Bioconjugate Chemistry

Article

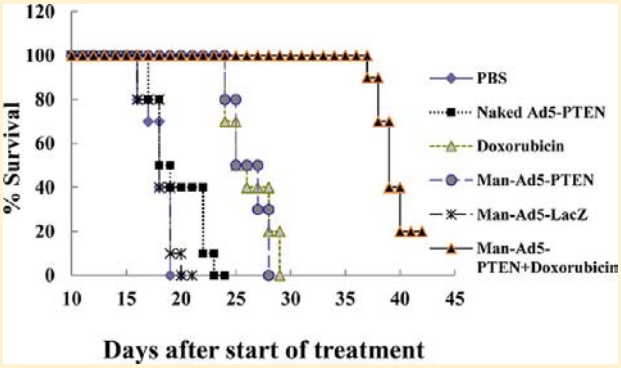
pubs.acs.org/bc

Mannan-Conjugated Adenovirus Enhanced Gene Therapy Effects on Murine Hepatocellular Carcinoma Cells *in Vitro* and *in Vivo*

Zhongbing Liu, Famin Ke, Chenggang Duan, Huan Lan, Juan Li, Cen Gao, Jinwei Li, and Zhirong Zhong*

Luzhou Medical College, Luzhou, Sichuan, P.R. China, 646000

ABSTRACT: The incidence of advanced hepatocellular carcinoma (HCC) is increasing worldwide, and its prognosis is extremely poor. For some patients for whom surgical treatments are not appropriate, one can only rely on chemotherapy. In the conventional chemotherapy, side effects usually occurred in most cases due to high toxicity levels. Moreover, the development of drug resistance toward chemotherapeutic agents often prevents the successful long-term use of chemotherapy for HCC. Gene therapy represents the exciting biotechnological advance that may revolutionize the conventional fashion of cancer treatment. Overexpression of phosphatase and tensin homologue (PTEN) in cancer cells carrying deletion/mutant type of it can induce the apoptosis of cancer cells and inhibit cell proliferation. In this work, in order to make full use of the high transfectivity of adenovirus, we managed to conjugate the polysaccharide mannan (polymannose) to the surface of the adenovirus chemically under appropriate oxidizing conditions to prepare the mannan-modified adenovirus (Man-Ad5-PTEN). The cytotoxicity and anticancer activity of Man-Ad5-PTEN were assessed *in vitro*. Reporter gene expression of LacZ transferred by Man-Ad5-LacZ was verified on mannose receptor-deficient NIH/3T3 cells versus mannose receptor-efficient macrophages. Hepatocellular carcinoma cell lines transduced by mannan-modified adenovirus were assayed for cell cycle, apoptosis, invasion, and migration. Further, we detected the antitumor effect on intraperitoneal H22 tumor-bearing mice treated by Man-Ad5-PTEN alone or combined with chemotherapeutic agent of doxorubicin. The results demonstrated that cell growth suppression was not observed in Chang normal hepatocyte cells and the cell killing by Man-Ad5-PTEN is tumor selective. Further, the results showed that the strategy of mannan conjugation could enhance adenovirus-mediated PTEN gene therapy effects on murine hepatocellular carcinoma cells *in vitro* and *in vivo*.



INTRODUCTION

Hepatocellular carcinoma (HCC) is a common primary malignant tumor of the liver.[1] The incidence of HCC is reportedly increasing worldwide[2] and the cases of advanced HCC are increasing in number.[3] The conventional therapy for HCC includes surgery, radiotherapy, and chemotherapy. Among those means, chemotherapy with cytotoxic agents is a common treatment for HCC.[4] Doxorubicin, a member of the anthracycline ring antibiotics, is arguably the most commonly used traditional chemotherapeutic drug for treating HCC, especially by means of transcatheter arterial chemoembolization.[5] Unfortunately, to achieve better chemotherapeutic outcomes, the high drug concentration and long-acting time within tumor tissues are highly required, which resulted in the high cytotoxicity to normal cells.[4] Therefore, clinical application of doxorubicin is restricted by its high toxicity, and also the resistance to doxorubicin is a common and representative obstacle to treat cancer effectively.[6] Meanwhile, it was reported that doxorubicin-based chemotherapy exhibited only modest antitumor activity with tolerable adverse effects in patients with advanced HCC[7] and systemic administration of doxorubicin provided a response rate only of 4% in a recent clinical trial involving a large number of HCC patients.[8] Therefore, new strategies to enhance the efficacy of doxorubicin to treat HCC are needed.

