Anti-tumor effect by inhibition of NF-κB activation using nafamostat mesilate for pancreatic cancer in a mouse model

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Abstract. Constitutive NF-κB activation plays a key role in the aggressive behavior of pancreatic cancer. We have reported that nafamostat mesilate, a serine-protease inhibitor, inhibited NF-κB activation and induced apoptosis in human pancreatic cancer cells. The aim of this study is to evaluate the therapeutic efficacy of nafamostat mesilate against pancreatic cancer. In vitro, nafamostat mesilate inhibited NF-κB activation of human pancreatic cancer cell line (Panc-1) by suppressing IκBα phosphorylation and induced caspase-8 mediated apoptosis. In vivo, Panc-1 was implanted into the back of nude mice. Five weeks after implantation, nafamostat mesilate was injected intraperitoneally as the treatment group (n=11) three times a week for six weeks, while the control group (n=13) received vehicle only. At the end of six-week treatment, the tumors grew up to 12.89±4.27 mm (mean ± SD) in the treatment group, and 17.93±4.45 mm in the control group, respectively. The tumor volume and weight of the treatment group were reduced by 43 and 61% as compared with the control group. The tumor growth was significantly inhibited in the treatment group (p<0.0001). Assays of primary tumors also indicated that nafamostat mesilate inhibited NF-κB activation by suppressing IκBα phosphorylation, resulting in caspase-8 mediated apoptosis. These results suggested that nafamostat mesilate has anti-neoplastic property against experimental pancreatic cancer.

Introduction

Pancreatic cancer is one of the most fatal human digestive cancers with an overall 5-year survival rate of only 1-4%, because of rapid tumor growth and high potential for distant metastasis. In addition, despite developments in diagnostic techniques and modalities, the majority of patients with pancreatic cancer are diagnosed at the advanced stage. Therefore, only 14% of patients are amenable to resection (1). Gemcitabine is currently the standard treatment for unresectable pancreatic cancer (2), but the therapeutic benefit of gemcitabine is limited (3). Besides, many patients can not receive sufficient amount of chemotherapy, because of rapid progression, deterioration of general condition, and significant adverse effects. Therefore, new therapeutic approaches, with minimal adverse effects and tolerance even in patients with poor general condition due to advanced cancer, should be developed to further improve the outcome of unresectable pancreatic cancer.

Recent studies have demonstrated that NF-κB plays an important role in the regulation of cell apoptosis, inflammation, and oncogenesis (4-7). Inhibition of NF-κB is considered as one of new treatment strategies for cancer patients (8-11). In addition, constitutive activation of NF-κB has been reported to play a key role in the aggressive behavior of pancreatic cancer (12-15). We have reported that nafamostat mesilate inhibits NF-κB activation by suppressing IκBα phosphorylation and induces caspase-8 mediated apoptosis of pancreatic cancer cells (16). Also, high efficacy of nafamostat mesilate combined with gemcitabine for pancreatic cancer has been demonstrated in animal experiments (17) that applied to clinical trials (18). Nafamostat mesilate, which is a serine-protease inhibitor (19,20), is widely used for treatment of pancreatitis (21), disseminated intravascular coagulation (22), and anti-coagulation in hemodialysis (23) in Japan, and has only minimal adverse effects such as hyperkalemia or hyponatremia (24-26).

To evaluate the potential of nafamostat mesilate for possible clinical application to treat unresectable pancreatic cancer, especially for patients in poor status, we explored antitumor and adverse effect of nafamostat mesilate in an experimental pancreatic cancer model.

Materials and methods

Reagents. Nafamostat mesilate was a kind gift from Torii Pharmaceutical Co., Ltd., (Tokyo, Japan), was dissolved to sterile distilled water (5 mg/ml) and stored at -20°C until use.
Cell line. Panc-1, a human pancreatic cancer cell line, was purchased from the American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco-BRL, NY, USA) and penicillin/streptomycin (Gibco-BRL). The cells were cultured at 37°C with 5% CO2.

