Human α-defensin-1 inhibits growth of human lung adenocarcinoma xenograft in nude mice

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Abstract

Human α-defensin-1 (HNP1), a small antimicrobial peptide, shows cytotoxicity to tumor cells *in vitro* and inhibitory activity for pathologic neovascularization *in vivo*. Here, we did a gene therapy with a plasmid that expresses a secretable form of HNP1 for assaying its antitumor activity. The expression and secretion of HNP1 were determined by reverse transcription-PCR and ELISA *in vitro*. We found that expression of HNP1 in A549 tumor cells caused significant growth inhibition. This effect is most likely cell autonomous, as a significant amount of recombinant HNP1 protein was found to be accumulated in the cytoplasm by immunohistochemical staining using an anti-HNP1 antibody and the supernatant containing secreted HNP1 failed to produce any noticeable antitumor activity. Flow cytometry and Hoechst 33258 staining showed that the number of apoptotic cells among the A549 cells expressing recombinant HNP1 proteins was significantly greater than that of the nontransfected control cultures, suggesting that this growth-inhibitory activity was due to an apoptotic mechanism triggered by the intracellular HNP1. The antitumor activity of intracellularly expressed HNP1 was also shown *in vivo*. Decreased microvessel density and increased lymphocyte infiltration were observed in tumor tissue from HNP1-treated mice through histologic analysis. These results indicate that intracellularly expressed HNP1 induces tumor cell apoptosis, which inhibits tumor growth. The anti-angiogenesis effect of HNP1 may contribute to its inhibitory activity *in vivo*, and HNP1 might involve the host immune response to tumor. These findings provide a rationale for developing HNP1-based gene therapy for cancer. [Mol Cancer Ther 2008;7(6):1588–97]

Introduction

The human α-defensins, also known as the human neutrophil peptides (HNP1-3), are small cationic peptides found in azurophilic granules (1). *In vitro*, HNP show cytotoxicity to various types of eukaryotic cells and tumor cells (2–4). It was proposed that HNP could bind to and damage cell membranes resulting in lethal damage (4, 5). Due to the increased permeability of cell membranes, HNP can also penetrate cells and cause a secondary injury that is likely required for tumor cell lysis (2). The mechanism of HNP-induced apoptosis involves release of cytochrome *c* from mitochondria, which is the key event of mitochondria-mediated apoptosis (6). Moreover, recent evidence shows that HNP are expressed in renal cell carcinomas and influence the proliferation of renal malignant cells and immune recognition (7) and also involved in host immune response to cervical human papillomavirus-associated neoplastic lesions (8). Based on these findings, we hypothesize that HNP may prove useful for cancer gene therapy.

In addition, HNP can regulate angiogenesis by affecting endothelial cell adhesion and migration in a fibronectin-dependent manner as well as endothelial cell proliferation (9, 10). Inhibition of angiogenesis through HNP1 would increase its antitumor effect, because antiangiogenesis has proven an effective strategy for treatment of cancer patients (11).

In this study, we construct a eukaryotic expression plasmid pSec-HNP1 to evaluate its antitumor effect *in vitro* and *in vivo*. Our data indicate that recombinant HNP1, expressed intracellularly by the pSec-HNP1, exhibits its antitumor activities through induction of apoptosis and likely inhibition of angiogenesis.

Materials and Methods

Tumor Cell Lines and Culture

The human lung adenocarcinoma cell line A549 and monkey kidney cell COS-7 (purchased from the American Type Culture Collection) were maintained in DMEM (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies) and antibiotics. The culture was maintained in 95% air-humidified atmosphere containing 5% CO_2 at 37°C.

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Construction of the pSec-HNP1 Vector

The nucleotide sequence encoding the mature region of HNP1 was amplified from total RNA of human peripheral blood lymphocytes by reverse transcription-PCR using the following primers: forward primer 5′-GCGGCCCAGC-CGGCCGCCTGCTATTGCAGAATA-3′ and reverse primer 5′-GAGATATCAGCAGCAGAATGCCCAGAGTC-3′. The amplified mature HNP1 fragment was cloned into expression vector pSecTag2B (Invitrogen), which contains a cytomegalovirus promoter and Igκ-chain leader sequence. The resultant recombinant plasmid was named pSec-HNP1 and verified by DNA sequencing. The empty vector (pSecTag2B) was used as a control and named pSecTag. The pSec-HNP1 and pSecTag were prepared with Endo-Free kits from Qiagen.

Detection of the Expression of HNP1 by Reverse Transcription-PCR and ELISA

To test the expression of HNP1 and the cytotoxicity of the products from transfected cells, COS-7 and A549 cells were transfected with pSec-HNP1 or pSecTag vector. Briefly, cells were plated on a six-well plate ($\sim 2 \times 10^5$ per well). When cultivated to 70% confluence, cells were transfected with 2 μg pSec-HNP1 and pSecTag using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's instruction. Forty-eight hours after transfection, the total RNA was then extracted with Trizol reagent (Invitrogen) and reverse transcription-PCR was done using One-Step Reverse Transcription-PCR Kit (Takara). The level of recombinant HNP1 in supernatant was determined by ELISA assay using HNP1-3 ELISA Test Kit (Hbt, HK317).

Detection of the Intracellular Expression of HNP1 in A549 Cells

After 24 and 48 h of pSec-HNP1 and pSecTag (2 μg) treatment, respectively, cells were fixed with cold acetone and then treated with 0.1% Triton X-100 to increase the membrane permeability. The cells were then incubated with mouse anti-HNP1 monoclonal antibody (1:1,000; Serotec MCA1465) to determine the intracellular expression of HNP1 in A549 tumor cells.

