p202, an Interferon-inducible Protein, Mediates Multiple Antitumor Activities in Human Pancreatic Cancer Xenograft Models[1]

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ABSTRACT

p202, an IFN-inducible protein, interacts with certain transcriptional activators leading to transcriptional repression. p202 expression has been associated with inhibition of cancer cell growth *in vitro* and *in vivo*. To examine a potential p202-mediated antitumor activity in pancreatic cancer, we used both ectopic and orthotopic xenograft models and demonstrated that p202 expression is associated with multiple antitumor activities that include inhibition of tumor growth, reduced tumorigenicity, prolonged survival, and remarkably, suppression of metastasis and angiogenesis. *In vitro* invasion assay also showed that p202-expressing pancreatic cancer cells are less invasive than those without p202 expression. That observation was supported by the findings that p202-expressing tumors showed reduced expression of angiogenic markers, such as interleukin 8 and vascular endothelial growth factor, and p202-expressing pancreatic cancer cells have reduced level of matrix metalloproteinase-2 activity, a secreted protease activity important for metastasis. Importantly, we demonstrated a treatment efficacy by using *p202*/SN2 liposome complex in a nude mice xenograft model, suggesting a feasibility of using the *p202*/SN2 liposome in future preclinical gene therapy experiments. Together, our results strongly suggest that p202 expression mediates multiple antitumor activities against pancreatic cancer and may provide a scientific basis for developing a *p202*-based gene therapy in pancreatic cancer treatment.

INTRODUCTION

Pancreatic cancer is highly aggressive and is a leading cause of cancer death in Western countries. The deadliness of this disease is illustrated by the prediction in 1999 that 28,600 new cases would be diagnosed and most of them would be fatal (1). The main reason for the extremely poor prognosis is the fact that patients often present with advanced stage at the time of diagnosis. The median survival varies between 4 and 6 months, and the 5-year survival rate is <2% (2). Currently, there is no effective treatment for this deadly disease because conventional chemotherapy and radiation treatments have had very limited success to improve patient survival (3). Therefore, novel treatment strategies against this disease are urgently needed.

p202 is an IFN-inducible protein, and its expression is associated with growth inhibition (4, 5). The findings that p202 interacts with cell cycle transcriptional regulators, such as E2F-1/DP-1, E2F-4/DP-1, AP-1 (c-Fos/c-Jun), and c-Myc, and represses their transcriptional activities have provided insight into the molecular mechanism by which p202 mediates growth inhibition (6–10). We have docu-

mented previously that p202 inhibits human cancer cell growth *in vitro* and suppresses tumor growth *in vivo* (11, 12). Furthermore, we showed that p202 expression sensitizes breast cancer cells to TNF-α-induced apoptosis. The mechanism responsible for the p202-mediated sensitization is likely attributable to the inactivation of TNF-α-induced NF-κB[4] by p202 (12). In light of the antiapoptotic role of NF-κB in TNF-α-mediated apoptosis (13), we have hypothesized that NF-κB inactivation by p202 leads to the abolishment of the antiapoptotic process and results in sensitizing cancer cells to TNF-α-induced apoptosis. On the other hand, the aberrant NF-κB activity has been implicated, at least in part, in tumorigenesis and the chemoresistant phenotype of certain human cancers including pancreatic cancer (14, 15). Thus, it is likely that *p202*-based gene therapy may be particularly useful in targeting tumors that contain such aberrant NF-κB activity. In this study, we tested that possibility in pancreatic cancer cells that possess the constitutively active NF-κB (16). We showed that, in addition to the growth inhibition *in vitro* and tumor suppression *in vivo*, p202 expression was found associated with suppression of metastasis and angiogenesis in an orthotopic pancreatic cancer xenograft model. Importantly, we demonstrated a treatment efficacy of using *p202*/liposome gene therapy in a pancreatic cancer xenograft model. Together, our results raise a possibility of using *p202*-based gene therapy strategy in pancreatic cancer treatment.

MATERIALS AND METHODS

Cell Culture. Human pancreatic cancer cell lines Capan-1, PANC-1, BxPC-3, AsPC-1, and CFPAC-1 were obtained from the American Type Culture Collection and maintained as recommended. Transfected cell lines were maintained in complete medium containing 500 μg/ml G418 (Life Technologies, Inc.).

Colony-forming Assay. Cells were transfected with a p202 expression vector (CMV-*p202*; in which *p202* cDNA is driven by CMV promoter) or an control vector (pcDNA3). Both plasmids contain the neomycin-resistant gene. Three weeks after transfection and G418 selection, cell colonies were stained by 0.5% crystal violet containing 20% ethanol.

Western Blot Analysis. Protein lysate was prepared with RIPA-B cell lysis buffer containing 20 mM Na$_2$PO$_4$ (pH 7.4), 150 mM NaCl, 1% Triton X-100, 100 mM NaF, 2 mM Na$_3$VO$_4$, 5 mM phenylmethylsulfonyl fluoride, 1% aprotinin, and 10 μg/ml leupeptin. Rabbit anti-p202 polyclonal antibody was kindly provided by Dr. Divaker Choubey (Loyola University, Chicago, IL). Donkey antirabbit IgG peroxidase (Jackson) was used as secondary antibody. Western blots were developed by enhanced chemiluminescence (ECL; Amersham).