Quantitative analysis of NF-κB activity. NF-κB is typically a heterodimer that consists of the p65 (RelA) and p50 proteins. In an inactive form of NF-κB proteins are sequestered in the cytoplasm with IkBα. Following IkBα phosphorylation, NF-κB proteins are released and translocated into the nucleus, where they activate transcription of target genes. For assessment of the NF-κB activity in Panc-1 cells treated with nafamostat mesilate, concentration of NF-κB p65 in the nuclear extracts was measured both in vitro and in vivo. Nucleic extracts of both nafamostat mesilate-treated and control Panc-1 cells were prepared using a nuclear extract kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer's protocol. For in vitro experiments, Panc-1 cells were incubated with nafamostat mesilate (320 μg/ml) as treatment group, or with distilled water as control group for 3 or 24 h. The nuclear extracts from both in vitro and in vivo experiments were assayed using an enzyme-linked immunosorbent assay (ELISA) kit (TransAM™ NF-κB; Active Motif) to detect and quantify the NF-κB activity according to the manufacturer's instructions. Briefly, 10 μg of nuclear extract protein was incubated for 1 h at 25°C in microwell coated with an oligonucleotide containing an NF-κB p65-binding consensus sequence. Next, the nuclear extract protein was incubated with rabbit anti-NF-κB p65 antibodies (1:1000 dilution) for 1 h at 25°C, followed by incubation with peroxidase-conjugated goat anti-rabbit IgG (1:1000 dilution) for 1 h at 25°C. The peroxidase activity was visualized by the tetramethylbenzidine reaction, and the optimal density was measured at 450 nm.

Western blot analysis. To evaluate the inhibitory effect of nafamostat mesilate on NF-κB signaling due to suppression of IkBα phosphorylation, and apoptotic effect for Panc-1 cells in vitro and in vivo, IkBα, phosphorylated IkBα, pro-caspase-8, and cleaved caspase-8 protein level in whole cell extracts of Panc-1 cells treated with nafamostat mesilate were determined by Western blot analysis. Protease inhibitor cocktail and phosphatase inhibitor cocktail tablets were purchased from Roche Diagnostics (Indianapolis, IN, USA). Pro- and cleaved caspase-8, IkBα, phosphorylated IkBα monoclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). For in vitro experiment, Panc-1 cells were incubated with nafamostat mesilate (320 μg/ml) as the treatment group, or with distilled water as control group for 3 or 24 h. Protein samples of nafamostat mesilate treated and control Panc-1 cells for SDS-PAGE were prepared according to the procedure previously described (27). These samples from both in vitro and in vivo experiments were resolved by SDS-PAGE on 4-20% acrylamide gradient gels using Tris-glycine buffer and transferred onto a nitrocellulose membrane. The blotted membranes were blocked with incubation in Tris-buffered saline (TBS) containing 0.1% casein, and 0.05% Tween-20® (MP Biomedicals, Solon, OH, USA) at room temperature for 2 h. Immunostaining was performed by incubating the blots in each primary antibody at appropriate dilution overnight. After brief washing, the membranes were incubated with the alkaline-phosphatase-labeled secondary antibody (Histofine, Nichirei, Tokyo, Japan) for 2 h and developed by using nitro blue tetrazolium/5-Bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) reagent (Bio-Rad, Hercules, CA, USA).

Cell proliferation assay. For evaluating the anti-tumor effect of nafamostat mesilate, the cell proliferation of Panc-1 cells after nafamostat mesilate treatment was measured. Panc-1 cells (1x10⁶) seeded into a 96-well plate were incubated with nafamostat mesilate (320 μg/ml) as treatment group, or with distilled water as control group for 24, 48 or 72 h. The cell proliferation was measured with Progma celltitter 96 Aquous One Solution Cell Proliferation Assay (Progma, Madison, WI, USA) following the manufacturer's instructions.