Trypan Blue Staining

As described previously (2), briefly, A549 cells were seeded in a six-well plate ($\sim 2 \times 10^5$ per well). When cultured to 70% confluence, cells were transfected with pSec-HNP1 (2 μg), pSecTag (2 μg), LipofectAMINE 2000, and left untreated, respectively. After 24 h, both attached and floating cells were harvested; 0.4% trypan blue (20 μL) was added to 20 μL cells and incubated for 5 min at room temperature. The stained cells were microscopically counted at five random high-power fields.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

Inhibition of cell growth was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded at a density of 1×10^4 per well in 100 μL culture medium into a 96-well plate. When cultured to 70% confluence, cells were transfected with pSec-HNP1 (0.5 μg), pSecTag (0.5 μg), LipofectAMINE 2000, and left untreated, respectively. After culturing for 48 h, MTT assay was done. Untreated cells served as the indicator of 100% cell viability.

Detection of Tumor Necrosis Factor-α

HNP1 can promote lymphocytes to release tumor necrosis factor-α (TNF-α) that may mediate apoptosis of tumor cells (12, 13). To exclude that HNP1 promote the release of TNF-α from A549, the TNF-α in supernatant was tested with a commercial detection kit.

Hoechst 33258 Staining

A549 cells were transfected with pSec-HNP1, pSecTag, LipofectAMINE 2000, or left untreated. As described previously (14), 48 h after transfection, cells were fixed for 20 min in 4% paraformaldehyde in PBS and then washed in PBS twice. Cells were stained with Hoechst 33258 for 5 min and washed with PBS. Finally, apoptosis was visualized with fluorescence microscope.

Flow Cytometry Assay

A549 cells including both attached and floating cells were harvested 48 h after transfection. Flow cytometric analysis was done to identify sub-G_1 cells/apoptosis cells. Briefly, cells were suspended in 1 mL hypotonic fluorochrome solution containing 50 μg/mL propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100, and cells were analyzed by a flow cytometer. Apoptosis cells appeared in the cell cycle distribution as cells with DNA content less than that of G_1 cells.

Evaluation of Antitumor Effect

Human A549 lung cancer cells (5×10^6) were implanted s.c. into the right flanks of 6- to 8-week-old female nude mice. When tumor diameter reached ~ 5 mm (23 days after inoculation), animals were randomly divided into three groups with five mice per group and were injected intratumorally and around tumor with pSec-HNP1 (100 μg), pSecTag vector (100 μg), or PBS (100 μL). The DNA was encapsulated in cationic liposome with a ratio of 1:3. Because we showed previously that liposome has no effect on tumor growth *in vivo* (15), we did not set the liposome group as a control. The DNA was administered once every 3 days in a volume of 100 μL for a total of five times, and the control injection in a volume of 100 μL PBS solution was also done at the same time point. Tumor volume was observed and tumor size was determined by caliper measurement of the largest and the smallest diameters once every 3 days. Tumor volume (V) was calculated using the formula: $V = 1 / 2 \times A \times B^2$, where A is the largest superficial diameter and B is the smallest superficial diameter. Experiments were terminated when tumors volume reached $\sim 2{,}000$ mm^3 in PBS group (~ 60 days after inoculation).

Histologic Analysis

Tumor tissues were harvested 48 h after the last treatment, and the sections were stained with H&E for histologic analysis. Three tumors per treatment group were analyzed. The expression of HNP1 was then determined by immunohistochemical staining with a mouse anti-HNP1 monoclonal antibody. Finally, the slides were viewed and photographed under the light microscope at ×200 magnification.

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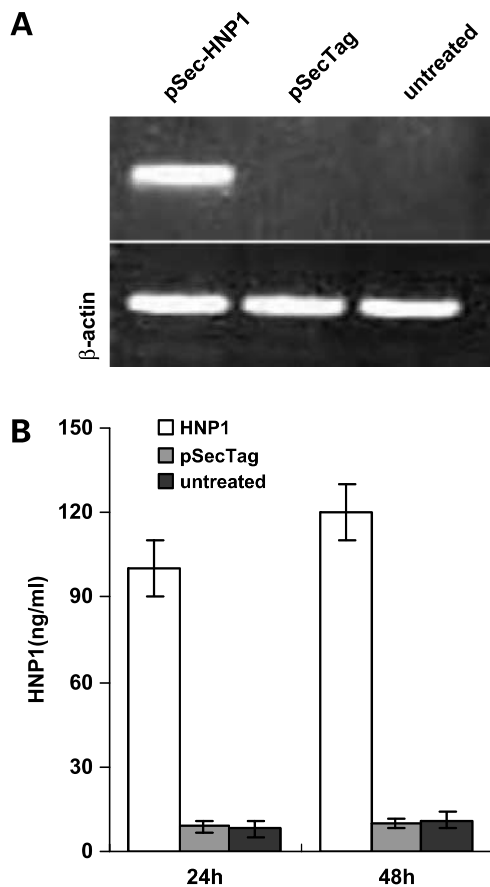

Figure 1. Expression of HNP1 *in vitro*. HNP1 was expressed on RNA level in COS-7 cells (**A**). The expression level of HNP1 in COS-7 cell supernatant was done by ELISA (**B**). The supernatants were collected 24 and 48 h after transfection. The concentration of HNP1 in supernatants 24 and 48 h after transfection were significantly higher than the pSecTag-transfected and untreated cells ($P < 0.01$). Mean ± SD.

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Assay in Tumor *In situ*

Tumor tissues were removed from tumor-bearing nude mice 48 h after the last treatment. Then, cell apoptosis analysis was done using a terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay according to the manufacturer's instructions (Roche Diagnostics). Three tumors per treatment group were analyzed. To determine the relationship between the expression of HNP1 and apoptosis of tumor cells *in vivo*, three tumor-bearing nude mice were administered 100 μg pSec-HNP1 only once. At 24, 48, and 72 h after injection, tumors were harvested. Then, the expression of HNP1 in tumor tissues was tested using immunohistochemical staining and the apoptosis induced by HNP1 was assayed by TUNEL.