Northern Blot Analysis. Total RNA was isolated from PANC-1, pcDNA3, p202-1, and p202-2 cells using a TRIZOL RNA isolation kit (Life Technologies, Inc.). Twenty μg of total RNA were separated by electrophoresis under denaturing conditions and then transferred to a Hybond N$^+$ membrane. Full-length *p202* cDNA isolated from CMV-*p202* plasmid by *Bam*HI digest was gel-purified and ^{32}P-labeled by using a random-labeling kit. Hybridization was performed at 65°C overnight in solution containing 1% BSA (w/v), 0.2 M sodium phosphate, 1 mM EDTA, 7% SDS (w/v), 15% formamide, and 40

[4] The abbreviations used are: NF-κB, nuclear factor-κB; CMV, cytomegalovirus; luc, luciferase; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; IL, interleukin.

7142

Table 1 *Number of colonies from transfection of pancreatic cell lines with p202 and control vector DNA*

Cell line	pcDNA3	p202	% of control
Capan-1	130	3	2.3
PANC-1	508	108	21.0
BxPC-3	4	1	25.0
AsPC-1	232	31	13.4
CFPAC-1	6	0	0

μg/ml salmon testes DNA (Sigma Chemical Co.). The blot was subsequently washed three times in 40 mM sodium phosphate, 1 mM EDTA, 1% SDS (w/v) at 65°C for 5 min/wash and twice at 70°C for 10 min/wash. The *p202* RNA (1.8 kb) was visualized by using a PhosphorImager.

Transfection and luc Assays. PANC-1, pcDNA3, p202-1, and p202-2 cells were transfected with 0.5 μg of κB-luc construct and 0.1 μg of the internal transfection control (pRL-TK; Promega). Forty-eight h after transfection, cells were harvested, and luc activity was measured using the dual luc assay system (Promega) according to the protocol supplied by the manufacturer. The κB-luc activity was normalized by the internal control luc activity of pRL-TK. To determine the p202 dose effect, PANC-1 cells were cotransfected with 50 ng of CMV-luc and an increasing amount (0, 0.5, or 2 μg) of CMV-*p202*. The total amount of DNA transfected at each p202 dose was kept constant (2.05 μg) by adding an appropriate amount of pcDNA3 vector. luc activity was measured 48 h after transfection. The relative activities were calculated by setting the luc activities obtained from transfections without CMV-*p202* (0 μg) at 100%. The data represent mean $\pm$ SD of two independent experiments.

Soft Agar Assay. Aliquots of cells (1 $\times$ 10^4) were mixed at 37°C with 0.5% agarose (Sea Plaque, low gelling temperature; FMC Bioproducts, Rockland, ME) in complete medium and gelled at 4°C for 15 min over a previously gelled layer of 1% agarose in complete medium in six-well dishes. After incubation for 3 weeks, 200 μl of 1 mg/ml *p*-iodonitrotetrazolium violet were added and incubated for an additional 24 h. Colonies were photographed using a Zeiss microscope and counted using computer software associated with the microscope.

Ectopic Tumorigenicity Assays in Nude Mice. Aliquots of cells (1 $\times$ 10^6) in 200 μl of PBS were injected s.c. on both sides of the abdomen of female nude mice, 4–5 weeks of age. Tumor sizes were measured with a caliper every week. The tumor volume was calculated using the formula: Volume = $S \times S \times L/2$, where S is the short length of the tumor in cm and L is the long length of the tumor in cm.

Orthotopic Tumorigenicity and Survival Assays in Nude Mice. Aliquots of cells (1 $\times$ 10^6) were suspended in 50 μl of PBS as single-cell suspensions. Nude mice were anesthetized with methoxyflurane and placed in the supine position. An upper midline abdomen incision was made, and the pancreas was exteriorized. Tumor cells were injected into the tail of the

pancreas, and the abdomen was closed using wound clips. Animals were sacrificed 3 months after tumor inoculation. Tumors in the pancreas were harvested and weighed. Livers were fixed in Bouin's solution for 24 h to differentiate the neoplastic lesions from the organ parenchyma, and the metastases on the surface of liver were counted with the aid of a dissecting microscope. For survival assays, daily survival of mice was monitored and recorded as dead or euthanized when the animals reached the moribund stage.

***In Vitro* Invasion Assay.** The procedure was followed as described previously (17), except for the following modification. The 24-well chamber with an 8-μm pore size polycarbonate filter (Costar Co., Cambridge, MA) was coated with Matrigel (Becton Dickinson Labware, Bedford, MA) according to the manufacturer's protocol.

Zymography. Cells were grown to 70% confluency in DMEM/F12 medium containing 10% FBS and switched to serum-free medium (17). After 2 days of incubation, the conditioned medium was collected, passed through a 0.22 μm filter, and then concentrated to a small volume on Centricon YM 30 filter units. Four μg of each sample were loaded on the gel. For the positive control, 0.25 μl of FCS was used. Zymography was performed using gelatin-embedded SDS gels as described previously (17).

