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New treatment strategy with nuclear factor-κB inhibitor for pancreatic cancer

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ABSTRACT

Background: Because of difficulties with early diagnosis, most patients with pancreatic cancer receive chemotherapy. The National Comprehensive Cancer Network guidelines (version 2.2015) suggest therapy with gemcitabine (GEM) plus nab-paclitaxel (nPTX) as a category 1 recommendation for metastatic pancreatic ductal adenocarcinoma. According to the results of many studies, the activation of chemotherapeutic agents-induced nuclear factor-κB (NF-κB) causes chemoresistance. Hence, we hypothesized that the addition of nafamostat mesilate (NM), a potent NF-κB inhibitor, to GEM/nPTX therapy could enhance the antitumor effect in the treatment of pancreatic ductal adenocarcinoma.

Materials and methods: In vitro, we assessed NF-κB activity and apoptosis under treatment with NM alone (80 μg/mL), with GEM/nPTX, or with a combination of NM and GEM/nPTX in human pancreatic cancer cell lines (PANC-1, MIA PaCa-2, and AsPC-1). *In vivo*, orthotopic pancreatic cancer mice (BALBc nu/nu) were divided into four groups: control ($n = 13$), NM ($n = 13$), GEM/nPTX ($n = 13$), and triple combination ($n = 13$). NM (30 mg/kg) was delivered intraperitoneally three times a week, and GEM/nPTX was injected intravenously once a week to orthotopic pancreatic cancer model mice. In the triple combination group, mice received NM followed by GEM/nPTX on the first day to avoid GEM/nPTX-induced NF-κB activation.

Results: In vitro and *in vivo*, NM inhibited GEM/nPTX-induced NF-κB activation, and a synergistic effect of apoptosis was observed in the triple combination group. Furthermore, tumor growth was significantly suppressed in the triple combination group compared with the other groups.

Conclusions: NM enhances the antitumor effect of GEM/nPTX chemotherapy for orthotopic pancreatic cancer by inhibition of NF-κB activation.

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Introduction

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States and the fifth in Japan.[1-3] Among patients with pancreatic ductal adenocarcinoma, the 5-y survival rate is worse compared with the rates for other malignant diseases.[2,4] Since 1997, the standard first-line treatment for patients with unresectable locally advanced or metastatic pancreatic ductal adenocarcinoma has been gemcitabine (GEM) therapy.[5] However, the therapeutic outcomes are not satisfactory, and several studies have reported 1-y survival rates of only 17%-23%.[5-7]

The Metastatic Pancreatic Adenocarcinoma Clinical Trial study showed that GEM plus nab-paclitaxel (nPTX) was tolerated and superior to GEM with statistical significance in overall survival, and this regimen is recommended in the National Comprehensive Cancer Network guidelines (version 2.2015) as a category 1 recommendation. However, the median overall survival is only 8.5 mo.[8]

Nuclear factor-κB (NF-κB) plays key oncogenic roles in angiogenesis, migration, invasion, proliferation, and chemoresistance in pancreatic cancer cells.[9-20] Most anticancer agents, including GEM and nPTX, induce the activation of NF-κB, which leads to chemoresistance. Therefore, inhibition of chemotherapy-induced NF-κB activation enhances the antitumor effect. We previously reported that nafamostat mesilate (NM), a synthetic serine protease inhibitor, inhibited NF-κB activation by inhibiting IκBα phosphorylation and induced apoptosis in pancreatic cancer cells *in vitro* and *in vivo*.[21-24] In Japan, NM has been used clinically for the treatment of disseminated intravascular coagulation and acute pancreatitis for more than two decades. We hypothesized that the addition of NM to GEM/nPTX chemotherapy for pancreatic ductal adenocarcinoma might enhance the antitumor effect in comparison with GEM/nPTX alone by inhibition of GEM/nPTX-induced NF-κB activation and induction of caspase-8-mediated apoptosis by NM.

Material and methods

Cell culture

Human pancreatic cancer cell lines PANC-1, MIA PaCa-2, and AsPC-1 were obtained from American Type Culture Collection (Rockville, MD). PANC-1 and MIA PaCa-2 were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (Gibco BRL, NY) and 1% penicillin and streptomycin (Gibco BRL). AsPC-1 was cultured in Roswell Park Memorial Institute 1640 medium (RPMI 1640) containing 10% fetal bovine serum (Gibco BRL) and 1% penicillin and streptomycin. The cells were incubated at 37°C in a humidified 5% CO_2 atmosphere.

