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Cell Biology International 33 (2009) 276–282

Cell Biology International

www.elsevier.com/locate/cellbi

Genetic transfer of PNAS-4 induces apoptosis and enhances sensitivity to gemcitabine in lung cancer

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Received 4 April 2008; revised 26 May 2008; accepted 29 November 2008

Abstract

PNAS-4 has been demonstrated to induce apoptosis in U2OS cells. To evaluate its feasibility as a new strategy for cancer therapy, we analyzed its anti-tumor effect with or without gemcitabine in A549 lung cancer cells. MTT assay, Hoechst 33258 staining and flow cytometric analysis were used to determine the cytotoxicity of PNAS-4 alone or plus gemcitabine. The anti-tumor efficacy was further investigated in vivo with nude mice. PNAS-4 plasmid/liposome complexes were injected by tail vein every 4 days. Gemcitabine was given ip on a weekly schedule for 4 weeks. PNAS-4 alone and plus gemcitabine induced apoptosis in A549 cells in vitro. The xenograft lung cancer treated with PNAS-4 retarded growth compared with the empty vector. The combination of PNAS-4 with gemcitabine induced anti-tumor activity accompanied by an increase in apoptotic cells compared with PNAS-4 or gemcitabine alone. No other obvious toxicity was found. PNAS-4 therefore suppresses tumor growth in vivo and enhances sensitivity to gemcitabine. This suggests that the PNAS-4 gene could be a potential candidate for lung cancer therapy alone or in combination with gemcitabine.

Keywords: PNAS-4; Gemcitabine; Apoptosis; Lung cancer; Gene therapy

1. Introduction

Lung cancer, one of the leading causes of deaths is treated by standard therapies which include surgical resection, radiation therapy, and chemotherapy alone or in combination. However, the overall 5-year survival rate remains at 15% (Jemal et al., 2006). Developing new therapies is important. Tumor formation and treatment response often involve apoptosis, and most therapeutic strategies tend to induce cell death, probably by apoptosis. Thus, genes related to apoptosis are targets in cancer gene therapy (Favrot et al., 1998).

PNAS-4 is a novel gene identified by large-scale sequencing, for which a function has not yet described. In microarray-based gene expression profile, the gene was upregulated in U2OSE6 cells (p53-independent) compared with U2OS cells (p53-dependent) treated with mitomycin C. Apoptosis significantly increased when U2OS cells overexpressed PNAS-4 (Filippov et al., 2005). Our data indicate that the PNAS-4-transfected A549 cells are susceptible to apoptosis. Therefore, PNAS-4 may be a new pro-apoptotic gene and is a good candidate for tumor therapy (Daniel, 2006; Yan et al., 2007).

Gemcitabine is a pyrimidine nucleoside analogue that inhibits DNA synthesis. It causes tumor regression by inducing apoptosis and is cytotoxic against a wide range of cancer in vitro and in vivo. Gemcitabine produced 20–25% of objective response in patients with advanced NSCLC (Hertel et al., 1990; Braakhuis et al., 1991). When combined with

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doi:10.1016/j.cellbi.2008.11.014

other cytotoxic drugs, the objective response is 28–54% (Crino et al., 1997; Comella et al., 2001; Leon et al., 2003).

We hypothesize that PNAS-4 plus gemcitabine has a profound efficacy in treatment of lung cancer. To test this hypothesis, the effects of PNAS-4 with or without gemcitabine were investigated in vitro and in vivo.

2. Materials and methods

2.1. Construction of recombinant plasmid and liposome preparation

pcDNA3.1 plasmid expressing human PNAS-4 protein was constructed in our laboratory. Briefly, the total RNA was isolated from HEK293 cells using the Trizol reagent (Invitrogen, USA) according to the manufacturer's instruction. RT-PCR was performed with the upstream primer GCG GAT CCA TGG GGG CTA ACC AGT TAG and the downstream primer CGC CTC GAG TTA TAG TTT AGT GTG GCG CCC based on the CDS sequence of the human PNAS-4 gene (GenBank accession No. NM_016076) (TaKaRa One Step RNA PCR kit, Japan). The RT-PCR product was cloned into *BamHI-XhoI* sites of pcDNA3.1 vector. Then, PNAS-4 cDNA derived from the pcDNA3.1–PNAS-4 was subcloned into *BamHI-XhoI* sites of the mammalian expression vector pVAX1. The recombinant pVAX1–PNAS-4 plasmid was confirmed by restriction digestion and DNA sequencing (Invitrogen). To prepare the plasmid, a Qiagen Endofree plasmid giga kit (Qiagen, Chatsworth, CA) was used according to the manufacturer's protocol. The pVAX1 was also purified as a control.