Immunohistochemistry. Tumor tissue sections (5 μm thick) of the formalin-fixed, paraffin-embedded specimens were deparaffinized in xylene and rehydrated in graded alcohol. The endogenous peroxidase was blocked by the use of 3% hydrogen peroxide in PBS for 12 min. The samples were incubated for 20 min at room temperature with a protein blocking solution containing 5% normal horse serum and 1% normal goat serum and in PBS. Samples were then incubated at 4°C in a 1:50 dilution of rabbit polyclonal anti-IL-8 antibody (Biosource International, Camarillo, CA) or 1:50 dilution of rabbit anti-VEGF antibody, followed by the incubation with peroxidase-conjugated antirabbit IgG at room temperature for 1 h, and with diaminobenzidene (Research Genetics) for 5 min. The sections were counterstained with Mayer's hematoxylin (Biogenex Laboratories, San Ramon, CA) and mounted with a Universal mount (Research Genetics). When examined under a microscope, a positive reaction was indicated by a reddish-brown precipitate in the cytoplasm or the nucleus. Tissue sections vessels in solid tumors growing in the pancreas of nude mice were determined under light microscope after immune staining of sections with anti-CD31 antibodies. Cryostat sections of tumors were fixed with 2% paraformaldehyde in PBS (pH 7.5) for 10 min at room temperature and processed for immunostaining as described above for paraffin-embedded tissues.

***p202* Gene Therapy Treatment in a Human Pancreatic Cancer Xenograft Model.** Aliquots of PANC-1 cells (1 $\times$ 10^6) in 200 μl of PBS were injected (per site) s.c. on both sides of the abdomen of female nude mice, 4–5 weeks of age. After tumors reached 5 mm in diameter, mice received treatment with the *p202*/SN2 complex through intratumor injection. For each injection, 15 μg of CMV-*p202* or the control vector, *i.e.,* a luc cDNA driven by a CMV promoter (CMV-*luc*), was complexed with 30 μg of SN2. CMV-*p202*/SN2
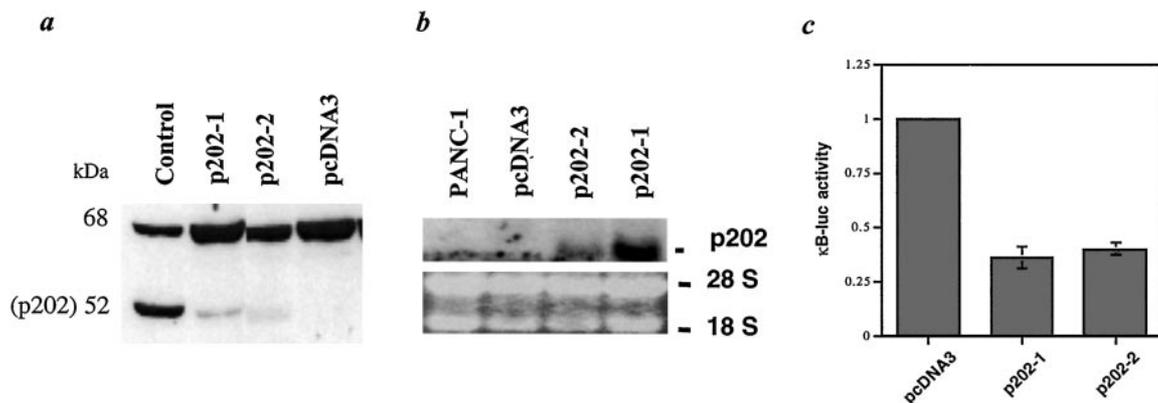

Fig. 1. Generation of the p202-expressing PANC-1 cells. *a*, two p202-expressing PANC-1 cell lines (p202-1 and p202-2) were generated, and p202 protein (M_r 52,000) expression was analyzed by Western blot using p202-specific antibody. The M_r 68,000 nonspecific band serves as an internal loading control. The positive control was AKR-2B cells stimulated by IFN-α (*Control*), and the vector (*pcDNA3*)-transfected cells served as negative control. *b*, p202 mRNA expression in p202-1 and p202-2 cells. Twenty μg of total RNA isolated from PANC-1, pcDNA3, p202-1, and p202-2 cells were analyzed by Northern blot using a full-length p202 cDNA as a probe. The p202-specific RNA (1.8 kb) is indicated. As internal loading controls, 18S and 28S rRNAs on the membrane after gel transfer were stained by ethidium bromide. *c*, p202 expression inhibits the NF-κB-mediated transcription. A *luc* gene driven by IκB promoter (κB-luc) was transfected into p202-1, p202-2, and vector control (*pcDNA3*) cells, followed by luc assay. The relative luc activity is shown with that in pcDNA3 cells set at 100%. *Bars,* SD.
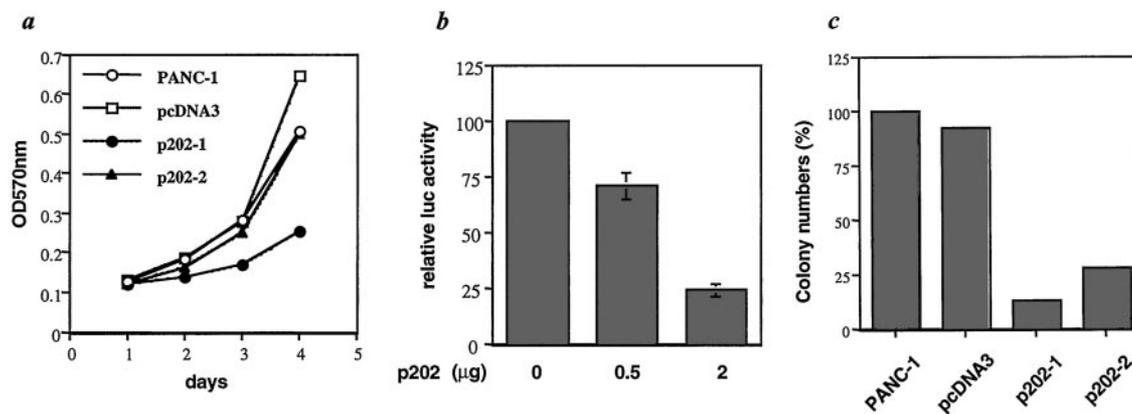
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Fig. 2. The p202-expressing PANC-1 cells exhibit a reduced growth *in vitro*. *a*, the growth rate of PANC-1 (parental), pcDNA3, p202-1, and p202-2 cells was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Each measurement was done in quadruplicates. *b*, the p202 dose effect on growth inhibition. PANC-1 cells were cotransfected with 50 ng of CMV-luc and an increasing amount (0, 0.5, or 2 μg) of CMV-p202. The relative activities were calculated by setting the luc activities obtained from transfections without CMV-p202 (0 μg) at 100%. The data represent means of two independent experiments; *bars,* SD. *c*, PANC-1, pcDNA3, p202-1, and p202-2 cells were grown in soft agar. The colony number was scored 3 weeks after seeding by *p*-iodonitrotetrazolium violet staining. The relative number is presented using that of the parental cells as 100%.