Reagents

NM was donated by Torii Pharmaceutical Co, Ltd (Tokyo, Japan) and was stored in a stock solution (5 mg/mL) in sterile water at −20°C. GEM was purchased from Eli Lilly Japan (Kobe, Japan). nPTX was purchased from Taiho Pharmaceutical Co, Ltd (Tokyo, Japan). Protease and phosphatase inhibitor cocktail tablets were obtained from Roche Diagnostics (Indianapolis, IN).

Antibodies

Monoclonal antibodies specific to cleaved caspase-8 and cleaved caspase-3 were obtained from Cell Signaling Technology (Beverly, MA). Anti-β-actin antibody was purchased from Sigma–Aldrich (St. Louis, MO).

In vitro *experiment treatment groups*

Based on a previous study, the concentrations of anticancer agents were determined.[25] PANC-1, MIA PaCa-2, and AsPC-1 cells were treated with NM (80 μg/mL; NM group), GEM (1000 nM, 494 nM, and 23.9 μM, respectively) and nPTX (500 nM, 683 nM, and 4.9 μM, respectively; GEM/nPTX group), GEM plus nPTX with NM (triple combination group), or vehicle-only (control group) for the appropriate time in each analysis. In triple combination group, the cells were treated with NM for 3 h before GEM/nPTX treatment.

Animals

Five-wk-old male nude mice (BALBc nu/nu) purchased from CLEA Japan Inc (Tokyo, Japan) were housed under specific pathogen-free conditions in a biologic cabinet at the Laboratory Animal Facility of the Jikei University School of Medicine. The animals were maintained in a 12-h light-dark cycle at a temperature of 22°C ± 2°C and humidity of 55± 5% in a room with filtered air supply. CLEA Rodent Diet CE-2, which is a good laboratory practice-compliant, standard rodent diet consisting mainly of vegetable protein with a proper balance of animal protein, was obtained from CLEA Japan, Inc (Tokyo, Japan) and given to the animals. The protocol of animal experiments was reviewed and approved by the Institutional Animal Care and Use Committee of the Jikei University (no. 26-005) and conformed to the Guidelines for the Proper Conduct of Animal Experiments of the Science Council of Japan (2006).

In vivo *experimental protocol*

The mice were anesthetized with isoflurane. The site of incision was chosen at left flank on the splenic silhouette. After making a 5-mm incision to enter the abdominal cavity without injury to the underlying organs, the spleen was gently mobilized, and the pancreas was delivered together with the spleen through the incision. A suspension of 5.0×10^6 PANC-1 cells in 50 μL of phosphate buffered saline was injected into the tail of the pancreas using a 29-ga needle. Once hemostasis was confirmed, the tail of the pancreas was placed in the abdomen, and the wound was closed in two layers. At 6 wk after injection, the animals were treated with intraperitoneal injection of NM (30 mg/kg) three times a week, or with intravenous (i.v.) injection of GEM (50 mg/kg) and/or nPTX (0.5 mg/kg) once a week. For control group, the equal amount of distilled water was injected intraperitoneal

