNK-specific inhibitors has greatly accelerated our understanding of this signaling pathway in hepatocyte apoptosis. One such inhibitor is an anthrapyrazolone inhibitor of JNK, SP600125, which shows 300-fold selective inhibition of JNK over extracellularly regulated kinases and p38 MAPKs, the closest kinase relatives of JNK (Bennett et al., 2001). Another class of newly developed JNK-specific inhibitors is peptide-based, such as a 20-amino-acid peptide derived from the JNK binding domain of JNK-interacting protein-1 (Bonny et al., 2001), which has recently been tested successfully in a model of FHF (Chang et al., 2006).

In this study, we first confirmed whether JNK activation was critical for GalN/LPS-induced hepatocyte apoptosis in vivo. We used SP600125 to show that liver injury is causatively coupled to JNK activation. Next, we investigated the mechanism of JNK in apoptosis signaling in the liver of GalN/LPS-treated mice.

Material and methods

Antibodies

Anti-phospho-JNK (#9251), anti-JNK (#9252), anti-phospho-c-*Jun* (#9261), anti-c-*Jun* (#9162), anti-Bad (#9292), anti-Bax (#2772) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-Bim/BOD (AAP-330) antibody was purchased from Stressgen (Victoria, BC). Anti-cytochrome *c* (clone 7H8.2C12), anti-Bcl-XL (clone 44), and anti-Bcl-2 (clone 7) antibodies were purchased from BD Biosciences Pharmingen (San Diego, CA). Anti-Bid (MAB860) and anti-β-actin (clone AC-15) antibodies were purchased from R and D systems (Minneapolis, MN) and Sigma (St. Louis, MO), respectively.

Animals and experimental protocols

Six-week-old male BALB/c mice (CLEA, Shizuoka, Japan) were maintained at the Animal Center of Niigata University School of Medicine under specific pathogen-free conditions. The in vivo experiments were performed according to the Niigata University School of Medicine Guidelines for the Care and Use of Laboratory Animals. For the preparation of mice with GalN/LPS-induced FHF, mice were given an intraperitoneal injection of GalN (800 μg/g body weight; Sigma), followed immediately by an intraperitoneal injection of LPS (100 ng/g body weight; Sigma). The mice were subcutaneously injected three times with SP600125 (50 mg/kg body weight; BIOMOL, Plymouth, PA) in PPCES vehicle (30% PEG–400/20% polypropylene glycol/15% Cremophor EL/5% ethanol/30% saline) at 6 and 2 h before and 2 h after GalN/LPS administration. Control mice were administered the GalN/LPS and then injected three times with PPCES vehicle alone. Serum and liver samples were obtained at 0, 2, 4, 6, 8, and 24 h after GalN/LPS administration ($n=5$ for each group).

Western blot analysis

Snap-frozen liver samples were lysed using ice-cold lysis buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1 mM Na_3VO_4, 50 mM NaF). Twenty to fifty μg of proteins were resolved on 10–15% sodium dodecyl sulfate polyacrylamide gels and transferred to Immobilon membranes (Millipore, Bedford, MA). After blocking, the filters were reacted with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. Peroxidase-labeled bands were visualized with an ECL detection system (Amersham Biosciences, Buckinghamshire, UK). For confirmation of equal loading, the blots were reprobed with anti-β-actin antibody. Quantitative analysis of bands was performed using the NIH Image software package (version 1.63).

Immunohistochemistry

Sections (4 μm thick) of formalin-fixed, paraffin-embedded tissues were deparaffinized with a xylene and ethanol series, and then stained with anti-phospho-c-*Jun* and anti-cytochrome *c* antibodies. Bound primary antibody was visualized by using a polymer-labeled enhancement system (Envision+System, Peroxidase; DakoCytomation).

Analysis of alanine aminotransferase, aspartate aminotransferase and TNF-α levels

Serum levels of liver enzymes, including alanine aminotransferase (ALT) and aspartate aminotransferase (AST), were determined using an automatic analyzer (SRL, Tokyo, Japan). Serum TNF-α (Mouse TNF-α mouse ELISA kit; Pierce Endogen, Rockford, IL) was determined by enzyme-linked immunosorbent assay according to the manufacturer's instructions.