Liposome was made as described previously (Lin et al., 2007). Briefly, liposomes containing lipids DOTAP (dioleyl trimethylammonium propane; Avanti Polar Lipids Inc., AL) and DOPE (dioleyl phosphatidylethanolamine; Avanti Polar Lipids Inc., AL) (1:1 molar ratio) were prepared in chloroform supplemented with methanol (3:1 volume ratio). The mixture was dried in a 100-ml round-bottomed flask and organic solvent was further removed under vacuum for 2 h. The lipid film was hydrated in 5% dextrose to give a final concentration of 5 mg/ml. The resulting liposome solution was vortexed for 1 min, sonicated for 10 min to form small unilamellar vesicles and stored at 4 °C.

In animals, the DNA/liposome complexes were formed by adding liposome solution to DNA solution at a ratio of 3 μg liposome to 1 μg DNA, gently mixing and incubating for 30 min at room temperature.

2.2. Cell culture and materials

Human A549 lung adenocarcinoma cell line was purchased from American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), at 37 °C in a humidified air atmosphere containing 5% CO_2. Gemcitabine obtained from Eli Lilly Company (Indianapolis, IN, USA) was diluted in 0.9% NaCl before use.

2.3. Western blot analysis

Briefly, A549 cells were plated in six-well plates at 2×10^5 cells/well, and transfected with 2 μg of plasmid and 5 μg lipofectamine™ 2000 (Invitrogen, USA) following the manufacturer's instructions. At 48 h, cells were harvested and lysed in ice-cold lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM phenylmethylsulphonyl fluoride (PMSF), 0.5 mM EDTA, 0.6 lM leupeptin, 2 lM pepstatin). Lysates were boiled in SDS sample buffer for 3 min and equal amounts of protein were run on 12% SDS–PAGE. Gels were electroblotted onto a PVDF membrane. The membrane blots were blocked at 4 °C in 5% nonfat dry milk, washed and probed with antihuman PNAS-4 polyclonal antibodies isolated from rabbit serum (unpublished data). Blots were washed and incubated with a HRP conjugated antirabbit IgG (Santa Cruz)., and specific protein bands were detected by ECL system (Pierce).

2.4. Cytotoxicity in vitro

Cell proliferation and viability were evaluated by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. Briefly, A549 cells were seeded into 96-well plates at 1×10^4 cells per well and incubated at 37 °C and 5% CO_2 in air in growth medium (RPMI-1640 containing 10% FBS) for 24 h. The DNA/liposome complexes were prepared in RPMI-1640 medium and left at room temperature for 30 min. The cells were incubated with the above complexes containing 100 ng DNA and 200 ng liposome per well for 24 h, then 100 μl of RPMI-1640 supplemented with FBS with or without gemcitabine in 100 nM was added. After 24 h, cell growth was measured by the MTT procedure. In addition, gemcitabine and medium were used as controls.

2.5. Apoptosis assay in vitro

Cells were plated at 2×10^5 cells per well in 6-well plates and incubated for 24 h before being treated with 1 μg DNA/2 μg liposome complexes with or without gemcitabine in. After 24 h, the cells were collected and analyzed (Wei et al., 1994). Briefly, cells were suspended in 1 ml fluorochrome solution (50 μg/ml propidium iodide, 0.1% sodium citrate plus 0.1% Triton X-100) and the cells were analyzed by a flow cytometer (ESP Elite, Beckman Coulter, Fullerton, CA). The percentage of apoptosis was estimated from the sub-G1 cell fraction.