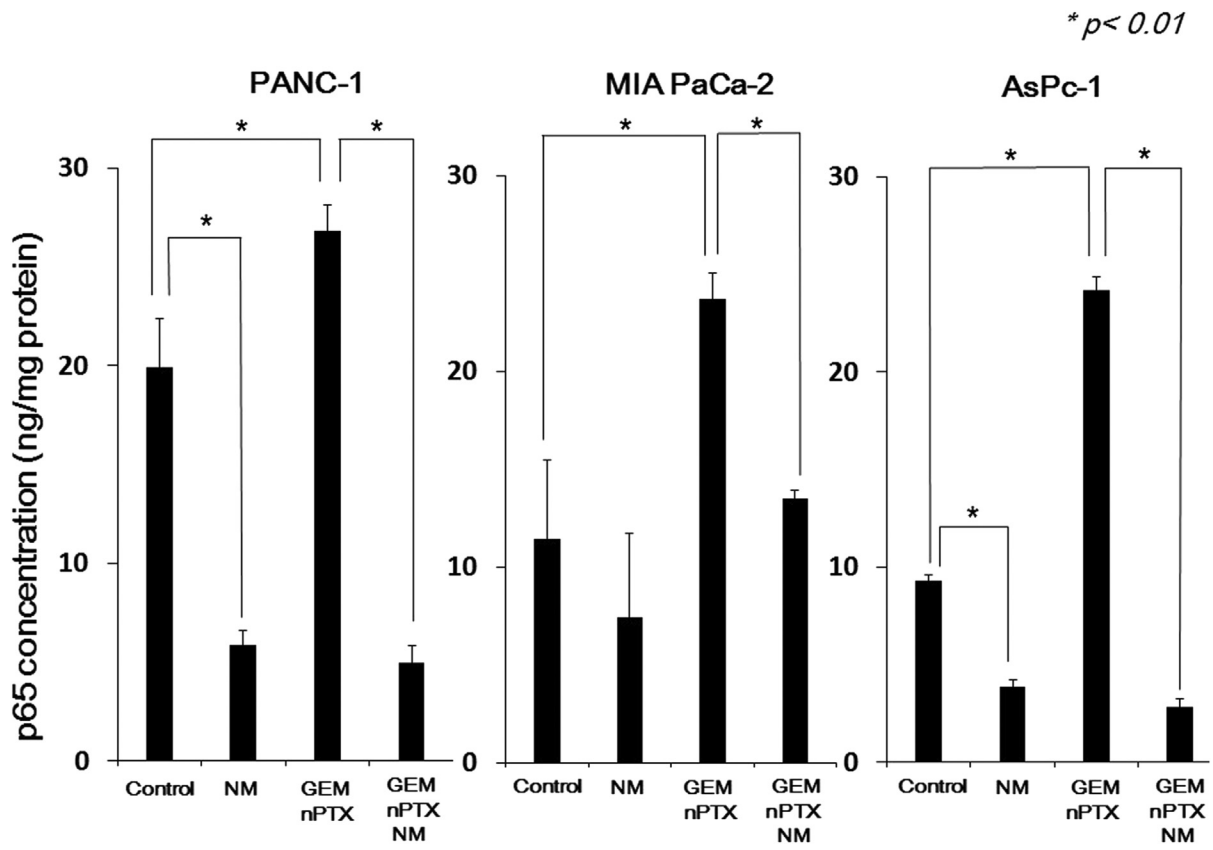

Fig. 1 – NF-κB p65 concentrations in the nucleus *in vitro*. The NF-κB concentrations were significantly higher in the GEM/nPTX group than those in the control group (PANC-1, $P < 0.001$; MIA PaCa-2, $P < 0.001$; AsPC-1, $P < 0.001$), and GEM/nPTX-induced NF-κB activations were significantly inhibited in the triple combination group (PANC-1, $P < 0.001$; MIA PaCa-2, $P < 0.001$; AsPC-1, $P < 0.001$).

three times and i.v. once a week. At 4 wk after treatment, the animals were sacrificed by cardiac puncture and cervical dislocation, and the tumor masses were removed from the normal pancreatic tissues.

Quantitative analysis of NF-κB activity

The NF-κB p65 concentrations in the nuclear extracts were measured. Nuclear extracts from both *in vitro* and *in vivo* experiments were prepared using nuclear extract kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. The cells were seeded into 10-cm dishes (5×10^6 cells/dish) and treated for 2 h. The nuclear extract proteins were assayed using an enzyme-linked immunosorbent assay kit (Trans AM NF-κB; Active Motif) to detect and quantify the NF-κB p65 activity according to the manufacturer's instructions.

Cell proliferation assay

The cells were seeded into 96-well plates (1×10^4 cells/well) and treated for 48 h. The cell proliferation was measured using a Celltiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) according to the manufacturer's instructions.

Annexin V assay

PANC-1, MIA PaCa-2, and AsPC-1 cells were seeded into 10-cm dishes (5×10^6 cells/dish) and treated for 24, 24, and 48 h, respectively. To evaluate the induction of apoptosis, Annexin V assay was performed using Annexin V-FITC Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and flow cytometry (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The data were analyzed with the Attune NxT Software.

Western blot analysis

PANC-1, MIA PaCa-2, and AsPC-1 cells were seeded into 10-cm dishes (5×10^6 cells/dish) and treated for 24, 24, and 36 h, respectively. This protocol for Western blot analysis was described in a previous study.[26] The lysate protein was extracted by 2% sodium dodecyl sulfate from whole cells after each treatment for appropriate time and from excised tumor after treatment *in vivo*. After incubating the blots in each primary antibody (1:1000 dilution) overnight, membranes were incubated with peroxidase-labeled secondary antibody (1:10,000 dilution, Histofine; Nichirei, Tokyo, Japan) for 2 h and detected by using Immunostar LD Chemiluminescent (WAKO chemical, Tokyo, Japan). Protein bands were detected using a