Liver histology and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling

The formalin-fixed livers were paraffin-embedded, and liver sections were stained with hematoxylin-eosin. Apoptotic hepatocytes were detected using the in situ terminal deoxynucleotidyl

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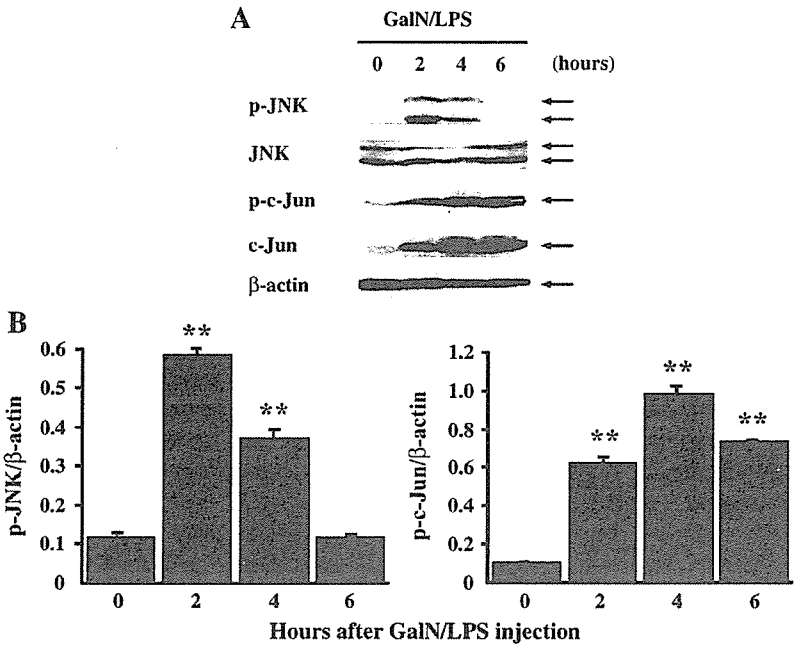

Fig. 1. (A) The profile of JNK activation in the livers of GalN/LPS-treated mice. Equal protein loading was confirmed using β-actin as a reference standard. (B) The graphs show relative values for levels of p-JNK or p-c-*Jun* normalized to β-actin after GalN/LPS administration. Data from three independent experiments are averaged and are represented as the means±SD. $^{**}P<0.01$, vs 0 h.

transferase-mediated dUTP nick-end labeling (TUNEL) method with a commercial kit (Takara, Tokyo, Japan) according to the manufacturer's protocol. Tissue sections were observed by fluorescence microscopy using a microscope (Axio Imager A1; Carl Zeiss MicroImaging, Inc.). Images were acquired using a digital camera (Axiocam MRc5; Carl Zeiss MicroImaging, Inc.) and Axiovision software (Carl Zeiss MicroImaging, Inc.) and then processed with the Photoshop software package (Adobe). As a negative control, PBS was substituted for the mixture of labeling safe buffer and terminal deoxynucleotidyl transferase.

DNA fragmentation assay

Chromosomal DNA was extracted from liver tissues using the DNeasy Tissue Kit (Qiagen, Maryland, USA) in accordance with the manufacturer s protocol. Two μg of each DNA was loaded onto 1.8% agarose gel containing ethidium bromide, electrophoresed in Tris acetate/EDTA buffer for 30 min at 100 V, and photographed under ultraviolet illumination.

Caspase-3 and -9 activities

Caspase-3 and -9 activities in liver tissues were measured using Caspase-3 and -9 Colorimetric Assay Kits (MBL, Nagoya, Japan), respectively, according to the manufacturer's instructions. Briefly, liver tissues were homogenized in cell lysis buffer included in the kit. The homogenates were centrifuged for 15 min at 15,000 rpm at 4 °C, and the supernatant (100 μg of proteins) was incubated with DEVD-*p*NA, LEHD-*p*NA substrates for caspase-3, and -9, respectively, in reaction buffer for 2 h at 37 °C. Absorbance was measured at 405 nm.

Reverse-transcriptase polymerase chain reaction

Total RNA was isolated using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. cDNA was synthesized from 1 μg of total RNA with an oligo$(dT)_{18}$ primer using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Penzberg, Germany). Next, the reverse transcriptase product was amplified with Takara Taq (Takara, Shiga, Japan). The primers used in this experiment were as follows: 5′-GGAAGACGC-TAGTGCTACAGA-3′, 5′-GACCTCCTTTGCCCAAGTTT-3′ for mouse Bad; 5′-GTATGTCGTGGAGTCTACTGGTGT-3′, 5′-TACTCCTTGGAGGCCATGTAGGCC-3′ for GAPDH. These polymerase chain reaction (PCR) primers were purchased from Japan Bio Services (Saitama, Japan). The PCR amplification was carried out in 30 cycles of denaturation (94 °C, 1 min), annealing (60 °C, 1 min), elongation (72 °C, 1 min) and with an additional 10 min of final extension at 72 °C. The PCR product was analyzed by running on 1.5% agarose gel.

Statistical analysis

Data are expressed as the means±SD. Student's *t* test was used for comparisons between 2 groups and differences at $P<0.05$ were considered significant. Kaplan–Meier survival analysis was used for survival data by log-rank test.