Immunohistochemical Analysis with Anti-CD31 Antibody

To explore whether antitumor effect of HNP1 involved the inhibition of angiogenesis, detection of vessel density in tumor tissues *in vivo* was done. An anti-CD31 antibody was used to determine vessel density. Three tumors per treatment group were analyzed 48 h after last treatment. Microvessel density was determined by counting the number of microvessels per high-power field of six random sections in each tumor.

Toxicity Evaluation

To investigate potential side effects or toxicity on mice during the treatment, they were observed continuously for relevant indexes such as weight loss, diarrhea, anorexia, skin ulceration, and toxic deaths. The tissues of heart, liver, spleen, lung, kidney, and brain were stained with H&E.

Statistical Analysis

SPSS 11.5 was used for statistical analysis. The statistical significance of results in all of the experiments was determined by Student's t test and ANOVA. The findings were regarded as significant if $P < 0.05$.

Results

Characterization of HNP1 and Cytotoxicity Test of Secreted HNP1

We transfected COS-7 cells with HNP1-expressing plasmid pSec-HNP1 and its expression was confirmed by reverse transcription-PCR (Fig. 1A). To determine whether HNP1 was secreted into the culture medium, we did ELISA assay. The results showed that in the supernatant from COS-7 cells the expression level of HNP1 was elevated to ~120 ng/mL by transfecting with pSec-HNP1, but transfection with pSecTag in COS-7 did not cause a change in HNP1 production (Fig. 1B). A549 tumor cells were then cultured with supernatants from COS-7 cells transfected with pSecTag vector or pSec-HNP1 or in serum-free DMEM for 48 h to test their effect on tumor cell growth. No significant differences were detected by flow cytometry and MTT (data not shown), which indicates that HNP1 at the tested concentration (120 ng/mL) does not have significant cytotoxic effect on A549 cells.

When A549 cells was transfected with pSec-HNP1, low level (<120 ng/mL) of HNP1 was detected in the supernatant from cultures of A549 cells (data not shown). The low-concentration HNP1 is insufficient to kill A549 cells (2, 6). However, significant amounts of dead cells were observed in the A549 cultures transfected with pSec-HNP1, whereas the cultures had fewer dead cells when transfected with the same vector without HNP1 insert. This observation suggests that the cell death may be associated with the intracellular expression of HNP1.

Apoptosis of A549 Cells Induced by Intracellularly Expressed HNP1

We hypothesize that the cell death of A549 cells might result from the apoptotic effect triggered by the intracellular accumulation of HNP1. To explore the possible relationship between the cell death and the recombinant HNP1, the intracellular expression of HNP1 in A549 cells was further analyzed. Our immunohistochemical study showed that HNP1 expression was almost uniformly distributed in the cytoplasm 24 and 48 h after transfection

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(Fig. 2A and B). Twenty-four hours after treatment, ~40% of the cells were observed to be positive for HNP1 expression and a small portion of cells were found dead (Fig. 2A), whereas the number of HNP1-postive cells decreased 48 h after treatment along with increased number of dead cells (Fig. 2B). In contrast, HNP1 expression and cell death were not observed in cells treated with pSecTag 48 h after the treatment and left untreated (Fig. 2C and D). One possible explanation is that most of HNP1-positive cells died and shed off the glass slide as time progressed. Therefore, compared with HNP1-positive cells observed 24 h after treatment, the number of HNP1-positive cells observed 48 h after treatment was fewer, suggesting that the intracellular HNP1 account for the A549 cell death.

Cell viability was also measured by MTT assay. The MTT assay results showed that the number of viable A549 cells was significantly decreased after the treatment of pSec-HNP1 (Fig. 2E; $P < 0.05$). We reasoned that the decrease in viable cells mainly resulted from the increased cell apoptotic effect caused by intracellularly expressed HNP1. To confirm the effect of HNP1 on cell membrane permeability, trypan blue internalization was used to stain the cells with increased membrane permeability. Twenty-four hours after transfection, attached and floating cells were harvested and mixed with 0.4% trypan blue for 5 min. The trypan blue permeates the cells with impaired membranes and stains the nuclear material blue. The mean percentage of stained cells to the total number of cells (~500 including stained and nonstained) in pSec-HNP1-treated group was higher than that in other groups (Fig. 2F; $P < 0.05$). The increase of cell membrane permeability suggested that intracellular HNP1 likely resulted in damages of membrane.

Immunohistochemical study also revealed that the A549 cell death occurred primarily among these HNP1-positive cells and through inducing apoptosis (Fig. 3A-*a*). Moreover, mitosis interruption was also observed in these cells