Apoptosis was also detected by Hoechst 33258 staining (Apoptosis-Hoechst staining kit, Beyotime Biotechnology, Chin). Briefly, after treated in six-plates, cells were fixed in 0.5 ml of methanol for 15 min. After rinsing in PBS twice, cells were stained with 1 μg/ml Hoechst 33258 in a dark chamber at room temperature for 10 min and rinsed again with PBS. Analysis was done by fluorescence microscopy using uv excitation at 348 nm and emission at 480 nm, apoptotic cells being characterized by pyknotic and fragmented nuclei emitting intense fluorescence (Lin et al., 2007).

2.6. Tumor model and treatment

A total of 2×10^6 A549 cells were injected into the right flanks of the BALB/c-nu/nu female mice (6–8 weeks). Mice were treated on day 8 after inoculation. They were randomly assigned to 5 groups (6 mice/group): (a) untreated; (b) LE: treated with 50 μg pVAX1/150 μg liposome complexes, iv by tail vein, 4 days a time for 4 weeks; (c) LG: treated with 50 μg pVAX1–PNAS-4/150 μg liposome complexes, iv by tail vein, 4 days a time for 4 weeks; (d) GEM: treated with 100 mg/kg gemcitabine, on day 10, ip weekly for 4 weeks; (e) LG–GEM: combine c with d. Tumor size was measured 5 days a time with calipers. Tumor volume was calculated according to the following formula: tumor volume $= ab^2/2$, where a is the largest dimension and b is the perpendicular diameter (Noda et al., 1999). Mice were killed in 2 months after inoculation. Blood was collected for evaluation of hepatic and renal functions and tumor samples were fixed in 4% formaldehyde. This work was approved by the institute's Animal Care and Use Committee.

2.7. Histology and apoptosis analysis

Tumors were fixed in 10% neutral buffered formalin solution and embedded in paraffin. Sections 3–5 μm were stained with hematoxylin and eosin. Fluorescent in situ terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay involved the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI, USA), following the manufacturer's protocol. The apoptotic index was calculated as the ratio of the apoptotic cell number to the total cell number in 5 different fields.

2.8. Statistical analyses

ANOVA and an unpaired Student's t-test were used in comparisons. Values of $P < 0.05$ were taken as significant.

3. Results

3.1. Recombinant pVAX1–PNAS-4 plasmid expression in vitro

Expression of recombinant plasmid was confirmed by RT-PCR (data not shown) and western blot assay in A549 transfected cells. A distinct band of ~21 kDa, corresponding to the size of PNAS-4, was visualized in the pVAX1–PNAS-4-transfected cells, but not in pVAX1-transfected and non-transfected cells (Fig. 1).

3.2. Reintroduction of PNAS-4 induction of apoptosis in A549 cells

PNAS-4 overexpression was followed by MTT assay. In Fig. 2 (top panel), reintroduction of PNAS-4 was cytopathic to A549 cell line compared to control groups, which featured as more floating, rounded cells. More cell debris was found in the group treated with

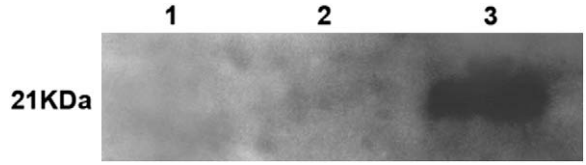

Fig. 1. Expression of PNAS-4 protein determined by Western blot. Recombinant human PNAS-4 protein was expressed as a single band of approximately 21 kDa in pVAX1–PNAS-4-transfected A549 cells (lane3), while no band was detected in empty plasmid (pVAX1) (lane2) transfected or untreated (lane1) tumor cells.

PNAS-4 plus gemcitabine. In MTT assay (bottom panel), cell viability was calculated as the ratio of absorbance value of treated groups to that of medium-treated group. Cell viability in the PNAS-4-transfected group was ~18% lower than in the empty vector control ($P < 0.05$; Fig. 2). This effect was enhanced by the addition of gemcitabine after treatment of PNAS-4 (gemcitabine 30%, PNAS-4 plus gemcitabine 64%).