Results

Activation of JNK in the liver of GalN/LPS-treated mice

To confirm that JNK activation occurred in the liver of GalN/LPS-treated mice, we examined phosphorylation of JNK and

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c-*Jun*, a major downstream target of JNK, by Western blot analysis (Fig. 1A). An increase of immunoreactivity was seen at 2 h after GalN/LPS administration for phosphorylated (p-) JNK, p-c-*Jun* and c-*Jun*. However, p-JNK immunoreactivity was not seen at 6 h, whereas p-c-*Jun* and c-*Jun* immunoreactivity remained markedly elevated by this time. Densitometric analysis revealed that the relative level of p-JNK normalized to that of β-actin was significantly enhanced beginning at 2 h after GalN/LPS administration, returning to the baseline level by 6 h, whereas that of p-c-*Jun* normalized to β-actin remained significantly elevated at 6 h (Fig. 1B).

Inhibition of JNK activity by SP600125 in the liver of GalN/LPS-treated mice

To test whether it was possible to modulate JNK activity in the liver of GalN/LPS-treated mice, a specific JNK inhibitor, SP600125 (50 mg/kg body weight), or vehicle (without SP600125) was administered 6 and 2 h before and 2 h after GalN/LPS administration. Western blot and densitometric analyses revealed that p-c-*Jun* levels were significantly attenuated at 2 (at the last administration of SP600125), 4, and 6 h after GalN/LPS administration by SP600125, whereas c-*Jun* levels were significantly attenuated at 6 h only after GalN/LPS administration by SP600125 (Fig. 2A and B). Similarly, immunohistochemistry showed that p-c-*Jun* expression was markedly decreased in the livers of SP600125-treated mice 4 h after GalN/LPS administration, compared with vehicle-treated mice (Fig. 2C). Additional studies showed that SP600125 did not affect other signaling pathways such as phosphorylation of ATF2 or degradation of IκB-α (data not shown). These findings suggest that SP600125 is specific and effective in blocking JNK activity in the liver of GalN/LPS-treated mice.

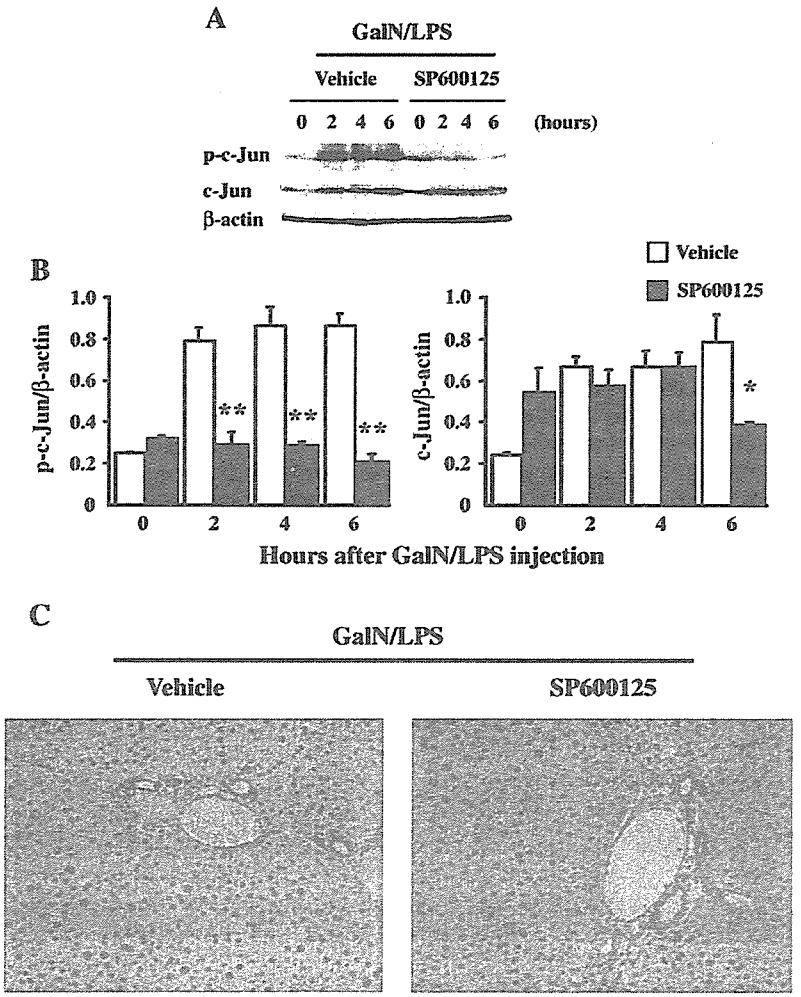

Fig. 2. Effect of SP600125 on JNK activation in the livers of GalN/LPS-treated mice. (A) Western blot analysis of p-c-*Jun* or c-*Jun* in the livers of SP600125-treated mice and vehicle-treated mice after GalN/LPS administration. Equal protein loading was confirmed using β-actin as a reference standard. (B) The graphs show relative values for levels of p-c-*Jun* or c-*Jun* normalized to β-actin after GalN/LPS administration. Data from three independent experiments are averaged and are represented as the means±SD. $^{*}P<0.05$, $^{**}P<0.01$, vs vehicle-treated mice. (C) Immunohistochemistry for p-c-*Jun* in the livers of SP600125-treated mice and vehicle-treated mice at 4 h after GalN/LPS administration.