Cell cycle analysis. For evaluating the induction of apoptosis by nafamostat mesilate, cell cycle analysis was performed. Panc-1 cells were incubated with nafamostat mesilate (320 μg/ml) as treatment group, or with distilled water as control group for 24 h. The cell cycle was analyzed by flow cytometry. In brief, the cells were harvested and 1x10⁶ were fixed with 70% (v/v) ethanol stored at -20°C until use. After centrifugation, the cell pellet was washed with phosphate-buffer saline (PBS). The cells were resuspended in PBS containing propidium iodide (50 μg/ml) and incubated at room temperature for 10 min, followed by incubation with 0.1% Triton® X-100 (MP Biomedicals) at room temperature for 2 h. For evaluating the induction of apoptosis by nafamostat mesilate, cell cycle analysis was performed. Panc-1 cells were incubated with nafamostat mesilate (320 μg/ml) as treatment group, or with distilled water as control group for 24 h. The cell cycle was analyzed by flow cytometry. In brief, the cells were harvested and 1x10⁶ were fixed with 70% (v/v) ethanol stored at -20°C until use. After centrifugation, the cell pellet was washed with phosphate-buffer saline (PBS). The cells were resuspended in PBS containing propidium iodide (50 μg/ml) and incubated at room temperature for 10 min, followed by incubation with 0.1% Triton® X-100 (MP Biomedicals) for 10 min. DNA content was determined with a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

Animals and xenograft pancreatic cancer model. Five-week old male nude mice (BALBc nu/nu), were purchased from CLEA Japan Incorporated (Tokyo, Japan). The animals were housed under specific pathogen-free conditions in a biological cabinet at the Laboratory Animal Facility of Jikei University School of Medicine. The animals were maintained with a 12-h light-dark cycle at a temperature of 22±2°C and 55±5% humidity in a room with a filtered air supply.

A mouse pancreatic cancer model was established by injection of Panc-1 cells (5x10⁶ cells) in 200 μl of PBS subcutaneously into the right side of the back of the animals. At five weeks after implantation, the animals were randomized into the following two groups; treatment group (n=11), intraperitoneally injected nafamostat mesilate (30 μg/g) three times a week for six weeks, and control group (n=13), intraperitoneal injection of the equal amount of vehicle three times a week for six weeks. The diameters of tumors in both groups were measured on the day of nafamostat mesilate or vehicle injection. At the end of the treatments, blood samples were taken, and animals were sacrificed. Subcutaneous tumors were excised, and the volume and weight were measured. Thereafter, paraformaldehyde-fixed and paraffin-embedded for immunohistochemistry and TdT-mediated dUTP-X nick end labeling (TUNEL) assay.
Nucleic and whole protein were extracted from the tumor tissue for ELISA and Western blot analysis for assessment of inhibitory effect for NF-κB activity and apoptotic effect for cancer cells by nafamostat mesilate treatment. These in vivo experimental protocols were approved by the animal committee of Jikei University School of Medicine.

**Histological studies.** Paraffin sections of tumor tissue were stained immunohistochemically using NF-κB p65 monoclonal antibody (Epitomics, Burlingame, CA, USA, 1:500) as a primary antibody and Dako Envision Kit/HRP as a secondary antibody (Dako, Carpenteria, CA, USA) for evaluating NF-κB activation. TUNEL assay with In Situ Cell Death Detection Kit, TMR red (Roche Diagnostics, Indianapolis, IN, USA) was performed for evaluating induction of apoptosis. These assessments followed the manufacturer’s instructions.

**Blood samples analysis.** The major adverse effects of nafamostat mesilate consist of hyperkalemia, hyponatremia and hepatopathy. To evaluate adverse effects, serum potassium, sodium, and alanine aminotransferase levels were measured.

**Statistical analysis.** Non-paired t-test and repeated measures ANOVA were used for statistical studies. All p-values were considered statistically significant when the associated probability was <0.05.