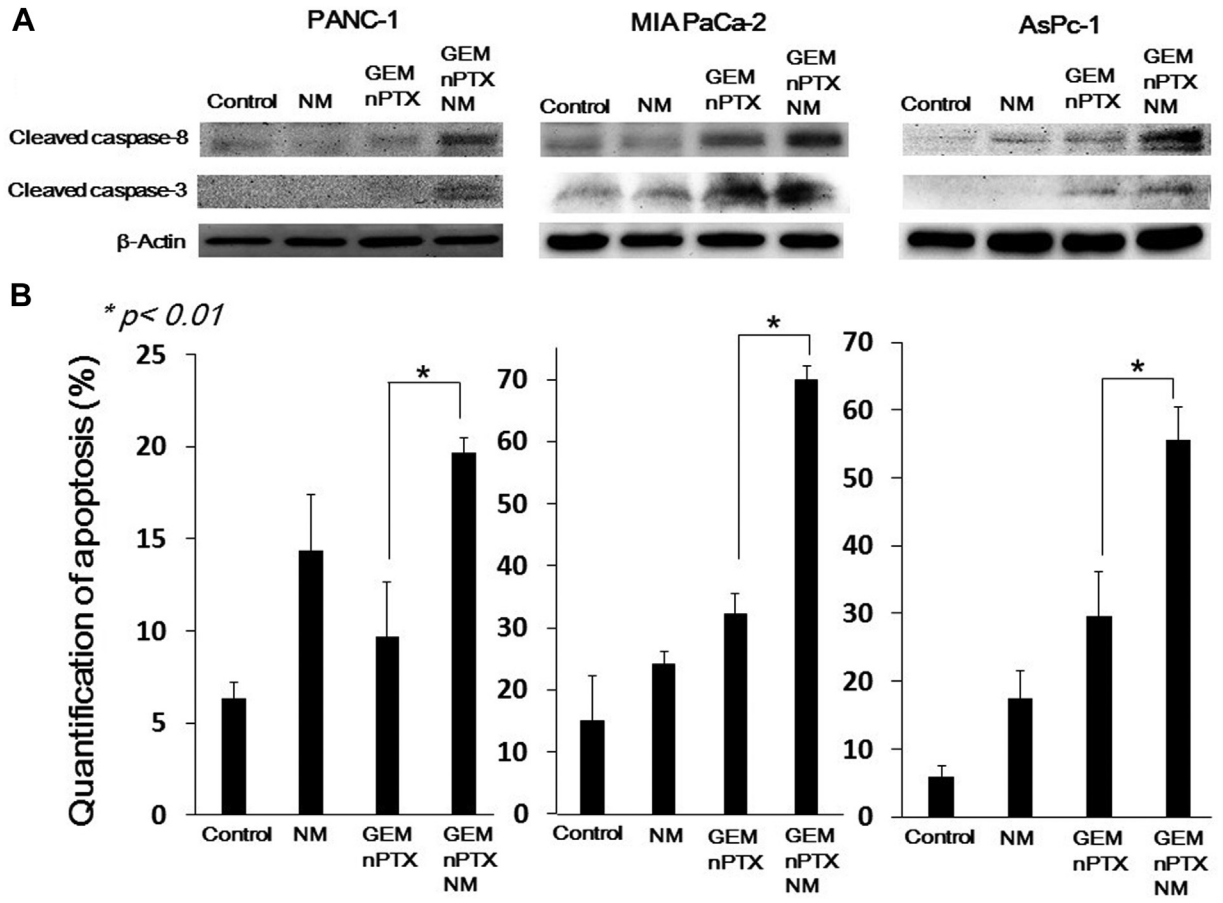

Fig. 2 – Apoptosis signaling and quantification of apoptosis *in vitro*. (A) In Western blot analysis, the levels of cleaved caspase-8 and cleaved caspase-3 in the triple combination group were greater than those in the other groups in all cell lines. (B) In Annexin V assay, quantifications of apoptosis of PANC-1, MIA PaCa-2, and AsPC-1 cells in the triple combination group were the greatest among the experimental groups, and those in the triple combination group were significantly greater than those in the GEM/nPTX group (PANC-1, 19.7 ± 0.9 *versus* 9.7 ± 2.9%; $P < 0.01$, MIA PaCa-2, 70.0 ± 2.1 *versus* 32.3 ± 3.2%; $P < 0.01$, AsPC-1, 55.6 ± 4.8% *versus* 29.6 ± 6.5%; $P = 0.01$).

ChemiDoc XRS + system and Image Lab Software (Bio-Rad, Hercules, CA). Cleaved caspase-8 and cleaved caspase-3 were investigated by Western blot analysis using cytoplasmic protein in PANC-1, MIA PaCa-2, and AsPC-1 cells.

TdT-mediated dUTP nick-end labeling assay

The TdT-mediated dUTP nick-end labeling (TUNEL) assay was performed using an In Situ Cell Death Detection Kit, Fluorescein (Roche diagnostics, Indianapolis, IN) to evaluate the induction of apoptosis. These measurements were performed according to the manufacturer's instructions.

Statistical analysis

Data were expressed as a mean ± SD. A nonpaired Student's *t* test (two-tailed) was used comparing two groups. One-way analysis of variance was used when comparing multiple groups followed by Tukey's post hoc test. All *P* values were considered statistically significant at < 0.05.