complex was administrated twice a week. Mice in the control group were injected with either CMV-*luc*/SN2 complex or SN2 alone. Tumor size and treatment related side effect were monitored twice a week. SN2 is a liposome-based, nonviral delivery system developed recently by our group.[5]

RESULTS

p202 Expression Inhibits Human Pancreatic Cancer Cell Growth *in Vitro*. To determine the growth-inhibitory activity of p202 in human pancreatic cancer cells, we performed a colony-forming assay on five human pancreatic cancer cell lines, *i.e.,* Capan-1, PANC-1, BxPC-3, AsPC-1, and CFPAC-1. [All of them are known to possess the constitutively active NF-κB (16).] As shown in Table 1, with all cell lines tested, the number of G418-resistant colonies in the *p202*-transfected cells was consistently fewer than that of the pcDNA3-transfected cells with a reduction ranged from 75% (BxPC-3) to 100% (CFPAC-1). This result suggests that p202 possesses a strong growth-inhibitory activity in human pancreatic cancer cells. Consistent with that observation, our effort to isolate p202 stable pancreatic cancer cell lines has yielded only 2 p202-expressing PANC-1 clones (*i.e.,* p202-1 and p202-2) of 20 G418-resistant clones screened by Western analysis. p202-1 expresses a higher level of p202 protein than p202-2 (Fig. 1*a*), and that correlates well with the levels of *p202* mRNA expression of these clones as determined by a Northern blot analysis (Fig. 1*b*). To test whether p202 expression inhibits NF-κB activity in the p202-expressing cells (12), we performed a gel-shift assay and found that the p202 expression level in p202-1 and p202-2 cells is efficient to abolish the DNA binding activity of NF-κB (data not shown). The reduced NF-κB DNA binding activity in p202-expressing cells was further confirmed by the reduced NF-κB-mediated promoter activity in p202-1 and p202-2 cells as compared with that in the control cells (pcDNA3; Fig. 1*c*). As shown in Fig. 2*a*, the growth rate of p202-1 cells is the slowest as compared with that of PANC-1, vector control (pcDNA3), and the low p202 expressor, p202-2 cells. The different levels of p202 expression may account for the different growth rates seen between p202-1 and p202-2 cells. This result is consistent with our previous observation that the extent of growth inhibition is p202 dose dependent (11). To further confirm that observation, we performed a transient transfection assay in which a fixed amount (50 ng) of CMV-*luc* was cotransfected with an increasing amount (0, 0.5, and 2 μg) of CMV-*p202* in PANC-1 cells.

Because the apparent luc activity is indicative of living cells, we showed that p202 expression caused overall growth inhibition in a dose-dependent manner (Fig. 2*b*). Under the same condition, no apparent apoptosis was observed as determined by flow cytometry analysis to detect sub-G_1 apoptotic cells (data not shown). Thus, our
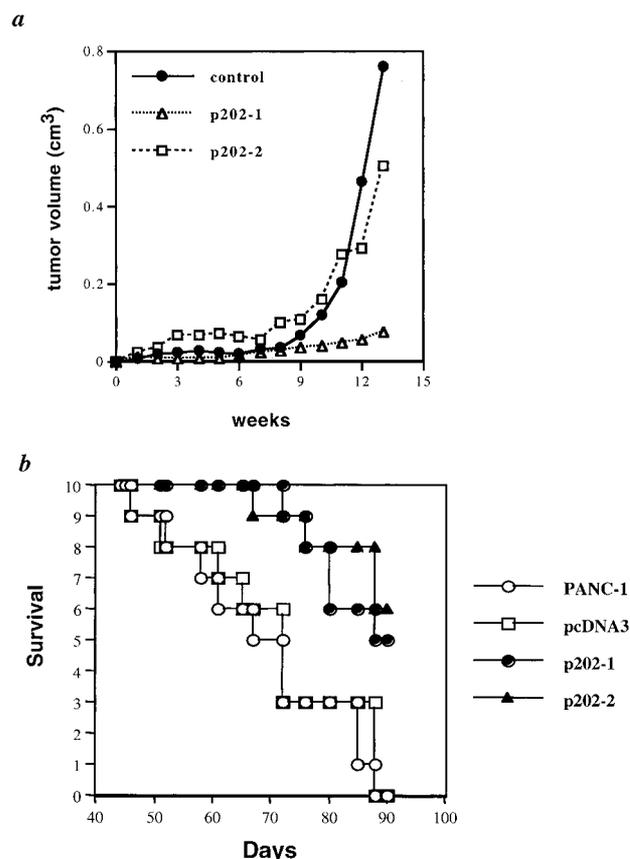