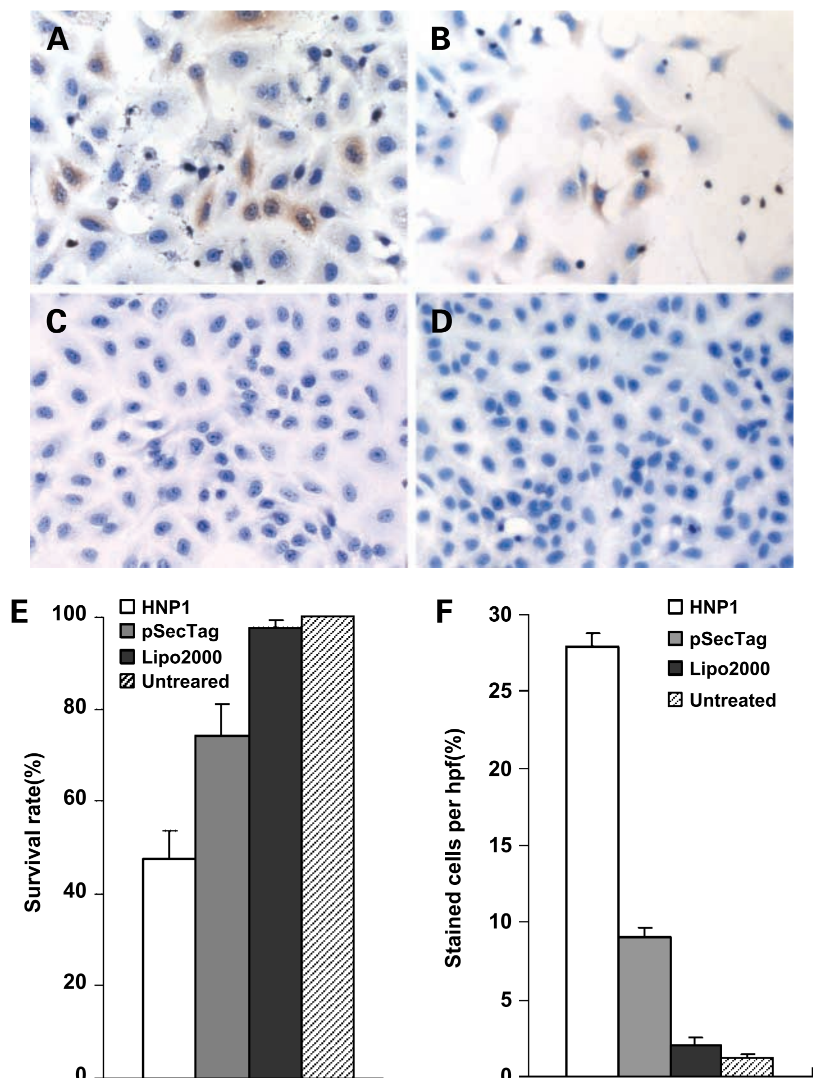

Figure 2. Intracellular expression of HNP1 inhibits the growth of A549 cells. After 24 and 48 h of transfection, immunohistochemical staining was done to determine the expression of HNP1 within cytoplasm. **A** and **B,** 24 and 48 h after pSec-HNP1 transfection. **C** and **D,** pSecTag-treated and untreated cells. **E,** inhibition of A549 cells growth *in vitro* was determined by MTT. The growth of pSec-HNP1-treated cells was significantly inhibited compared with the growth of cells that underwent other treatments. **F,** trypan blue internalization was done to detect the changes of membrane permeability induced by HNP1. Mean ± SD. $P < 0.05$. The experiments repeated at least three times.

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expressing HNP1 (Fig. 3A-*b*). Therefore, we speculate that the intracellularly expressed HNP1 directly causes the apoptosis in the A549 tumor cells. Subsequently, apoptosis was evaluated by Hoechst 33258 staining. A549 cells transfected with pSec-HNP1 were stained with Hoechst 33258 and then microscopically examined for evidence of apoptosis (Fig. 3B). Condensed nuclei and internucleosomal DNA fragmentation, which are characteristic of apoptosis, were observed in a large number of cells treated with pSec-HNP1 (Fig. 3B-*a*), whereas only a few pSecTag-treated cells showed similar signs (Fig. 3B-*b*). There was no significant apoptosis in control groups treated with LipofectAMINE 2000 (Fig. 3B-*c*) and medium only (Fig. 3B-*d*). Quantitative assessment of sub-G_1 cells by flow cytometry was further done to estimate the number of apoptotic cells. As shown in Fig. 3C, there was an apoptotic peak before the normal G_1 peak of cell cycles in pSec-HNP1 group and the percentage of apoptotic cells was significantly higher in the pSec-HNP1-transfected cells than that in control groups. These results suggest that the intracellular HNP1 could directly induce apoptosis in A549 cells *in vitro*. In addition, increased apoptosis was also detected in pSecTag-treated cells reflected the cytotoxic effect from the DNA-liposome complex, which was in accordance with the previous study (16).

Inhibition of Tumor Growth by HNP1 *In vivo*

The A549 s.c. xenograft model was employed to analyze the therapeutic potential of pSec-HNP1. Intratumoral injection of pSec-HNP1 was done because intracellularly expressed HNP1 directly induces tumor cell apoptosis. The result showed that treatment with pSec-HNP1 resulted in a significant regression of established tumors compared with PBS treatment ($P < 0.01$) and pSecTag treatment ($P < 0.05$), respectively, after the fourth treatment (Fig. 4A).

Histologic analysis was subsequently used to explore the role of HNP1 *in vivo*. We did H&E staining on the tumor tissue sections from animals sacrificed at 48 h after the last treatment. Although the tumor size from pSec-HNP1-treated mice was smaller than that from other groups, the local necrosis was still observed in the tumor tissue. Interestingly, lymphocyte infiltration, characterized as cluster-like aggregation, was found within the interspaces of tumor tissue from pSec-HNP1-treated mice, whereas lymphocyte infiltration was rarely observed in pSecTag- and PBS-treated mice (Fig. 5A).

The expression of HNP1 in tumor tissues was confirmed by immunohistochemical staining with mouse anti-HNP1 monoclonal antibody 48 h after the final administration. The expression of HNP1 was found to be positive in the cytoplasm of some tumor cells in pSec-HNP1-treated mice, whereas no sign of expression of HNP1 was identified in