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Suppression by SP600125 of lethality in mice with GalN/LPS-induced FHF

To assess the beneficial effect of JNK inhibition on lethality in mice with GalN/LPS-induced FHF, survival studies were performed. The survival rate of vehicle-treated mice was only 14% (1 of 7) at 24 h after GalN/LPS administration. In sharp contrast, the survival rate of SP600125-treated mice was improved to 80% (4 of 5) at 24 h after GalN/LPS administration ($P<0.05$; Fig. 3).

The changes in serum ALT (vehicle-treated mice, 1935±1274 IU/L; SP600125-treated mice, 262±59 IU/L) and AST (vehicle-treated mice, 1229±486 IU/L; SP600125-treated mice, 485±38 IU/L) levels by GalN/LPS administration were more marked in vehicle-treated mice than in SP600125-treated mice ($P<0.05$ for each; Fig. 4A).

It has been reported previously that the serum TNF-α level increases rapidly and markedly in GalN/LPS-treated mice, reaching a maximal value within 1 to 2 h after GalN/LPS administration (Chang et al., 2006; Wang et al., 2006; Nakama et al., 2001). To clarify whether SP600125 exerts its effect by blocking TNF-α production, we measured the serum TNF-α level 2 h after GalN/LPS administration. We observed that SP600125 treatment did not suppress the serum TNF-α level (vehicle-treated mice, 2657±1420 pg/mL; SP600125-treated mice, 2480±284 pg/mL) in GalN/LPS-treated mice (Fig. 4B).

Reduction of hepatocyte apoptosis by SP600125 in mice with GalN/LPS-induced FHF

To confirm the preventive effects of SP600125 against liver injury, we assessed the liver architecture histopathologically after GalN/LPS administration. Apoptotic hepatocytes with intralobular hemorrhage were observed, beginning 6 h after GalN/LPS administration (data not shown). At 8 h after GalN/LPS administration, severe liver damage, including numerous apoptotic hepatocytes and massive necrosis with intralobular hemorrhage, was observed in the livers of vehicle-treated mice, whereas this was hardly observed in the livers of SP600125-treated mice (Fig. 5A).

To obtain further evidence that SP600125 prevents hepatocyte apoptosis, apoptotic hepatocytes were detected by TUNEL staining. A large number of TUNEL-positive hepatocytes were

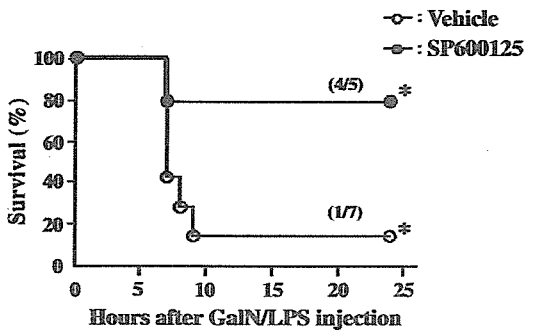

Fig. 3. Effect of SP600125 on the survival curve of GalN/LPS-treated mice. *$P<0.05$, vs vehicle-treated mice.

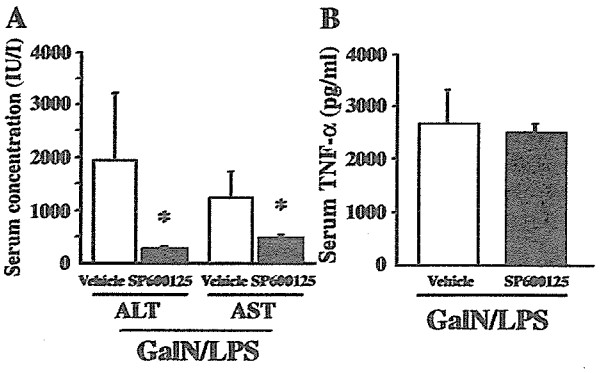

Fig. 4. (A) Effect of SP600125 on serum ALT and AST levels of mice at 6 h after GalN/LPS administration. (B) Effect of SP600125 on serum TNF-α levels of mice at 2 h after GalN/LPS administration. Each value represents the means± SD for 5 mice. *$P<0.05$, vs vehicle-treated mice.

seen in the livers of vehicle-treated mice 8 h after GalN/LPS administration; however, only a few TUNEL-positive hepatocytes were present in the livers of SP600125-treated mice even 8 h after GalN/LPS administration (Fig. 5B).