Fig. 3. p202 expression mediates antitumor activity *in vivo*. *a*, tumorigenicity assay. PANC-1 (*control*), p202-1, and p202-2 cells were s.c. implanted in the abdomen of 4–5-week-old female nude mice (five mice/group). Tumor sizes were measured with a caliper every week. The tumor volume was calculated using the formula: Volume = $S \times S \times L/2$, where S is the short length of the tumor in cm and L is the long length of the tumor in cm. *b*, mice bearing p202-expressing PANC-1 tumors in pancreas exhibited a longer survival rate. PANC-1, pcDNA-3, p202-1, and p202-2 cells were injected orthotopically into mouse pancreases. The time of death was recorded, and the survival rate was calculated as the percentage of the surviving animals (the starting animal number was set at 100%).

7144

Table 2 *Growth and metastasis of PANC-1 human adenocarcinoma cells in nude mice[a]*

Lines	Orthotopic growth (pancreas)		Gross hepatic metastasis	
	Incidence	Median (tumor weight, g)	Incidence	Median (number)
PANC-1	5/5	0.30 (0.12, 0.20, 0.30, 0.51, 0.63)	2/5[b]	0 (0, 0, 0, 3, 4)
pcDNA3	5/5	0.22 (0.08, 0.13, 0.22, 0.32, 0.46)	1/5[c]	0 (0, 0, 0, 0, 5)
p202-1	1/5	0 (0, 0, 0, 0, 0.06)[d]	0/5	0 (all 0)[d]
p202-2	2/5	0 (0, 0, 0, 0.045, 0.05)[d]	0/5[e]	0 (all 0)[d]

[a] Othortopic tumor transplantation (1×10^6 cells/mouse) was performed by intrapancreatic injection. Mice were killed after 3 months.

[b] Two mice were found with peritoneal dissemination.

[c] One mouse was found with peritoneal dissemination.

[d] $P < 0.01$ as compared with control groups.

[e] One mouse was found dead before the experiment terminated with no tumor and no metastasis.

results support the idea that the level of p202 expression is proportional to its growth-inhibitory activity. Consistent with the ability of p202 to suppress transformation phenotype (11, 12), both p202-1 and p202-2 cells showed a significant reduction in the number of soft-agar colonies as compared with that of the control cell lines, *i.e.,* PANC-1 and pcDNA3 (Fig. 2*c*). Our results thus indicate that p202 is a potent growth inhibitor in suppressing pancreatic cancer cell growth.

p202 Expression Suppresses Tumorigenicity in Ectopic and Orthotopic Pancreatic Cancer Xenograft Models. To determine whether p202 expression could mediate an antitumor effect on pancreatic cancer cells *in vivo*, we first examined whether p202 suppresses the tumorigenicity of PANC-1 cells. p202-1 and p202-2 cells were s.c. injected into nude mice ,and tumor growth was monitored thereafter. As shown in Fig. 3*a*, p202-1 tumors grew significantly slower than the vector-transfected PANC-1 tumors. p202-2 tumors (which express less p202 than p202-1 tumors) on the other hand

started to show a modest growth reduction 12 weeks after injection as compared with that of the control tumors. Similar to p202-mediated growth inhibition, the extent of antitumor activity observed here appears to be dependent on the level of p202 expression. To further examine the p202-mediated antitumor activity in an organ (pancreas)-specific environment, we injected p202-1 and p202-2 cells directly into mouse pancreas. Three months after injection, mice were sacrificed, and tumor growth was measured. We found that although PANC-1 tumors and vector control tumors grew readily in mouse pancreas at 100% frequency (five of five), p202-1 and p202-2 cells are tumorigenic at a much lower frequency, *i.e.,* 20% (one of five) and 40% (two of five), respectively (Table 2). Furthermore, the average tumor size (measured by weight) of the control tumors was about five times that of p202-1 or p202-2 tumors. When the survival rate was measured, we observed that mice bearing p202 tumors had a longer survival than those bearing control tumors without p202 expression (Fig. 3*b*). In particular, mice bearing p202-1 or p202-2 tumors had 50% survival at 90 days after implantation as opposed to the mice bearing either PANC-1 or vector control tumors that showed 0% survival at the same time. Our results clearly demonstrated a potent antitumor activity of p202 in orthotopic pancreatic cancer xenograft models but to a lesser extent in the ectopic environment.