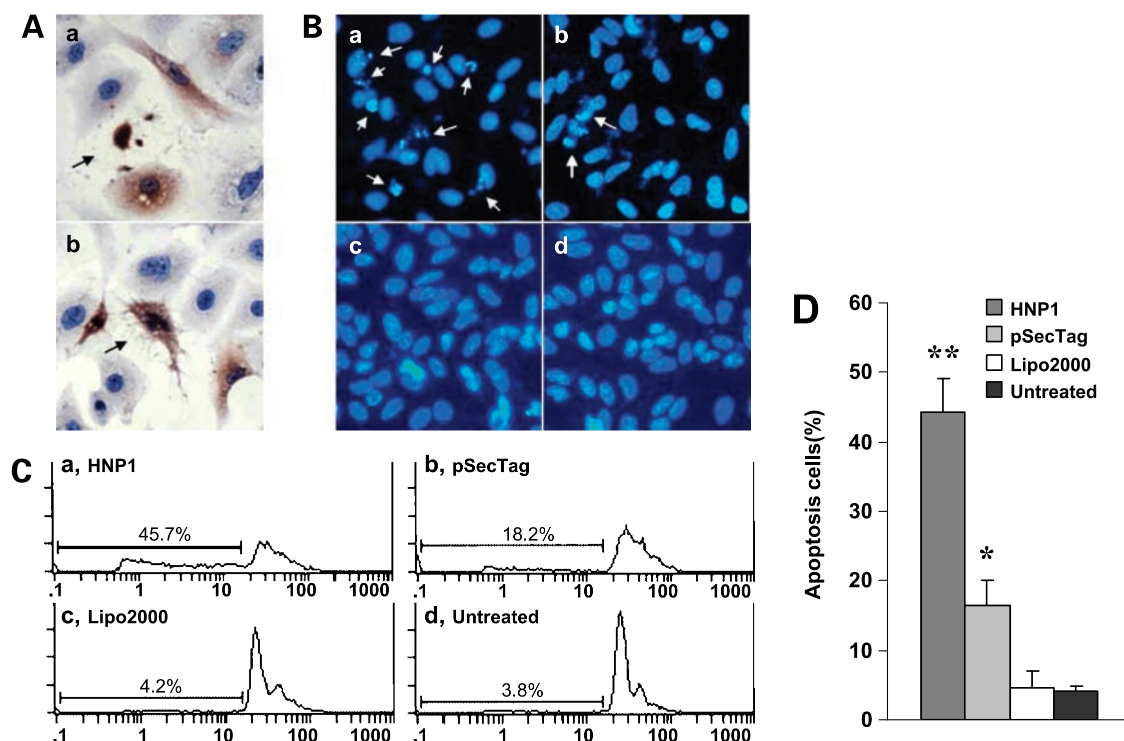

Figure 3. Induction of apoptosis by intracellular HNP1 *in vitro*. The immunohistochemical staining of A549 cells revealed that apoptosis (**A** – **a**) and mitosis disturbance (**A** – **b**) might be induced by pSec-HNP1. Next, Hoechst 33258 staining was done (**B**). **a** and **b**, pSec-HNP1-treated and pSecTag-treated cells (*arrows*; apoptotic cells); **c** and **d**, cells treated by LipofectAMINE 2000 and medium only. Quantitative assessment of apoptotic cells was further done by flow cytometry. **C**, representative flow cytograph from three separate experiments. **D**, mean ± SD apoptosis. The experiments repeated at least three times.

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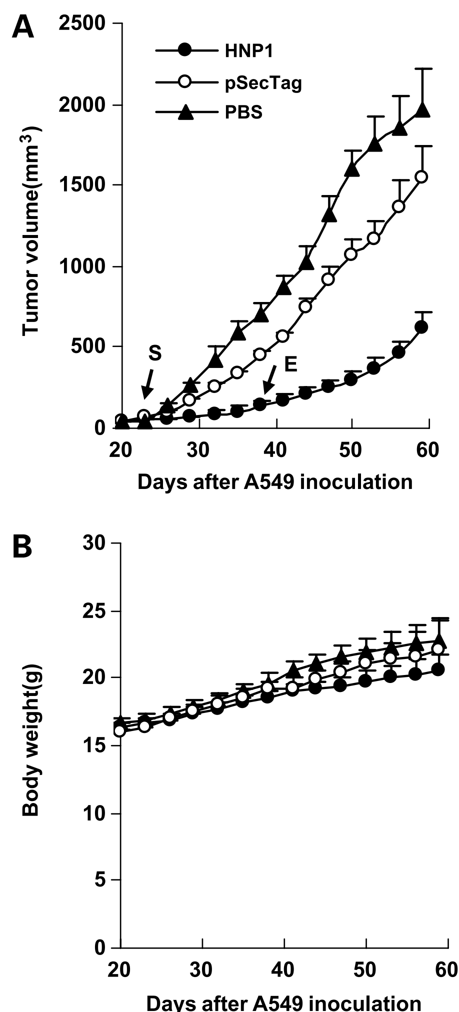

Figure 4. Antitumor effects of HNP1 *in vivo* and toxicity-dependent weight gain retardations. **A,** tumor-bearing nude mice were treated as described with pSec-HNP1, pSecTag, and PBS, respectively (*S,* start of treatment; *E,* end of treatment). The treatment with pSec-HNP1 resulted in significant inhibition of tumor growth versus pSecTag and PBS controls ($P < 0.05$). **B,** body weights of tumor-bearing mice were plotted at 2-d intervals and the curve of the HNP1 group paralleled very closely to that of the control pSecTag and PBS groups with no significant differences among them ($P > 0.05$). Mean ± SD.

the cells from tumor tissues in pSecTag- and PBS-treated mice (Fig. 5A). On the contrary, tumor cells in the HNP1-negative region from the pSec-HNP1-treated mice almost grew normally.

Inducing Apoptosis by HNP1 *In vivo*

To evaluate whether the expression of HNP1 induce apoptosis *in vivo*, immunostaining and TUNEL assay in tumor *in situ* was done with the tumor tissues harvested at 48 h after the last treatment. The results of immunostaining revealed that HNP1 was expressed in tumor cells and secreted into tumor interstitium. Interestingly, we found that some apoptotic tumor cells with internucleosomal DNA fragmentation did not express HNP1 (Fig. 5A). One rational explanation for this is that the secreted HNP1 generates a ''bystander'' effect to adjacent tumor cells resulting in increased apoptosis. The TUNEL assay showed that the number of apoptotic cells increased significantly in tumor tissue from pSec-HNP1-treated mice (Fig. 5A and B) and slightly increased apoptotic cells were observed in tumor tissue from mice treated with pSecTag compared with that from PBS-treated mice; this most likely due to the *in vivo* cytotoxicity of the complex of pSecTag and liposome.