Results

NF-κB in nucleus, cleaved caspase-8 and cleaved caspase-3 levels, apoptosis, and cell proliferation in the experimental groups in vitro

As shown in Figure 1, the concentrations of NF-κB p65 in the nuclear extracts of PANC-1, MIA PaCa-2, and AsPC-1 cells treated with NM were significantly lower than those in the control group (PANC-1, $P < 0.001$; AsPC-1, $P < 0.001$), and NF-κB of MIA PaCa-2 cells tended to be lower than that in the control group ($P = 0.202$). The NF-κB concentrations were significantly higher in the GEM/nPTX group than those in the control group (PANC-1, $P < 0.001$; MIA PaCa-2, $P < 0.001$; AsPC-1, $P < 0.001$), whereas those in the triple combination group were significantly lower than those in the GEM/nPTX group (PANC-1, $P < 0.001$; MIA PaCa-2, $P < 0.001$; AsPC-1, $P < 0.001$).

In each cell line, both cleaved caspase-3 and cleaved caspase-8 were most upregulated in the triple combination groups (Fig. 2A).

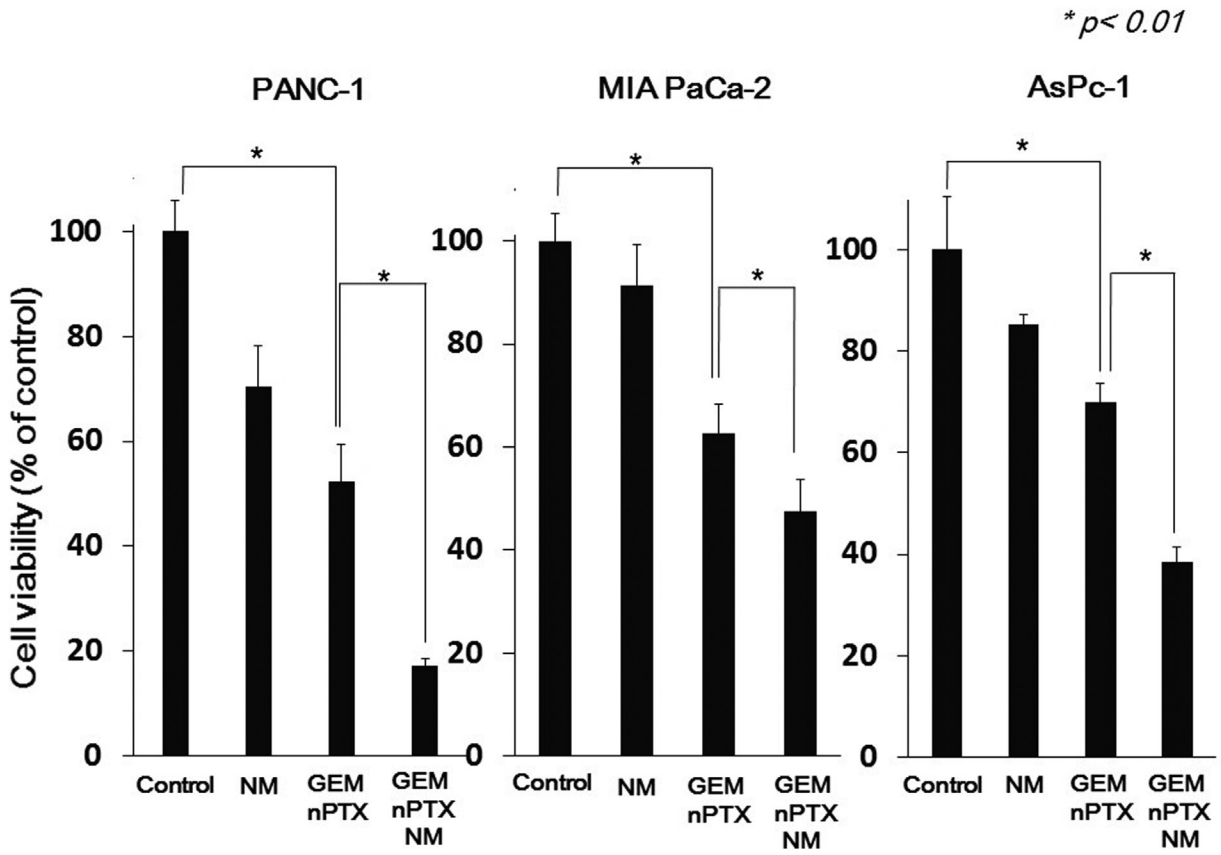

Fig. 3 – Cell proliferation *in vitro*. In the cell proliferation assay, cell viabilities of PANC-1, MIA PaCa-2, and AsPC-1 cells in the triple combination group were significantly lower than those in the GEM/nPTX group (PANC-1, 17.3 ± 1.3 *versus* 52.4 ± 7.0%; $P < 0.01$, MIA PaCa-2, 47.5 ± 6.2 *versus* 62.6 ± 5.8%; $P < 0.01$, AsPC-1, 38.6 ± 2.8 *versus* 70.0± 3.7%; $P < 0.01$).