p202 Expression Suppresses Metastasis in Pancreatic Tumors. Upon examining the orthotopic pancreatic tumor xenograft model, we observed liver metastasis in 40% (two of five) and 20% (one of five) of mice bearing either PANC-1 tumors or vector control tumors, respectively. In contrast, we found no detectable liver metastasis in mice bearing p202 tumors (Table 2). This result suggests a possible antimetastasis function of p202 in pancreatic cancer cells. To test that possibility *in vitro*, we used a double-chamber assay (17) in which the test cells were grown in the top chamber, and the bottom chamber was
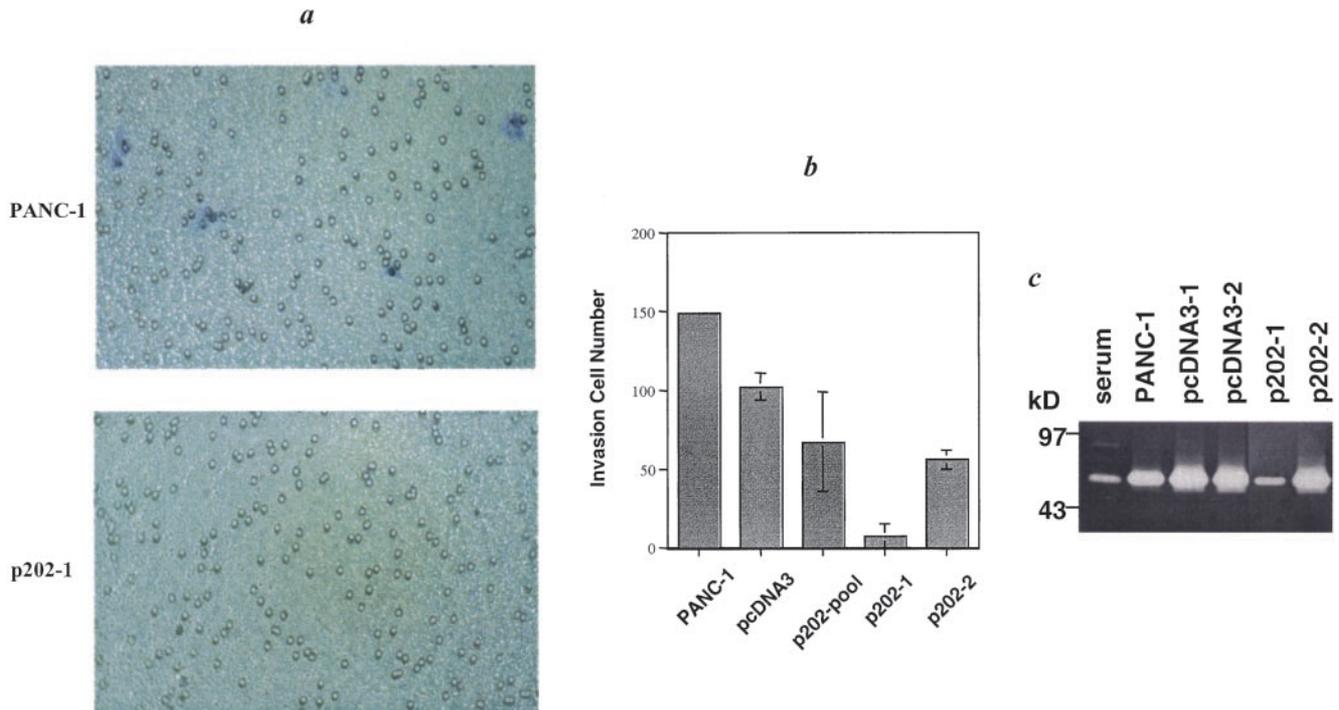

Fig. 4. p202 expression inhibits cell invasion *in vitro*. Cells were tested for their invasiveness by using a double-chamber assay in which a Matrigel-coated membrane was used to separate two chambers. PANC-1, pcDNA3, a pool of p202-transfected clones (p202-pool), p202-1, and p202-2 cells were seeded in the top chamber with lower chamber filled with laminin (30 μg/ml)-containing conditioned medium. Seventy-two h after seeding, the cells that had migrated on the bottom side of the membrane were scored by Giemsa's stain, as indicated by the *blue* cells (*a*); the quantification of the cells that migrated is shown *b*. *Bars,* SD. *c*, p202 expression is associated with a reduced level of secretory MMP-2 in the medium. Cells [PANC-1, two vector-transfected cell lines (pcDNA3-1 and pcDNA3-2), p202-1, and p202-2] were grown in the serum-free medium. The secreted MMP-2 activity was assayed by zymography. After 24 h incubation, the conditioned medium was harvested and concentrated before being subjected to SDS-PAGE, in which the gel was imbedded with 1.5% gelatin. The MMP-2 activity in serum served as a positive control.