Previous studies have shown that the cytotoxicity of HNP1 is concentration dependent. However, it was difficult to quantitatively determine the relationship between intracellular concentration of HNP1 and apoptosis *in vivo*. Thus, we qualitatively analyzed the correlation between the apoptosis level and the intracellular expression level of HNP1 through TUNEL assay and immunohistochemical staining *in vivo*. We observed a strong correlation between the HNP1 expression and apoptosis in tumor cells at 24 and 48 h after the intratumoral injection of pSec-HNP1. Subsequently, the number of HNP1-positive cells and apoptotic cells was observed to decrease 72 h after injection (Fig. 5C). These findings further support that the intracellular expression of HNP1 induces the apoptosis of tumor cells both *in vitro* and *in vivo* and also suggest that the apoptosis-promoting effect of HNP1 depends on the level of intracellular HNP1.

Inhibition of Tumor Angiogenesis

Previous studies have shown that HNP1 has an effect on angiogenesis via affecting endothelial cell in pathologic retinal neovascularization and inflammation (9, 10). To investigate its role in tumor angiogenesis, we did immunohistochemical staining with anti-CD31. The microvessel density was quantified as a measure of angiogenesis in tissue sections. Lower microvessel density was observed in tumor tissues from mice treated with pSec-HNP1 (Fig. 6A) compared with those from control groups (Fig. 6B and C). This result suggests that inhibition of angiogenesis might also play a role in the induction of antitumor activity.

Toxicity Observation

To evaluate the health status of mice treated with pSec-HNP1 injection, weight of mice was monitored once every 3 days throughout the whole experiment and considered a variable for evaluation of systemic well-being, anorexia, or cachexia. It was plotted at regular intervals and no significant differences in weights were found among the three groups (Fig. 4B). No adverse effects in other gross measures such as skin ulcerations or toxic death were observed in pSec-HNP1 group. Furthermore, toxic pathologic changes in liver, lungs, kidneys, spleen, brain, or heart were not detected by microscopic examination.

Discussion

In this study, we constructed a plasmid encoding the mature HNP1 peptide to explore the *in vitro* and *in vivo* antitumor effect of HNP1. Our study indicate that HNP1 can be effectively expressed in tumor cells and the intracellularly expressed HNP1 directly results in

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apoptosis of tumor cells with significant inhibition of tumor growth *in vivo* by intratumoral administration.

Our initial intention was to treat tumors *in vivo* through intratumoral administration with recombinant pSec-HNP1, with the expectation that we would observe indirect paracrine cytotoxicity to tumor cells resulting from a relatively high concentration of secreted HNP1. This expectation was based on the conclusion of previous studies, which showed that HNP1 is cytotoxic to tumor cells at high concentration (17, 18). However, the *in vitro* experiments in our study indicate that the recombinant failed to generate high concentration of HNP1 in the supernatant and that the supernatant from COS-7 or A549 cells transfected with pSec-HNP1 did not show significant cytotoxicity to tumor cells despite that the growth inhibition and increased apoptosis of tumor cells after transfection with pSec-HNP1 were observed by MTT and flow cytometry. Thus, we speculate that it is intracellular HNP1 rather than the secreted HNP1 that exerts cytotoxicity on tumor cells. We then observed through immunohistochemical staining that HNP1 proteins were almost uniformly distributed in cytoplasm accompanied with apoptosis and morphologic changes in HNP1-positive cells. These findings suggest that intracellularly expressed HNP1 can directly induce apoptosis of tumor cells, which is consistent with previous investigation showing that HNP1 first damages the membrane integrity of the tumor cells that results in increased cell membrane permeability. HNP1 then penetrates the cells and subsequently causes a secondary injury. This second-phase injury is likely required for tumor cell lysis (2).

In fact, the activation of HNP1 peptide needs proteolytic removal of an anionic ''propiece'' of ~40 residues, which protects HNP1-inducing cells (19). In our study, the mature HNP1 gene fragment without an anionic ''propiece'' directly fuses with Igκ-chain leader sequence in pSecTag plasmid to obtain the secretory mature HNP1 with cytotoxic activity. This fusion without ''propiece'' actually makes the fusion peptide keep cytotoxicity, the characteristics of the mature peptide. Although the concentration of HNP1 in supernatant tested by ELISA was not of sufficient concentration to exert cytotoxicity, the result of immunohistochemical staining still suggests that HNP1 peptide can be expressed and shows a cytotoxic effect on tumor cells. Because the cytotoxicity of HNP1 depends on the concentration, one reasonable explanation for these results is that