The type of cell death shown by Hoechst 33258 staining, as shown in Fig. 3, were morphological changes of characteristic of apoptosis, including pyknotic and fragmented nuclei. In addition, the sub-G1 population estimated from the number of apoptotic cells by flow cytometry showed that 26.8% apoptotic cells were caused by PNAS-4 treatment in contrast to 15.2% with the empty vector. Treatment with PNAS-4 at the presence of gemcitabine dramatically increased the percentage of apoptotic cells from 28.6% (treatment with gemcitabine only) to 61.4%.

3.3. Inhibition of tumor growth of A549 in nude mice

For PNAS-4 and combination treatment in vivo, we used subcutaneous A549 tumors in nude mice. On day 60 after tumor cells inoculation, the inhibition of tumor growth increased mostly in the group treated with PNAS-4 compared with the untreated group ($p = 0.023$), pVAX1 alone ($p = 0.038$; Fig. 4,). More importantly, most tumor inhibition occurred in the group treated with PNAS-4 plus gemcitabine ($p < 0.05$), compared with the group treated with PNAS-4 or gemcitabine alone.

3.4. Increased tumor apoptosis in mice with PNAS-4 treatment, especially combined treatment

To investigate the mechanism of tumor growth inhibition, we examined the microscopic morphology of tumors. Many apoptotic cells could be found in the PNAS-4-treated group and combination-treated group, while other groups showed more tumor cells with apparently enlarged nuclei (Fig. 5). The phenomenon was further verified by TUNEL assay. There were more apoptotic cells (in green) within residual tumors treated with PNAS-4 and the gene plus gemcitabine (Fig. 6A). Apoptotic index was highest in the combined group (Fig. 6B).

3.5. Side effects analyses

We investigated the toxicity of the treatment by measuring the body weights of mice every 5 days. No obvious signs of