7145

filled with conditioned medium containing a chemoattractant, *e.g.,* laminin. A Matrigel-coated membrane was used to separate the two chambers. To migrate from the top chamber to the bottom chamber, cells must digest away the reconstituted basement membrane matrix by producing secretory proteases, such as MMPs, and then penetrate through pores on the membrane. Thus, this assay somewhat mimics a typical metastatic process, and the number of cells found on the bottom side of the membrane [which can be visualized by Giemsa staining, *i.e.,* blue cells (Fig. 4*a*)] is indicative of the metastatic potential of the test cells. On the basis of these criteria, we found that p202-1 possesses the least metastatic potential among the cell lines tested (Fig. 4*b*). p202-2 and p202-pool cells (*i.e.,* the pooled *p202*-transfected clones) have slightly lower metastatic potential than that of PANC-1 or vector control cells (Fig. 4, *a* and *b*). Because MMP-2 (M_r 72,000) is one of the important MMPs secreted by cancer cells during the metastatic process (18), we examined whether MMP-2 expression is altered in p202-1 and p202-2 cells by using a zymography to analyze the MMP-2 activity in each cultured medium. As shown in Fig. 4*c*, the level of MMP-2 secreted by p202-1 cells is greatly reduced, but PANC-1, vector control (pcDNA3-1 and pcDNA3-2), and p202-2 cells maintain a high level of secreted MMP-2. Although the MMP-2 level is not significantly reduced in p202-2 cells, it is likely that the low p202 expression level accounts for the difference in both the MMP-2 activity and the *in vitro* invasiveness between p202-1 and p202-2 cells. The MMP-2 activity in serum serves as a positive control. Together, our *in vivo* and *in vitro* results support the idea that p202 expression suppresses metastatic potential of pancreatic cancer cells.

p202 Expression Suppresses Angiogenesis in Pancreatic Tumors. It has been well documented that tumor growth and metastasis require persistent growth of new blood vessel (neovasculature; Ref. 19). To examine whether the reduced tumorigenicity of p202-expressing pancreatic cancer cells is associated with a reduced angiogenesis, we analyzed the formation of neovasculature in p202-1 tumors and PANC-1 tumors obtained from the orthotopic pancreatic cancer xenografts. Fig. 5 shows that the number of blood vessels [stained by antibody against a blood vessel marker, *i.e.,* CD31 (20)] was significantly reduced in p202-1 tumor as compared with PANC-1 tumor. Because the expression of angiogenic factors such as IL-8 and VEGF are critical for the onset of angiogenesis (19), we examined a possible correlation between the expression of these proteins and the reduced angiogenesis in p202-1 tumors. Using immunohistochemical analysis with antibody specific to IL-8 or VEGF, we showed that p202-1 tumor has much reduced IL-8 and VEGF protein staining (dark gray color) as compared with that of PANC-1 tumor (Fig. 5). These results strongly suggest that p202 expression in pancreatic tumors is associated with suppression of angiogenesis.

***p202*/Liposome Treatment Suppresses Tumor Growth in a Pancreatic Cancer Xenograft Model.** On the basis of the strong antitumor activity of p202 in human pancreatic cancer cells described above (Fig. 2 and Table 2), we tested a potential therapeutic effect of *p202* gene therapy treatment in a s.c. pancreatic cancer model. Briefly, the mice bearing s.c. PANC-1 tumors were treated by intratumor injection of CMV-*p202*/SN2 complex twice a week for 8 weeks. (SN2 is a lipid formula developed in our laboratory, and when complexed with DNA, it enhances *in vivo* and *in vitro* transfection efficiency.[5]) The control groups consisted of tumor-bearing mice treated with SN2 alone (SN2) or CMV-*luc*/SN2 complex. As shown in Fig. 6, although there was no significant difference in tumor growth between SN2 and CMV-*luc*/SN2 complex treatment groups, the CMV-*p202*/SN2-treated tumors exhibited a slower growth rate than that of the control treatments. This proof-of-concept experiment clearly shows a feasi-
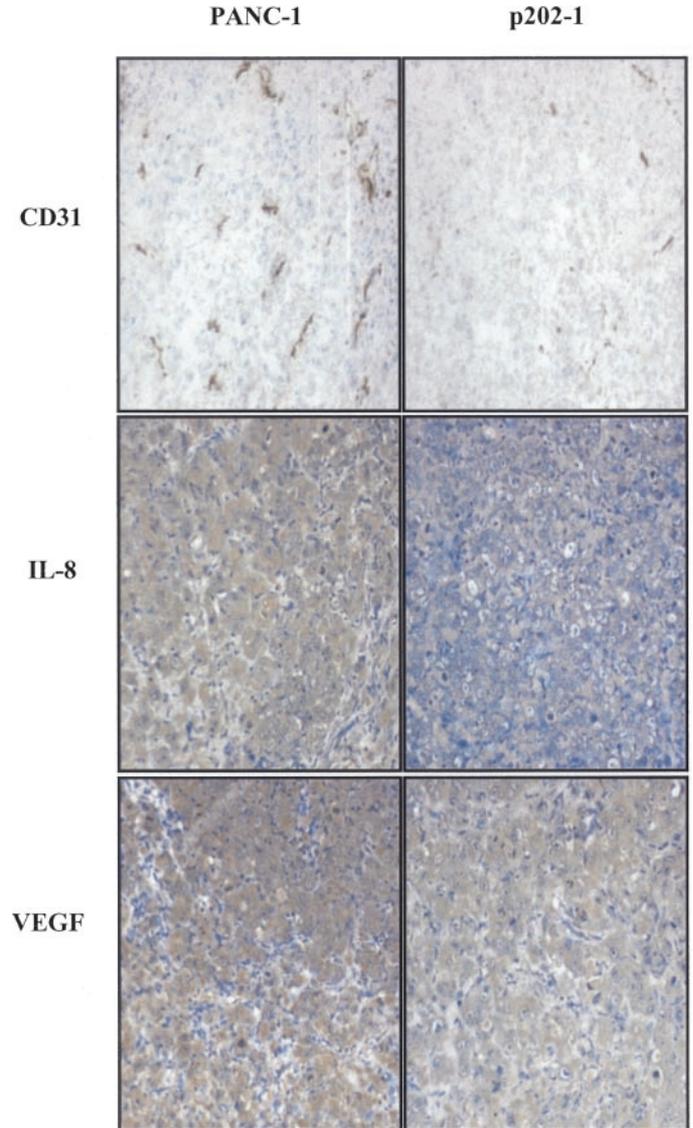