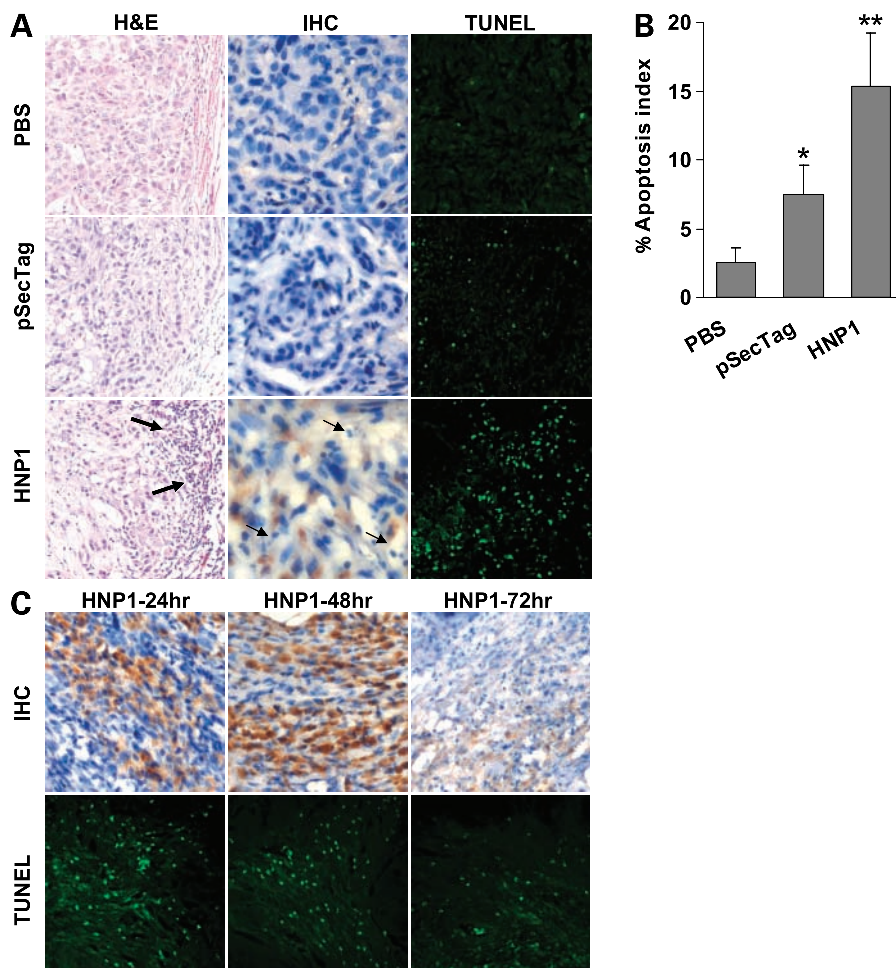

Figure 5. Histologic analysis and detection of apoptosis in tumor tissues. **A,** tumor tissues were analyzed by immunohistochemical analysis and TUNEL assay. Increased lymphocyte infiltration (*wide arrows*) was observed in the margin and interspaces of tumor tissues from pSec-HNP1-treated mice compared with control mice treated with pSecTag and PBS. The expression of HNP1 was only observed in the tumor tissue from mice treated with pSec-HNP1. Apoptosis also happens in tumor cells with low or without expression of HNP1 (*narrow arrows*). Moreover, TUNEL assay showed that intratumoral administration with pSec-HNP1 results in significantly increased apoptosis versus controls ($P < 0.05$). **B,** apoptosis of tumor cells was related with the intracellular expression level and time of HNP1.

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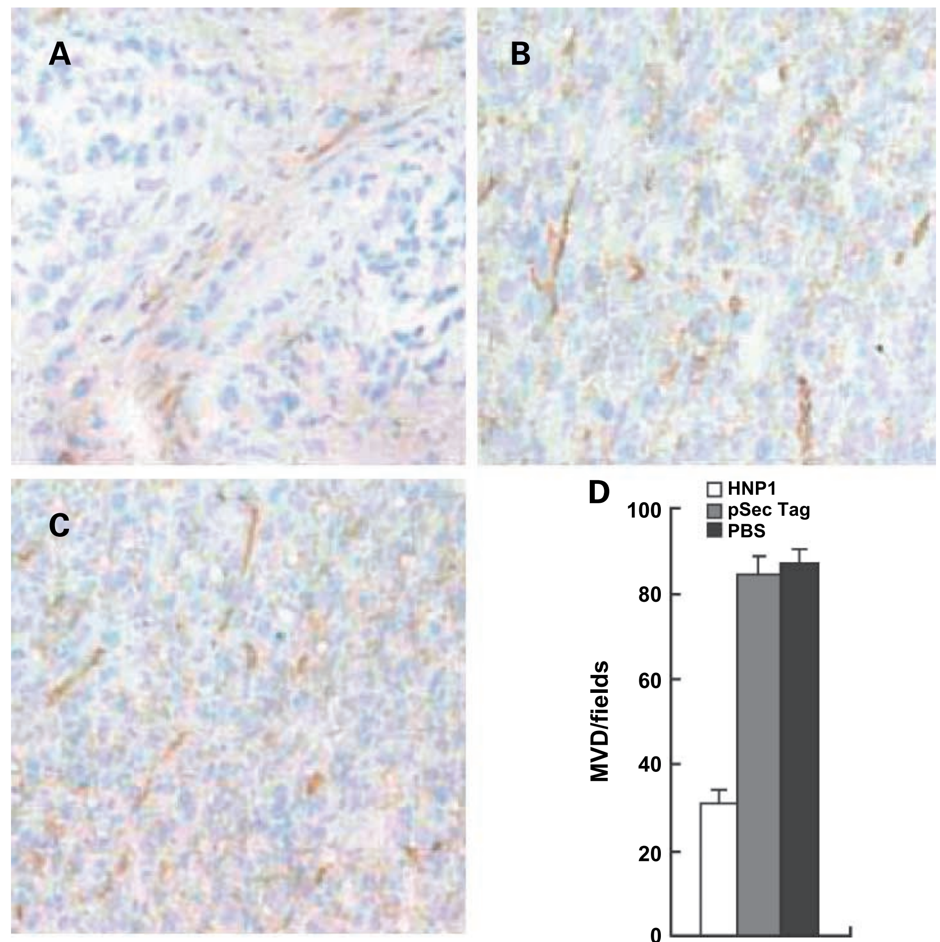

Figure 6. Inhibition of angiogenesis *in vivo*. Nude mice were treated with pSec-HNP1 (**A**), pSecTag (**B**), and PBS (**C**). Frozen sections of tumor tissue were tested by immunohistochemical analysis with anti-CD31 antibody. Vessel density of tumor tissue from pSec-HNP1-treated mice indicated a significant decrease compared with control groups (**D**; $P < 0.05$). Mean ± SD.

HNP1 can be secreted when the concentration of HNP1 fusion peptide is produced at a relatively low level in pSec-HNP1 transfected cells, and the increased intracellular concentration of fusion HNP1 can subsequently induce the HNP-1-producing cells apoptosis.