NM significantly enhanced apoptosis induced by GEM/nPTX in all cell lines (PANC-1, $P = 0.001$; MIA PaCa-2, $P < 0.001$; AsPC-1, $P = 0.001$; Fig. 2B).

Cell viabilities of PANC-1, MIA PaCa-2, and AsPC-1 cells in the triple combination group were significantly lower than those in the GEM/nPTX group (PANC-1, $P < 0.001$; MIA PaCa-2, $P = 0.009$; AsPC-1, $P < 0.001$; Fig. 3). In addition, those in the GEM/nPTX group were significantly lower than those in the control group (PANC-1, $P < 0.001$; MIA PaCa-2, $P < 0.001$; AsPC-1, $P < 0.001$; Fig. 3).

Antitumor effects of each treatment in vivo

The tumor weight were 237 ± 171 mg in the triple combination group, 404 ± 217 mg in the GEM/nPTX group, 409 ± 194 mg in the NM group, and 560 ± 380 mg in the control group. The tumor weight in the triple combination group was significantly lower than that in the GEM/nPTX groups (Fig. 4B; $P = 0.039$). Animal body weight loss at sacrifice in the triple combination group was comparable with those in the control and GEM/nPTX group (data not shown).

NF-κB activity, cleaved caspase-8 and cleaved caspase-3 expression in resected pancreatic tumor

The NF-κB concentration in the GEM/nPTX group was significantly greater than that in the control group ($P = 0.001$). The NF-κB concentration in the triple combination group was significantly lower than that in the GEM/nPTX group ($P < 0.001$). Upregulation of both cleaved caspase-8 and cleaved caspase-3 was observed in the triple combination group (Fig. 5B).

Apoptosis in resected tumor treated with each agent

To evaluate apoptosis in the resected tumor, TUNEL staining was used. Apoptotic cells were stained as bright green cells in sections after TUNEL staining. The number of apoptotic cells was the greatest in the triple combination group compared with the other groups (Fig. 5D). Furthermore, NM significantly enhanced apoptosis induced by GEM/nPTX treatment.

Discussion

GEM, a cytotoxic pyrimidine analog, is one of the standard treatments for pancreatic ductal adenocarcinoma. However, its effect on survival is limited. Combination therapy with a second cytotoxic agent has so far proven to be largely ineffective.[5,27] Recent reports in mice and humans suggested that these poor responses to treatment were attributable to poor drug delivery, the highly desmoplastic and hypovascular nature of the cancer, or metabolic inactivation of therapeutic

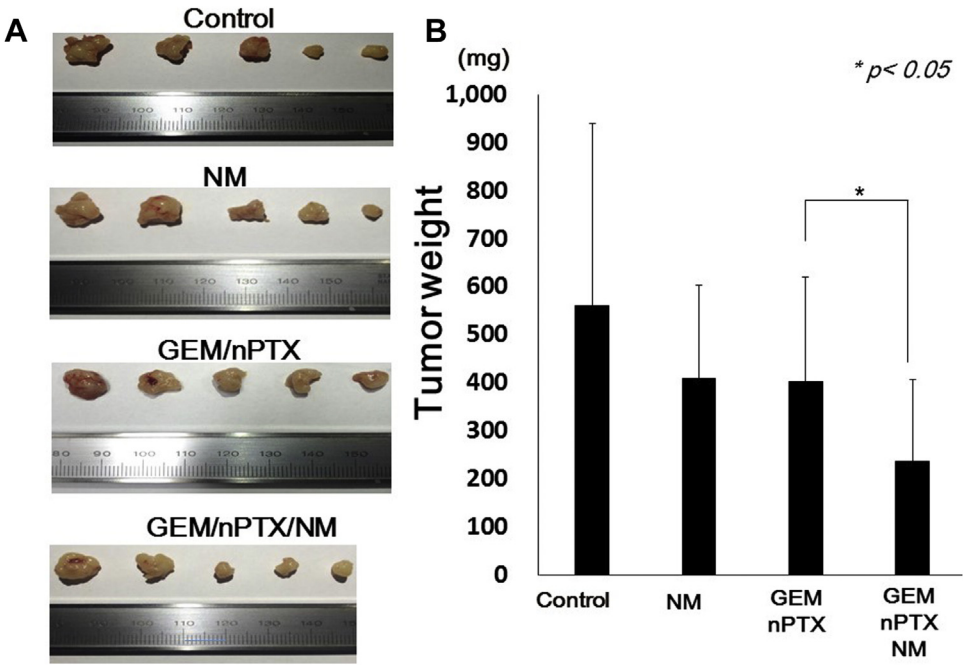

Fig. 4 – The tumor growth in orthotopic mouse model of pancreatic cancer. (A) The photographs of the excised tumor of the orthotopic model in the four experimental groups. (B) The tumor weight of the triple combination group was the lowest and significantly lower than the GEM/nPTX group ($P = 0.039$). (Color version of figure is available online.)