Consistent with these histopathological observations and the TUNEL assay, DNA fragmentation was detected in the livers of vehicle-treated mice 6 h after GalN/LPS administration, whereas this was not detected in the livers of SP600125-treated mice (Fig. 5C).

Attenuation of the mitochondria-mediated apoptosis pathway by SP600125 in mice with GalN/LPS-induced FHF

Not only the death receptor-mediated (TNF/Fas ligand-mediated) apoptosis pathway but also the mitochondria-mediated apoptosis pathway plays a critical role in hepatocyte apoptosis. In this study, we examined whether JNK activity contributed to mitochondrial damage and to activation of the downstream apoptosis cascade after GalN/LPS administration. First, the release of cytochrome *c* into the cytosolic fraction, an indicator of compromised mitochondrial integrity, was assessed using immunohistochemistry. In untreated livers, cytochrome *c* appeared in the cytoplasm as a punctate (mitochondrial) staining pattern (data not shown). Six h after administration, cytochrome *c* in the hepatocytes appeared as a diffuse (cytosolic) staining pattern, whereas the punctate staining pattern was still prominent in the hepatocytes of SP600125-treated livers at 6 h after GalN/LPS administration (Fig. 6A). In line with its demonstrated inhibitory effect on the mitochondria-mediated apoptosis pathway, SP600125 diminished the activation of both caspase-9 and caspase-3 after GalN/LPS administration (Fig. 6B and C). These results suggest that JNK activity is required for the mitochondrial damage-mediated activation of cell death effectors, including caspase-9 and caspase-3.

Downregulation of bad expression and inhibition of bid cleavage by SP600125 in the livers of GalN/LPS-treated mice

Bcl-2 family proteins serve as critical regulators of the mitochondria-mediated apoptosis pathway, functioning as either

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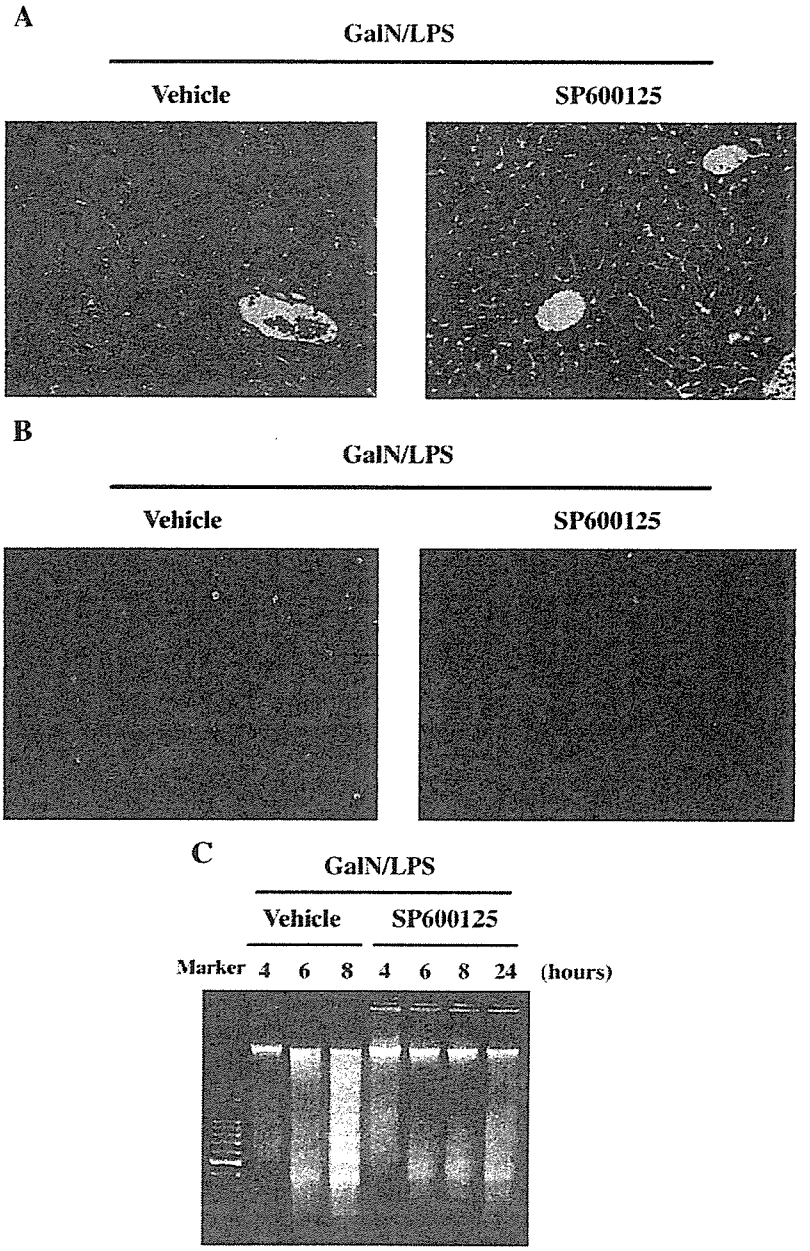