**Results**

**Inhibition of NF-κB activity by nafamostat mesilate in vitro.** In assessment of the NF-κB activity using ELISA, concentration of NF-κB p65 in the nuclear extracts of Panc-1 cells treated with nafamostat mesilate was significantly lower than those in control group (p=0.0001, Fig. 1a). In Western blot analysis, concentration of phosphorylated IκB· was lower, and concentration of IκB· was higher in Panc-1 cells treated with nafamostat mesilate, in comparison with those in control group (Fig. 1b). These results showed that NF-κB activity was inhibited due to suppressed IκB· phosphorylation in Panc-1 cells treated with nafamostat mesilate.

**Inhibition of cell proliferation by nafamostat mesilate in vitro.** In the cell proliferation assay, cell viability of Panc-1 cells treated with nafamostat mesilate was significantly lower than those in control group at each exposure time, including 24, 48 and 72 h (75.58±7.96%, *p=0.0030, 56.47±4.97%, **p<0.0001, 40.95±5.85%, ***p<0.0001, Fig. 2). In addition, cell viability of Panc-1 cells treated with nafamostat mesilate was decreased exposure time-dependently (p<0.0001).

**Induction of apoptosis by nafamostat mesilate in vitro.** In FACS analysis, cell counts of M1 period in nafamostat mesilate-treated cells was significantly lower than those in control cells at each exposure time, including 24, 48 and 72 h (75.58±7.96%, *p=0.0030, 56.47±4.97%, **p<0.0001, 40.95±5.85%, ***p<0.0001, Fig. 2). In addition, cell viability of Panc-1 cells treated with nafamostat mesilate was decreased exposure time-dependently (p<0.0001).

**Antitumor effect of in vivo nafamostat mesilate treatment.** Fig. 4a shows tumors in both control and nafamostat mesilate-treated tumors at the end of study, respectively. As to
changes in the tumor diameter, the initial diameter at five weeks after implantation in control group was 8.01±3.24 mm, which gradually grew to 17.93±4.45 mm at the end of six weeks.
weeks. In contrast, the tumor diameter in nafamostat mesilate treatment group was 7.5±2.88 mm, which gradually grew to 12.89±4.27 mm, but significant slower than those of the control group (p<0.0001) (Fig. 4b). Resected tumor volumes and weights in control group were 2.779.5±1.658.48 mm³ and 1.445.23±885.43 mg, respectively. In contrast, those in the treatment group were 1.190.78±903.49 mm³ and 878.64±692.17 mg. Both tumor volumes and weights in treatment group were significantly smaller than those of control group (p=0.0048, Fig. 4c, p=0.0496, Fig. 4d). These data showed that nafamostat mesilate treatment had inhibitory effect on the tumor growth of pancreatic cancer in an experimental mouse model.

Inhibition of NF-κB activity by nafamostat mesilate in vivo.
In assessment of the NF-κB activity using ELISA, concentration of NF-κB p65 in the nuclear extracts of nafamostat mesilate-treated tumor was significantly lower than those in control (p=0.0383, Fig. 5a). In Western blot analysis, concentration of phosphorylated IκBα was lower, and concentration of IκBα was higher in nafamostat mesilate-treated tumors, in comparison with those in control (Fig. 5b). In immunohistochemical staining, reduced nuclear translocation of NF-κB p65 was shown in nafamostat mesilate-treated tumors, in comparison with control tumors (x200).

Induction of apoptosis by nafamostat mesilate in vivo.
In Western blot analysis, concentration of pro-caspase-8 was lower, and concentration of cleaved caspase-8 was higher in nafamostat mesilate-treated tumors, in comparison with those in control (Fig. 6a). In TUNEL staining, TUNEL-positive cells were greater in nafamostat mesilate-treated tumors (Fig. 6b). These results showed that nafamostat mesilate treatment induced a caspase-8 mediated apoptosis in vivo, which is in accordance with the results of the in vitro experiment.