Apoptosis is an important mechanism by which cells undergo death to control cell proliferation or as a response to cell damage, including cellular membrane, cellular organelle, and DNA damage. The apoptotic pathways include the extrinsic (cytoplasmic) and intrinsic (mitochondrial) pathways. The extrinsic or cytoplasmic pathway is triggered through the Fas death receptor, a member of the TNF receptor superfamily (20). The intrinsic or mitochondrial pathway, when stimulated, leads to the release of cytochrome *c* from the mitochondria and activation of the death signals (21). High concentration of HNP1 has been shown to be cytotoxic to many kinds of tumor cells *in vitro*, which can directly induce apoptosis of tumor cells (7, 17, 22). Previous investigations indicated that the apoptosis induced by HNP involved both pathways. On the one hand, HNP1 can promote lymphocytes to release TNF-α that could result in the death of tumor cells through receptor-mediated apoptosis (12, 13). On the other hand, HNP also mediate apoptosis by the mitochondrial pathway (6). In present study, apoptosis was observed in A549 cells with the expression of HNP1 and increased apoptosis was detected in pSec-HNP1-treated A549 cells by Hoechst 33258 staining and flow cytometry *in vitro*. The mechanism may involve the mitochondrial pathway because HNP1 expressed in tumor cells directly induced apoptosis of HNP1-positive cells and there was no TNF-α in the supernatant (data not shown). In addition, increase in permeability of A549 cells after transfection with pSec-HNP1 was detected by trypan blue staining, although the supernatant, including HNP1, was insufficient to result in injury of the cell membrane. The change of membrane permeability is likely due to the intracellular HNP1, which interferes with cell energy metabolism and possibly also influences the cytoskeletal function (4, 23). Therefore, the change in cell membrane permeability probably plays a partial role in antitumor effect induced by HNP1 *in vitro*. Subsequently, significant inhibition of tumor growth was observed in tumor-bearing nude mice after intratumoral administration of pSec-HNP1 and augmented induction of apoptosis was also detected in tumor tissues from pSec-HNP1-treated mice. The immunohistochemical staining of tumor tissues 24, 48, and 72 h after the final treatment indicates that the expression level of HNP1 correlates with

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apoptosis level *in vivo*. Based on these findings, we infer that the intracellularly expressed HNP1 can induce apoptosis of tumor cells *in vitro* and *in vivo*, and this should be the primary mechanism of antitumor effect of HNP1.

Angiogenesis plays a central role in the growth and metastasis of primary solid tumors and is a complex biological process regulated by several growth factors and components of the extracellular matrix, including fibronectin and its receptor the integrin $\alpha_5\beta_1$ (24), vascular endothelial growth factor, and vascular endothelial growth factor receptors. Inhibition of integrin $\alpha_5\beta_1$ could exhibit distinct antiangiogenic effect against tumor (25). HNP can bind to fibronectin, modify lipoprotein metabolism, and inhibit plasminogen activation (26, 27). Moreover, HNP can form a ternary complex with fibronectin and $\alpha_5\beta_1$ integrin and further affect endothelial cell adhesion, migration, and proliferation (10). HNP have also been shown to attenuate endothelial permeability and proliferation stimulated by the vascular endothelial growth factor and fibroblast growth factor *in vivo* (9). In this study, a lower density of vessels was found in tumor tissues treated with pSec-HNP1 by anti-CD31 staining, which indicates that tumor angiogenesis is effectively inhibited. Thus, inhibition of tumor angiogenesis could be another potential mechanism for antitumor effect by HNP1. We presumed that the inhibitory effect on angiogenesis likely depended on secreted HNP1, because HNP1 can be secreted into tumor interstitium.

HNP1 also shows chemotactic activity to human monocytes (28), T cells (29), and immature dendritic cells and is selectively chemotactic for resting $CD4/CD45RA^+$ and $CD8^+$ T cells (30, 31). HNP1-3 are reported to increase the production of TNF-α and interleukin-1 while decreasing the production of interleukin-10 by monocytes (13). It is known that increased TNF-α can lead to induction of apoptosis in tumor cells (32). In addition, decreased interleukin-10 likely attenuates interleukin-10-mediated immune suppression (33). In this study, a large number of infiltrated lymphocytes were detected in tumor tissues in pSec-HNP1-treated nude mice, implying that the chemotactic activity of HNP1 was remained in nude mice, which was likely related with the high concentration of interstitial HNP1 secreted from HNP1-positive tumor cells. This finding suggests that HNP1 might be involved in immune response of host to tumor. However, the defect of T lymphocytes in nude mice indicates that infiltration of lymphocytes is not associated with T-cell mediating specific antitumor immunity. Therefore, HNP1 could be further explored as a candidate for cancer immunogene therapy.

In previous studies, the antitumor effect of HNP1 was identified primarily with purified HNP1 protein *in vitro* but have not been well explored *in vivo* mainly due to the lack of efficient manufacture of mature HNP1 peptide (34, 35) and, more importantly, due to the inhibition to cytotoxicity of HNP1 through serum proteins. The present studies show that cancer gene therapy by the intratumoral delivery of plasmid DNA encoding HNP1 could effectively inhibit tumor growth in A549 xenograft model. The antitumor effect depends on the intracellular expression of HNP1, which directly induces apoptosis in tumor cells, and might involve antiangiogenesis through locally secreted HNP1. The results suggest that gene therapy with *de novo* expression of HNP1, by introducing mature peptide *in vivo*, could provide an attractive alternative. Moreover, induction of apoptosis has been established as an effective strategy to induce death in cancer cells or to sensitize them to cytotoxic agents and radiation therapy (31). The increase of cell membrane permeability and promotion of tumor cell apoptosis mediated by HNP1 may provide a potential use of HNP1 as a sensitizer for cancer chemotherapy by promoting penetration of chemotherapeutic drugs into the tumor cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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