Fig. 5. (A) Histological examination of the liver in mice at 8 h after GalN/LPS administration with or without SP600125 (hematoxylin-eosin, ×100). (B) Detection of apoptotic hepatocytes in the liver of mice at 8 h after GalN/LPS administration with or without SP600125 (TUNEL, ×100). (C) DNA fragmentation in the livers of mice after GalN/LPS administration with or without SP600125.

inhibitors (e.g., Bcl-2, Bcl-XL, Bcl-w and Mcl-1) or promoters (e.g., Bax, Bid, Bad and Bim) of cell death (Gross et al., 1999). JNK modulates some Bcl-2 family proteins on multiple levels: transcriptional, post-transcriptional, and post-translational (Yamamoto et al., 1999; Putcha et al., 2001; Yu et al., 2004). We therefore examined Bcl-2 family protein expression by Western blot analysis in the livers of SP600125- and vehicle-treated mice after GalN/LPS administration (Fig. 7A). Among prosurvival members of the Bcl-2 family, there was no detectable expression of Bcl-2 protein in the livers of both vehicle- and SP600125-treated mice after GalN/LPS administration, and no differences in the protein level of Bcl-XL in the livers between vehicle- and SP600125-treated mice after GalN/LPS administration. On the other hand, among proapoptotic members of the Bcl-2 family, there were no differences in the protein level of Bim and Bax in the livers between vehicle- and SP600125-treated mice after GalN/LPS administration. Bid cleavage started in the livers of vehicle-treated mice 6 h after GalN/LPS administration, and SP600125 blocked Bid cleavage, as observed in other in vivo models (Uehara et al., 2005). Interestingly, there was no change in the protein level of Bad in the livers of vehicle-treated mice after GalN/LPS administration. However, the protein level was downregulated in the livers of SP600125-treated mice beginning at 2 h after GalN/LPS

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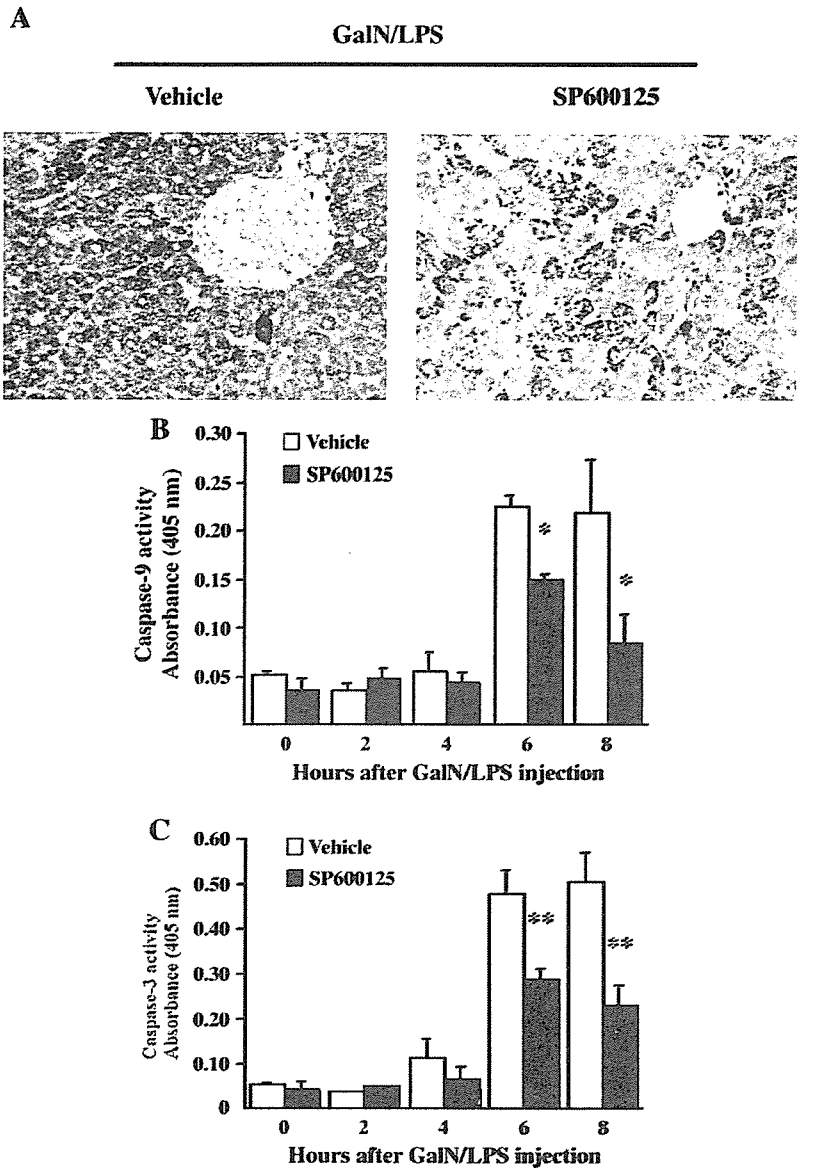