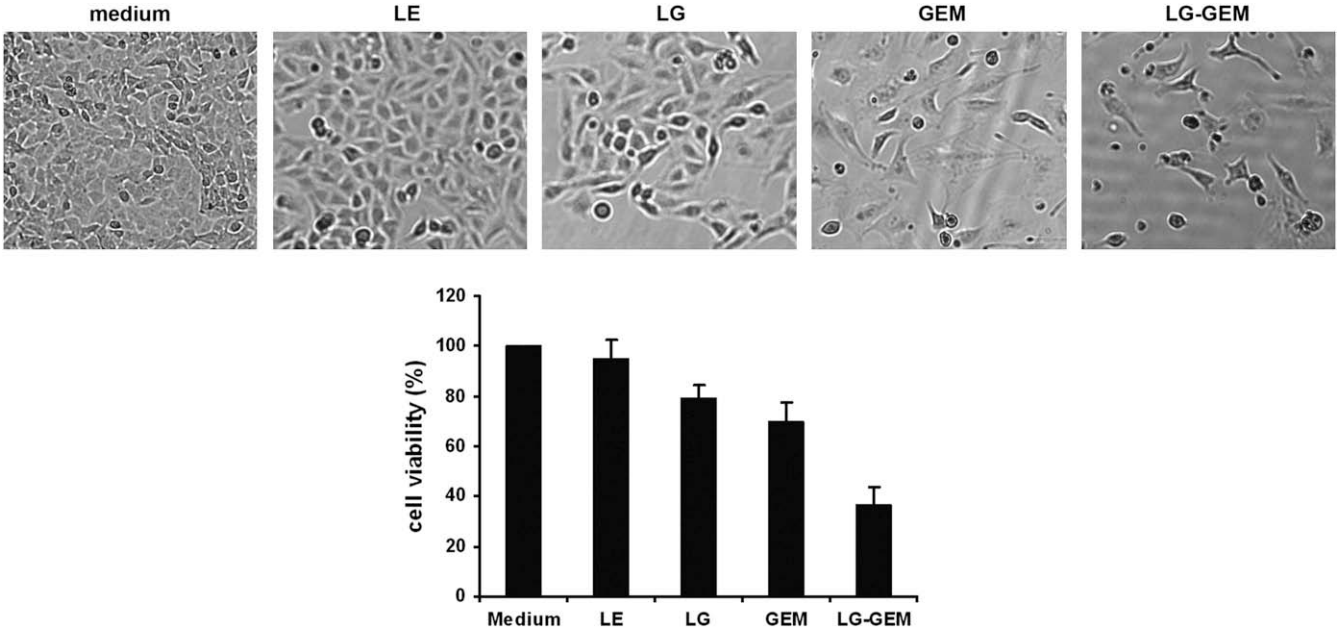

Fig. 2. Cytotoxic effect of PNAS-4 with or without gemcitabine on A549 cells. Mophological changes of A549 cells were recorded under phase-contrast microscope (×100). Top panel: Medium, medium-treated only; LE, treated with pVAX1 plasmid; LG, treated with PNAS-4 plasmid; GEM, treated with gemcitabine in 100 nM; LG−GEM, after 24 h incubation with PNAS-4, treated with gemcitabine in 100 nM. In MTT assay (Bottom panel), data represent means from 5 samples of viable cells in three independent experiments; bars, SD.

adverse results were observed in body weight, behavior or feeding. The serum ALT (alanine aminotransferase) and AST (aspartate aminotransferase) levels were among normal range, as also creatine levels. In addition, hematoxylin and eosin staining sections of liver, kidney, and lung were observed under microscope. No organ hemorrhage was found (data not shown).

4. Discussion

Cancer gene therapy has two broad goals (Favrot et al., 1998); one is to revert the genetic profile of malignancy within tumor cells by apoptosis or differentiation. The other is to increase sensitivity to current therapies. This study suggests

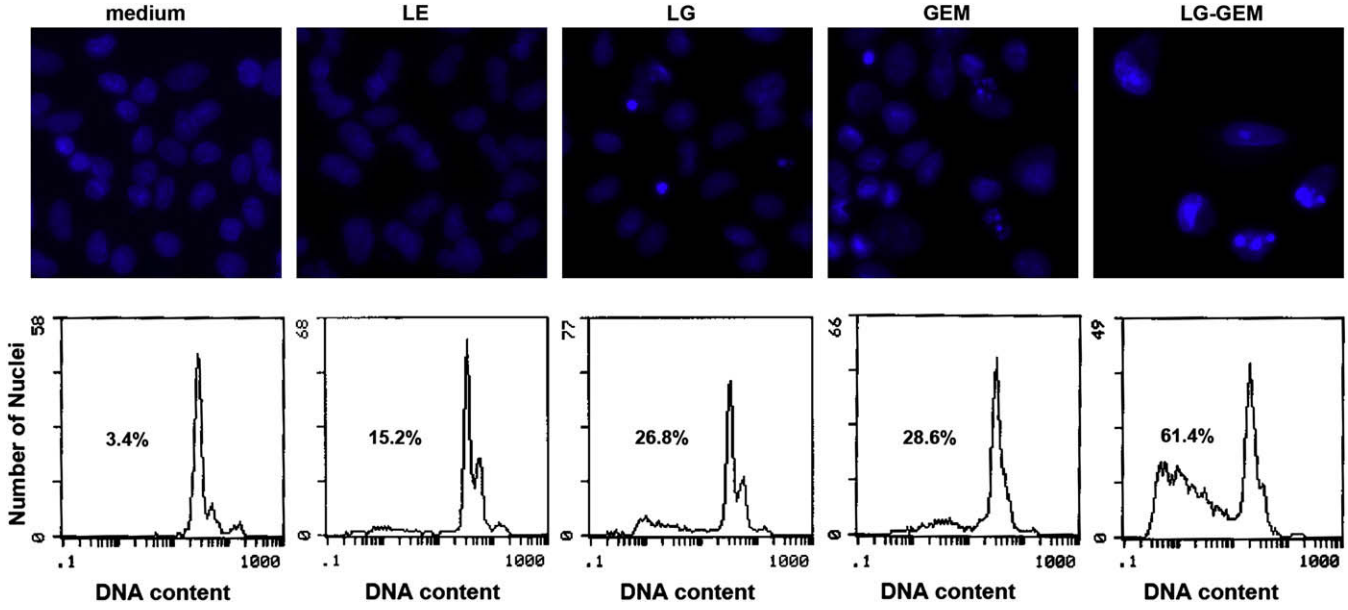

Fig. 3. Induction of apoptosis by re-introduce of PNAS-4 in vitro. A549 cells treated with the indicated agents in 6-wells were examined by fluorescent microscopy after Hoechst staining (×100). Condensed and fragmented nuclei were present in larger numbers in the PNAS-4-transfected cells (LG), gemcibabine-treated cells (GEM) and the cells treated by both (LG−GEM), but not the empty-vector-transfected cells (LE) and medium-treated cells. The percentage of apoptosis estimated from the sub-G1 cell fraction was 61.4% (LG−GEM), 26.8% (LG), 28.6% (GEM), 15.2% (LE) and 3.4% (medium) respectively. DNA fluorescence histograms of PI-stained cells were representative of three independent experiments.