Fig. 5. p202 expression is associated with reduced angiogenesis and down-regulation of IL-8 and VEGF. PANC-1 and p202-1 tumors excised from the pancreas of the orthotopic xenografts were analyzed by immunohistochemical staining for vascularity (CD31 staining) and IL-8 and VEGF protein expression.

bility of using CMV-*p202*/SN2 complex to achieve a therapeutic effect on an ectopic pancreatic cancer xenograft model.

DISCUSSION

In this report, we showed that p202 expression resulted in a growth inhibition of pancreatic cancer cells *in vitro* and *in vivo*. The enforced expression of p202 correlates well with the inactivation of the otherwise constitutively active NF-κB. This observation is significant because persistent NF-κB activity was associated with antiapoptosis and chemoresistance in human cancers (14, 15), and that sets the stage for us to test whether p202 expression can sensitize these pancreatic cancer cells to chemotherapy treatment. To date, very few chemodrugs are found beneficial in treating pancreatic cancer (3), and the constitutive activation of NF-κB is likely to contribute to such chemoresistant phenotype. It is therefore possible that a *p202*-based gene therapy may restore chemosensitivity and lead to a potential *p202*/chemo-drug combined treatment for pancreatic cancer. Experiments are under way to test this hypothesis.
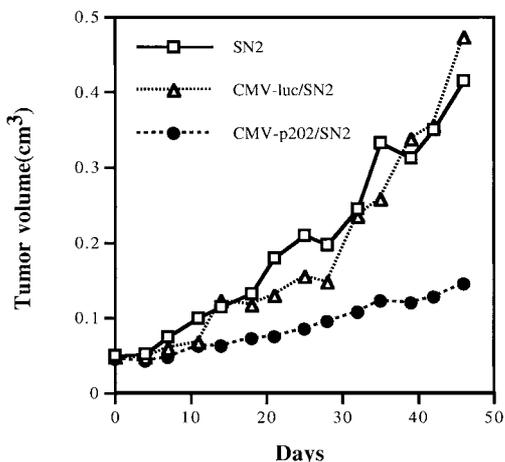
Fig. 6. Antitumor effect of p202/SN2 complex by intratumoral injection in a s.c. xenograft model. Tumors were produced by s.c. implanting PANC-1 cells into both flanks of each nude mouse. Tumor-bearing mice were divided into three treatment groups (five mice/group and two tumors/mouse): SN2 liposome alone (*SN2*), *CMV-luc/SN2*, and *CMV-p202/SN2*. SN2 (30 μg) with or without DNA (15 μg) in 100 μl of PBS was injected twice a week into each tumor. Tumors were measured twice a week after treatment began, and the average tumor volume per treatment group at the indicated time is presented.

We observed a more pronounced p202-mediated antitumor activity (*i.e.,* reduced tumor growth and prolonged survival) in the orthotopic pancreatic cancer xenograft model than that in a s.c. ectopic xenograft model. Although the reason for the differential therapeutic effect remains unknown, it does indicate that the orthotopic xenograft model is not only a more relevant but also a better model to evaluate future treatment efficacy of *p202*-based gene therapy than the s.c. ectopic model. We also observed a decreased vascularity (CD31 staining) and a decreased level of angiogenic factors, *e.g.,* IL-8 and VEGF, in p202-expressing tumors. Thus, our results suggest that p202 inhibits the expression of angiogenic factors and that in turn leads to suppression of angiogenesis. Remarkably, we observed that mice bearing p202-expressing tumors apparently lacked liver metastasis, and this result correlates with a reduced invasiveness and a reduction in MMP-2 expression *in vitro*. Together, our data present the first evidence to suggest that p202 expression is associated with suppression of both angiogenesis and metastasis.

Finally, we showed a significant therapeutic effect using CMV-*p202*/SN2 treatment in an s.c. pancreatic cancer xenograft model. It nevertheless implicates a feasibility of using a *p202*-based gene therapy treatment for pancreatic cancer. Given that the orthotopic model is a better system to study the efficacy of p202 antitumor activity as indicated in this report, it is possible that CMV-*p202*/SN2 treatment

may yield even better antitumor activity in an orthotopic xenograft model. Success of a *p202*-based gene therapy in orthotopic model will pave the way for future experiments to determine the efficacy *p202*/chemo-drug combined treatment in preclinical gene therapy settings.

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