Fig. 6. (A) Immunohistochemistry for cytochrome *c* in the livers of SP600125-treated mice and vehicle-treated mice at 6 h after GalN/LPS administration (×200). Effect of SP600125 on caspase-9 (B) and -3 (C) activities in the livers of mice after GalN/LPS injection. The results are presented as the mean±SD for 5 samples. $*P<0.05$, $**P<0.01$, vs vehicle-treated mice.

administration. To determine whether SP600125 altered the expression of Bad mRNA, we examined the expression of the mRNA by reverse-transcription-PCR in the livers of SP600125- and vehicle-treated mice after GalN/LPS administration (Fig. 7B). Consistent with the alteration in the protein levels of Bad, the expression of Bad mRNA was also markedly downregulated in the livers of SP600125-treated mice beginning at 2 h after GalN/LPS administration.

Discussion

As apoptosis of hepatocytes is the major underlying cause of FHF, a better understanding of this molecular mechanism has important implications in devising strategies for treating FHF. The JNK signaling pathway is one of the most important apoptosis-signaling pathways, and is activated by various forms of liver injury (Trautwein et al., 1998; Bendinelli et al., 1996; Bradham et al., 1997; Schattenberg et al., 2006; Chang et al., 2006). Consistent with previous report (Wang et al., 2006), we observed that GalN/LPS administration led to prolonged hepatic JNK activation. The duration of JNK activation is thought to be a critical factor determining cell proliferation or apoptosis; that is, transient JNK activation leads to cell proliferation, whereas sustained JNK activation causes cell apoptosis (Chen et al., 1996). In the liver, transient and modest JNK activation is required for liver regeneration (Schwabe et al., 2003), whereas sustained JNK activation promotes cell death (Schattenberg et al., 2006; Maeda et al., 2003). The sustained

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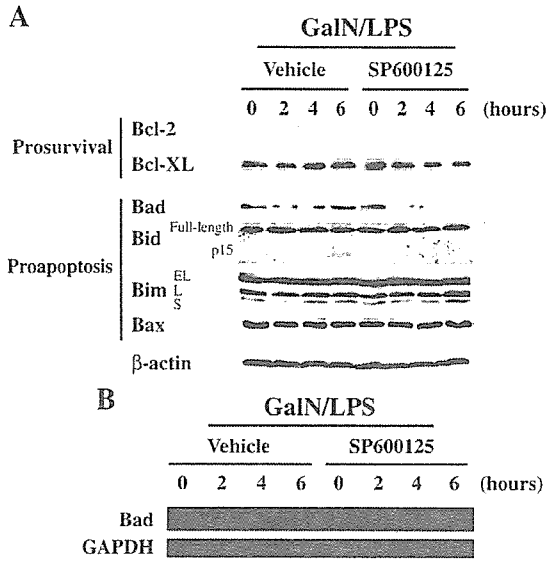

Fig. 7. Effect of SP600125 on Bcl-2 protein family expression in the livers of mice after GalN/LPS administration by Western blot analysis (A) and reverse-transcription PCR (B). All experiments were performed at least 3 times, and representative results are shown.

activation of JNK in mice with GalN/LPS-induced FHF may have similarly triggered a cell death response in hepatocytes in vivo.

Our study demonstrated that SP600125 blocked p-c-*Jun* in the livers of GalN/LPS-treated mice. One possible target for p-c-*Jun* in the AP-1 complex is the TNF-α gene itself, which contains an AP-1 binding site on its promoter (Becker et al., 1999). In the hepatic I/R model (Uehara et al., 2005) and liver transplantation model (Uehara et al., 2004), SP600125 decreased hepatocyte apoptosis, resulting in improved survival. In the former, SP600125 decreased the hepatic TNF-α level, whereas in the latter it did not, although TNF-α was a critical cytokine in the liver injury produced in both models. In the present study, SP600125 was able to suppress hepatocyte apoptosis and prolong the survival of mice with GalN/LPS-induced FHF without suppressing the increased level of serum TNF-α. This difference observed among the studies may have been due to differences in experimental design. This finding in GalN/LPS-induced FHF suggests that SP600125 suppresses GalN/LPS-induced hepatocyte apoptosis via its interaction with the downstream targets of TNF-α.