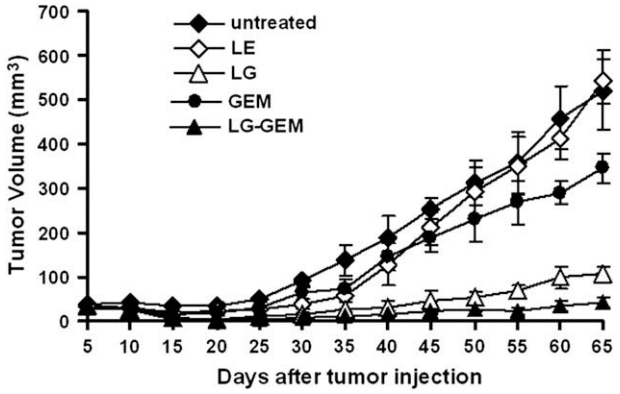

Fig. 4. Suppression of tumor growth in A549 xenograft by PNAS-4 alone or in combination with gemcitabine. Tumor volume was measured every 5 days. untreated, untreated mice bearing tumors; LE, liposome-pVAX1-treated mice bearing tumors; LG, liposome-PNAS-4-treated mice bearing tumors; GEM, gemcitabine-treated mice bearing tumors; and LG–GEM, liposome-PNAS-4 plus gemcitabine-treated mice bearing tumors.

that PNAS-4 could inhibit the growth of A549 lung cancer and enhance the effect of gemcitabine by inducing apoptosis. These were confirmed by reduced tumor gowth, lots of apoptotic bodies in morphology, characteristically condensed nuclei in Hoechst staining, sub-G1 cells in flow cytometric assay as also a lot of green fluorescence in the TUNEL assay. In contrast to gemcitabine, PNAS-4 was more potent in vivo than in vitro. Other processes, such as cell cycle arrest and angiogenesis, may be involved in tumor inhibition in vivo. Dubrez et al. (2001) demonstrated that cell cycle arrest was sufficient for p53-mediated tumor regression. Angiogenesis plays an important role in both tumor growth and metastasis (Folkman, 1990). The most frequently used gene in therapeutic approaches is p53, which plays a central role in the regulation of apoptosis. In clinical trials, it has shown promise either by itself or in combination with cisplatin (Opalka et al., 2002). In animal models, transduced p53 generally induced tumor growth retardation in nude mice bearing tumors of breast, colon, head and neck, and human lung malignant cells (Favrot et al., 1998). Other recent inducers of apoptosis, such as Bax and caspases, are also hopeful (Hollstein et al., 1991). The above apoptosis-inducing regulatory genes in the treatment of malignancies yield promising anti-tumor activity in vitro and in vivo, accompanied by low general toxicity. In accordance with the tolerant side effects, our results demonstrated no significant organ-related toxicity after gene transfer.

We have no idea of the exact molecular mechanism underlying the suppression of A549 lung cancer implanted in nude mice by PNAS-4, but the p53-independent pathway may be involved. In U2OSE6 cells (lacking p53) treated with mitomycin C, PNAS-4 was upregulated compared with U2OS cells (expressing p53), demonstrating the gene could be involved in the p53-independent pathways by genotoxic damage (Filippov et al., 2005). It is important to identify novel pro-apoptotic gene independent of p53 as the latter loses its normal function in ~50% of malignancies (Fulda and Debatin, 2004). PNAS-4 may have a role in apoptotic induction in tumors with mutated p53.