Adverse effects of in vivo nafamostat mesilate treatment.
In post-therapeutic blood sample results, serum potassium levels of nafamostat mesilate-treated and control group were 5.07±0.67 mmol/l and 4.6±0.16 mmol/l, respectively (p=0.1160). Serum sodium levels of treatment and control
group were 152±1.73 mmol/l and 152±2.45 mmol/l, respectively (p=1.0000). Serum alanine aminotransferase levels of treatment and control group were 36±7.69 IU/l and 43.75±13.72 IU/l, respectively (p=0.2380). Blood test results related to known nafamostat mesilate adverse effects, such as hyperkalemia, hyponatremia and hepato-pathy, were comparable between the two groups.

Discussion

NF-κB regulates the expression of genes involved in inflammatory cytokines, adhesion molecules and anti-apoptotic proteins. Therefore, the NF-κB inhibitor may suppress proliferation, invasion, metastasis and chemoresistance in tumors. Several reports described anti-tumor effect of protease inhibitor as an NF-κB inhibitor. Tsuzuki et al and Chih et al reported that nafamostat mesilate inhibited NF-κB activation in inflammation (28,29). Kimura et al reported that nafamostat mesilate inhibited liver metastasis and invasion of colon cancer cells in a mouse model (30), and Outa et al reported that nafamostat mesilate inhibited growth and invasion of pancreatic cancer cells by blocking tumor-associated trypsinogen into protease-activated receptor-2 in vitro (31,32). Uchima et al reported gabexate mesilate, a kind of protease inhibitors, prevented the invasive potential of pancreatic cancer cells and liver metastasis in nude mice (33). Yoon et al reported gabexate mesilate reduced the invasion and metastasis of colon cancer cells by inhibiting matrix metalloproteinases in vitro and in vivo (34).

Several drugs, which play roles as NF-κB inhibitors, have also been used for treatment of malignancies. However, these drugs have problems in clinical application, including adverse effects. Bortezomib, which inhibits proteasome resulting in inhibition of NF-κB, is used for refractory and relapsed multiple myeloma in clinical medicine (35), but also inhibits reduction of unnecessary ubiquitin proteins. Curcumin, which is a yellow pigment present in the rhizome of turmeric has been shown to suppress NF-κB activation (36), and a phase II trial of curumin for advanced pancreatic cancer has been reported (37). However, curcumin is reported to impair function of tumor suppressor p53 in colon cancer cells (38). Resveratrol, a component of grapes, berries and peanuts, has been demonstrated to be a potent blocker of the NF-κB pathway (39) that enhances anti-tumor activity of gemcitabine in orthotopic mouse model of human pancreatic cancer (40). In the viewpoint of drug administration, resveratrol is available only orally, and has strong action for tumors in direct contact with the drug, such as skin and gastrointestinal tract tumors (41). Recent therapeutic advances in chemotherapy for malignancy allow patients with advanced cancer to live longer than before. Consequently, developments of therapeutic approach with less invasiveness and adverse effects for patients with poor general condition due to advanced cancer are needed to maintain and improve quality of life. Nafamostat mesilate is widely used for patients with poor performance status and organ function due to pancreaticitis, disseminated intra-vascular coagulation or chronic renal failure, with minimal adverse effects. Therefore, nafamostat mesilate has a potential to become a new therapeutic option for cancer patients. In addition, because nafamostat mesilate is administered via infusion, serum drug concentration is not affected by alimentary absorption.

In conclusion, we demonstrated anti-tumor effect of nafamostat mesilate via inhibition of NF-κB activation by...
suppression of IkBo phosphorylation, and induction caspase-8 mediated apoptosis in vitro and in vivo without definite adverse effects. Nafamostat mesilate treatment may be therapeutic application for patients with advanced pancreatic cancer.

References