Our results strongly suggest that JNK activity is required for activation of the mitochondria-mediated apoptosis pathway. Supporting evidence included clearly demonstrated prevention of cytochrome *c* release, and caspase-9 and caspase-3 activation by SP600125 after GalN/LPS administration. To further elucidate the underlying mechanisms, we examined the effects of JNK on the expression of Bcl-2 family proteins, which govern the mitochondria-dependent pathway of apoptosis. Among these proteins, there is direct evidence that hepatocyte-specific Bcl-XL-deficient mice develop spontaneous and continuous apoptosis in hepatocytes (Takehara et al., 2004); that is, hepatocytes cannot survive without expressing Bcl-XL, and this molecule must be a crucial apoptosis antagonist of hepatocytes since these cells do not express Bcl-2. Consistent with a recent study using GalN/LPS-treated *jnk2* null mice (Wang et al., 2006), in the present study SP600125 had no effect on the steady-state expression of Bcl-XL after GalN/LPS administration. The influence and function of prosurvival Bcl-2 family proteins, including Bcl-XL, are regulated by BH3-only proteins, which are proapoptotic members of the Bcl-2 family (Huang and Strasser, 2000). To date, eight members of the BH3-only protein family have been characterized: Bad, Bid, Bik, Bim, Bmf, Hrk, Noxa, and Puma. BH3-only proteins, except for Noxa, are capable of binding to Bcl-XL and neutralizing its function (Baskin-Bey and Gores, 2005). A recent study using knockout mice has shown that ablation of *jnk2*, but not *jnk1*, inhibits Bid cleavage, but has no effect on the steady-state

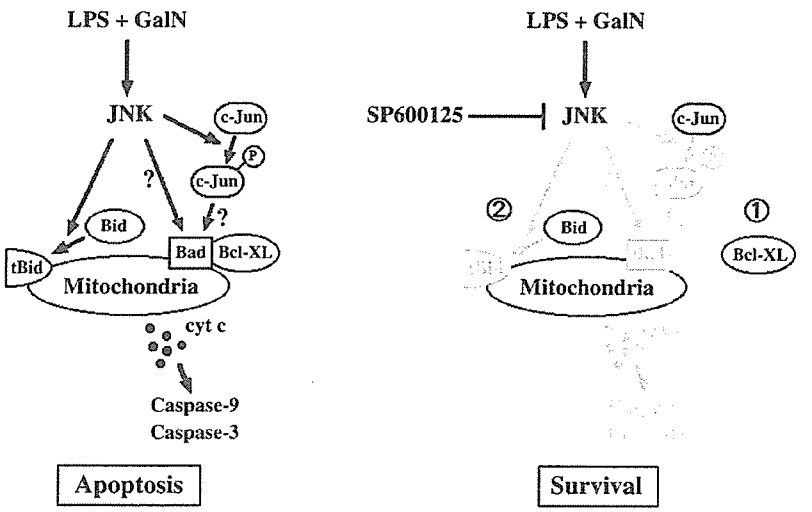

Fig. 8. Schematic model for the mechanism by which SP600125 suppresses GalN/LPS-induced hepatocyte apoptosis. Inhibition of JNK activity by SP600125 induces downregulation of Bad expression, increasing the relative amount of functional Bcl-XL protein (in the early stage; ①), and inhibiting the cleavage of Bid (in the late stage; ②), thereby preventing hepatocyte apoptosis in the liver of mice with GalN/LPS-induced FHF.

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expression of Bad, Bax and Bim after GalN/LPS administration (Wang et al., 2006). In the present study, we found that SP600125 downregulated the expression of Bad in the early period following GalN/LPS injection and prevented Bid cleavage in the late period, but had no effect on the steady-state expression of Bax and Bim. Because SP600125 is a potent and specific inhibitor of all JNK isoforms (JNK1, JNK2 and (neural specific) JNK3), our results appear to support the possibility that the functions of both JNK1 and JNK2 are redundant. Indeed, the mortality of GalN/LPS-treated mice administered SP600125 was markedly lower than that of GalN/LPS-treated *jnk1* or *jnk2* null mice (Wang et al., 2006).

There is some evidence for the function of Bad and Bid in the liver; absence of Bad markedly protects primary hepatocytes from glucose-withdrawal-induced apoptosis (Danial et al., 2003), and administration of GalN/LPS to *Bid*-deficient mice offered only partial protection (Zhao et al., 2001). Therefore, it appears that modulation of not only one but also two molecules by SP600125 plays a critical role in GalN/LPS-induced hepatocyte apoptosis. These results permit us to speculate that SP600125 causes an increase in the relative amount of functional Bcl-XL protein, which is freed from Bad (in the early stage) and truncated Bid (in the late stage), thereby preventing hepatocyte apoptosis in the liver of mice with GalN/LPS-induced FHF (Fig. 8). The mechanism involved in the modulation of these BH3-only proteins by JNK blockade requires further study.

In summary, this study has confirmed the critical role of JNK signaling in liver injury induced by GalN/LPS administration in vivo. It is also suggested that SP600125, which inhibits hepatocyte apoptosis by modulating BH3-only proteins, may be a useful therapeutic tool for FHF.

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