agents.[28,29] nPTX, a water-soluble albumin-bound formulation of paclitaxel, has been reported to disrupt the pancreatic ductal adenocarcinoma stromal architecture in tumor xenografts and induce reactive angiogenesis, resulting in increased perfusion and delivery of GEM.[30] It is suggested that nPTX produces the antitumor effects by itself and decreases the levels of cytidine deaminase, the primary GEM catabolic enzyme, through the induction of reactive oxygen species to stabilize GEM and sensitize the pancreatic cancer cells to combination treatment.[31]

On the other hand, most cytotoxic chemotherapeutic agents, including GEM and paclitaxel, activate NF-κB,[17-20,32] which leads to chemoresistance in cancer.[14,15,23] The inhibitor-of-apoptosis (IAP) proteins, such as c-IAP1 and c-IAP2, are regulated by NF-κB, and they inhibit caspase-8-mediated apoptosis.[33] Therefore, inhibition of GEM/nPTX-induced NF-κB activation could be a novel chemotherapeutic approach for pancreatic cancer. Various NF-κB inhibitors have been investigated to block a series of NF-κB signaling cascades.[34] We have already demonstrated that NM, which is not what is referred to as a cytotoxic agent, suppresses tumor growth through the inhibition of NF-κB activation in pancreatic cancer cells by inhibiting phosphorylation of IκBα.[22,23] Uwagawa *et al.*[21] reported that NM inhibited NF-κB activity in a dose-dependent manner. We previously reported that GEM, tumor necrosis factor-alpha gene delivery, and ionizing radiation activate NF-κB of pancreatic cancer, and NM can inhibit those agents-induced NF-κB activation.[23,24,35] In this study, NM could inhibit GEM/nPTX-induced NF-κB activation, which was higher than NF-κB activation induced by GEM alone (data not shown) and significantly enhanced the anticancer effect of GEM/nPTX on pancreatic cancer cells. Moreover, NM had an additive antitumor effect of GEM/nPTX on orthotopic pancreatic cancer mice. Similar to subcutaneous cancer model of previous studies,[22,24] NM inhibited GEM/nPTX-induced NF-κB activation and induced caspase-8-mediated apoptosis. To clarify the effect of NM, GEM, and nPTX in human pancreatic cancer cell line, we examined the effect using nude mice. The effect in immunocompetent mice on tumor progression is important. Our previous study showed that NM inhibited not only NF-κB activation but also interleukin-8 production.[36] The correlation of these proinflammatory cytokines and tumor progression in immunocompetent mice needs to be examined with or without NM in the future.

In clinical, as the half-life of NM by i.v. injection is short in human body, it is difficult to keep a same blood concentration as *in vivo* study in human. Therefore, we inserted the arterial catheter into common hepatic artery through a side hole in the celiac artery and continuously administered NM to pancreatic cancer together with intravenously injected anticancer agents.

Several large phase 3 studies for unresectable advanced pancreatic cancer have been conducted worldwide. However, only three regimens were able to achieve improvement in overall survival in comparison with GEM monotherapy. The Metastatic Pancreatic Adenocarcinoma Clinical Trial study, which is the most recently reported, is a phase 3 study of GEM and nPTX for metastatic pancreatic cancer.[8] The occurrence rate of severe adverse effects was less than that observed with FOLFIRINOX, reported in the ACCORD11 study.[28] In our previous phase I study of NM with GEM for patients with unresectable pancreatic cancer, only one of 12 patients (8%) developed grade 3 neutropenia, and no patient experienced