In combination, both the p53-dependent and -independent pathways may be involved. Gemcitabine has an anti-tumor effect on A549 cells that have a functional p53 (Zhao et al., 2006). A role of p53 function in gemcitabine-mediated cytotoxicity has been established in vitro (Chen et al., 2000). Reintroduction of p53 restores pathways that are necessary for an improved response to gemcitabine (Cascalló et al., 2005). However, the anti-tumor effect resulting from gemcitabine was about 30% (compared with untreated group) on the current schedule. By introduction of gene PNAS-4 this effect was greatly potentiated. P21, known to be a downstream effector of p53, is responsible for p53-mediated growth-suppressing activity (Eastham et al., 1995). The p53-independent induction of p21 expression is associated with the induction of terminal growth arrest and differentiation (Dolnikov et al., 2000). E2F-1, another target of p53, has an anti-tumor efficacy in a variety of tumors by apoptosis through p53-dependent and -independent mechanisms (Elliott et al., 2002). Therefore, the above two should be investigated further to elucidate the underlying mechanisms for combination therapy. In addition, the enhanced anti-tumor effects of gemcitabine by gene transfer of PNAS-4 may correlate with the Bcl-2 family. In apoptosis signaling pathways, Bcl-2 protein family members are the key regulators. Bcl-2 and Bcl-xL overexpression delay the onset of apoptosis induced by several cytotoxic drugs including gemcitabine (Kurdow et al., 2005). Inhibitors of Bcl-2 and Bcl-xL could efficiently knockdown Bcl-2 protein levels, resulting in direct inhibition of growth (Oltersdorf et al., 2005).

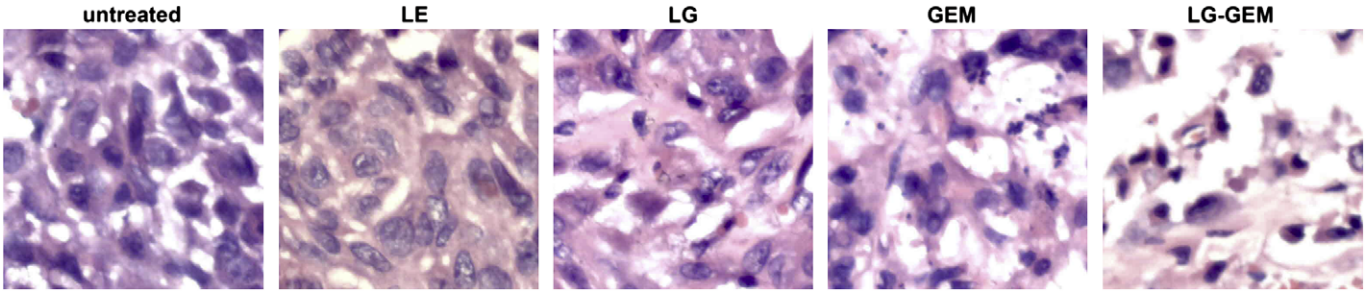

Fig. 5. Morphological changes in H.E. sections under light microscope (×400). Apoptotic cells were rare in paraffin sections of the control groups (untreated group, LE, liposome-pVAX1-treated group and GEM, gemcitabine-treated group), whereas apoptotic bodies were scattered but obvious throughout the sections of the PNAS-4-treated group (LG) and the combination-treated group (LG–GEM).

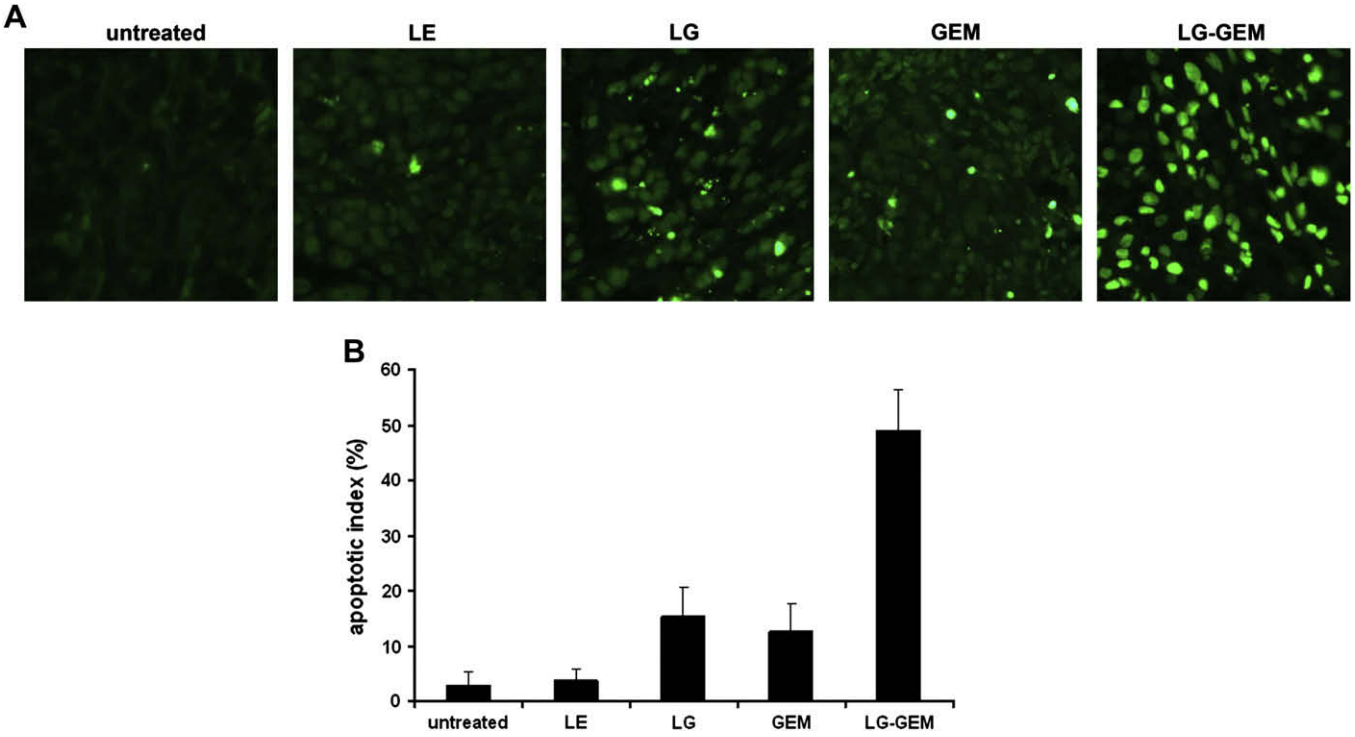

Fig. 6. Induction of apoptosis in A549 lung cancer xenograft. (A) Histological sections of tumors were stained using the TUNEL kit and detected under immunofluorescence microscope (×200). untreated, untreated group; LE, liposome-pVAX1-treated group; LG, liposome-PNAS-4-treated group; GEM, gemcitabine-treated group and LG–GEM, the combination-treated group. (B) Apoptotic index showed an increase in the PNAS-4-treated group, especially in the combination-treated group compared with the control groups; bars, SD.

In addition, non-viral gene transfection as a delivery system provides a safer alternative to viral systems in gene therapy. In this context, cationic liposomes represent the most common tools used in vitro and vivo gene therapy experiments (Hirko et al., 2003). DOTAP/DOPE liposome, one of the most widely used type of cationic liposomes, was used in this study. The non-specific anti-tumor activity observed in the pVAX1-treated group results from an inflammatory response following injection of liposome-DNA complex (Dubrez et al., 2001). The cationic liposome delivery system was safe, and side effects such as fever, nausea, vomiting and discomfort, were tolerable. Compared to viral vector in gene therapy, its low immunogenicity allows repeated administration, increasing overall efficiency of gene delivery and therapeutic efficacy. Multiple preclinical studies and clinical trials have demonstrated the therapeutic efficacy and minimal side effects of liposomal gene therapy for cancers (Ueno et al., 2002; Day et al., 2006).

In conclusion, the gene PNAS-4 may be useful for clinical tumor treatment, either alone or in combination with cytotoxic drugs. The current findings provide a potentially promising approach for the treatment of lung cancer.

Acknowledgements

The authors thank Yang Hanshuo, Xie Gang for technical help, Dr. Gou Lantu, Yuan Zhu for helpful discussions. This work was supported by the National Basic Research Program of China grants (No. 2004CB518800).

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