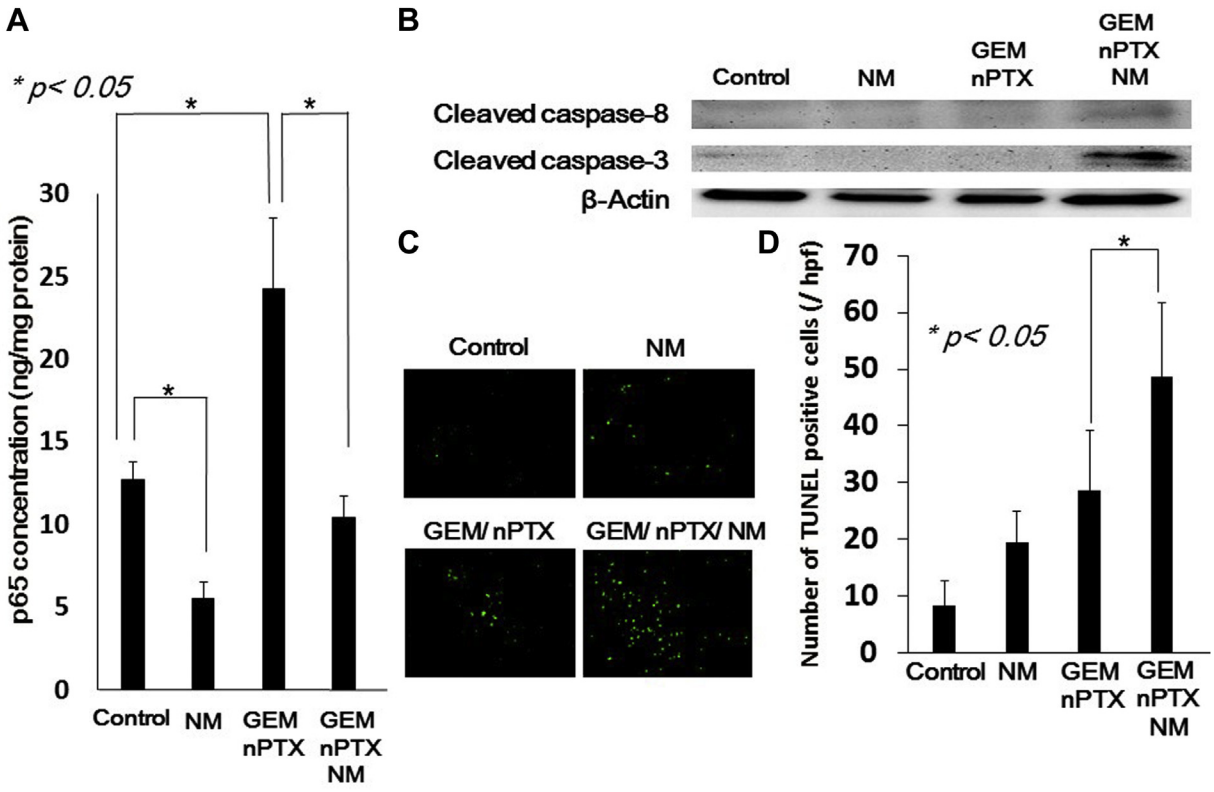

Fig. 5 – NF-κB activity in the nucleus and apoptosis in the orthotopic mouse model of pancreatic cancer. (A) The NF-κB concentration of excised tumor tissues in the GEM/nPTX group was significantly greater than that in the control group ($P = 0.001$), and GEM/nPTX-induced NF-κB activation was inhibited in the triple combination group ($P < 0.001$). (B) In Western blot analysis, the levels of cleaved caspase-8 and cleaved caspase-3 in the triple combination group were greater than those in the other group. (C) TUNEL staining of excised tumors for the experimental groups (×200). (D) The triple combination group had the greatest number of apoptotic cells among the four experimental groups, and the numbers of apoptotic cells of the triple combination group was significantly greater than those of the GEM/nPTX group (48.6 ± 13.2 *versus* 28.6 ± 10.7; $P < 0.01$). (Color version of figure is available online.)

either grade 2 or 3 nonhematologic adverse events.[37] Moreover, our phase 2 clinical study in patients with unresectable pancreatic cancer demonstrated that combination treatment with GEM and NM provided both acceptable overall survival and other clinical advantages such as dose reduction of opioid analgesic consumption and a healthy weight gain during the treatment.[38] Given these results, this combination chemotherapy would be expected to be a useful novel chemotherapy regimen. We plan on applying this combination chemotherapy for patients with metastatic pancreatic cancer or local advanced and borderline resectable pancreatic cancer as neoadjuvant and adjuvant chemotherapy.

In summary, NM enhanced GEM/nPTX-induced apoptosis by inhibiting NF-κB activation in pancreatic ductal adenocarcinoma *in vitro* and *in vivo*. Triple combination therapy with GEM, nPTX, and NM could be a new strategy to add to the clinical approaches for pancreatic ductal adenocarcinoma.

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Authors' contribution: Critical revision of the article for important intellectual content was done by T.H. Conception and design of the study was developed by T.U., H.S., and T.O. Collection and assembly of data were carried out by T.H., Y.S., N.S., R.I., and K.H. Final approval of the article was done by K.Y.

Disclosure

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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