Gene therapy represents the exciting biotechnological advance that may revolutionize the conventional fashion of cancer treatment. Phosphatase and tensin homologue deleted from chromosome ten (PTEN), a kind of tumor suppressor gene the same as P53 gene, is located on human chromosome 10q23.3.[9,10] PTEN was found to be inactivated in many malignant tumors. Moreover, frequent deletions and somatic mutations of PTEN have been reported in glioblastoma, endometrial cancer, prostate cancer, and small cell lung cancer.[11−16] The induction of PTEN was reported to arrest cell cycle at the G1/G0 or G2/M phase through both Akt-dependent and -independent mechanisms.[17−20] Overexpression of PTEN in cancer cells carrying deletion/mutant type of PTEN can induce the apoptosis of cancer cells and inhibit cell proliferation. Based on these reports, we try to investigate the tumor-suppressive effect of PTEN on murine hepatocellular carcinoma cells *in vitro* and *in vivo*.

Received: May 1, 2013
Revised: July 23, 2013
Published: July 26, 2013

   dx.doi.org/10.1021/bc400215a | *Bioconjugate Chem.* 2013, 24, 1387−1397

However, an efficient gene transfer system is the key problem for carrying the PTEN gene. Up to now, a wide variety of vectors including viral and nonviral gene delivery systems have been used for experimental purposes. Among those viral vectors, adenoviruses can infect virtually many cell types either in dividing and nondividing periods[21−23] with no integration into the host genome and broad tissue or cell tropisms. So, adenovirus has been widely used as vectors for gene therapy and genetic vaccine. In our previous study, we also demonstrated that adenovirus could deliver genes to cancer cells or airway epithelia with high efficiency.[24−26] Additionally, an ideal gene carrier system should transfer genes efficiently to specific cell types with minimal toxicity to nontarget cells.[27] Because of the broad host range of adenovirus, they usually lack tissue specificity and have low safety. Therefore, it is necessary to ameliorate the therapy strategy through improving the cancer-targeting ability of adenovirus vector. According to the published studies, in order to specifically increase transfection efficiency of the delivery system to targeted cell populations, various targeting ligands such as antibodies, growth factors, and so on have been conjugated to polymers or lipids.[28,29] It was reported that mannose receptors (MR) are extensively expressed in macrophages and in liver nonparenchymal tissues; the introduction of mannose residues may enhance the endocytosis mediated by these receptors.[30−32] In this work, in order to enhance the gene therapeutic effect, we managed to couple the polysaccharide mannan (polymannose) to the surface of the adenovirus chemically under appropriate oxidizing conditions to prepare the mannan-modified adenovirus (Man-Ad5-PTEN). Reporter gene expression of LacZ transferred by Man-Ad5-LacZ was verified on MR-deficient NIH/3T3 cells[33] versus MR-efficient macrophages.[34] The cytotoxicities and anticancer activity of Man-Ad5-PTEN were also assessed *in vitro*. Further, we also detected the antitumor effect on intraperitoneal H22 tumor-bearing mice treated by Man-Ad5-PTEN alone or combined with the chemotherapeutic agent of doxorubicin.

MATERIALS AND METHODS

Mice and Cell Culture. Male SD rats weighing 200 ± 20 g and Kunming male mice, six- to seven-week-old, were obtained from the Laboratory Animal Center of Luzhou Medical College, Luzhou, China. All animal experiments were approved by the Animal Ethics Committee of Luzhou Medical College, and all procedures with animals were conducted according to the guidelines of the Local Animal Use and Care Committees of Luzhou and executed according to the National Animal Welfare Law of China.

The mouse hepatoma H22 cell line (syngenic to the Kunming strain of mice), Chang normal hepatocyte, NIH/3T3 cells, and human embryonic kidney 293 (HEK 293) cells were from Chinese Academy of Sciences (Shanghai, China) and maintained in our laboratory. H22 cells were cultured in RPMI 1640 medium (Hyclone Corporation, Utah, USA) and the other cell line including Chang normal hepatocyte, NIH/3T3 cells, and HEK 293 were cultured in DMEM medium (Hyclone Corporation, Utah, USA) supplemented with 10% fetal bovine serum (Hyclone Corporation, Utah, USA), 100 units/mL penicillin, and 100 μg/mL streptomycin and cultured at 37 °C in 5% CO_2/95% air.

Preparation of Mannan-Modified Adenovirus. The origin or construction of Ad5-LacZ and Ad5-PTEN were as same as the previous report.[35] Both Ad5-LacZ and Ad5-PTEN were amplified in HEK 293 cells and purified by two rounds of CsCl (Amersco, American) density gradient centrifugation. The genome copy number of adenovirus stock was determined by Taqman real-time PCR and the adenovirus titer was detected using plaque-forming assay on HEK 293 cells (PFU/mL).

To prepare the mannan-modified adenovirus, mannan was dissolved into 0.1 M phosphate buffer (pH 6.0) with concentration of 25 mg/mL and oxidized with sodium periodate (0.01 M, Sigma, St Louis, MO, USA) for 60 min at 4 °C followed by dialysis (molecular weight cut off, 1000) at 4 °C for 5 h in bicarbonate buffer (pH 9.0). The resulting oxidized mannan was mixed with Ad5-PTEN and incubated overnight at room temperature to perform the conjugation of mannan and adenovirus (Man-Ad5-PTEN).

Harvesting and Culture of Macrophages. Before the experiment, 5 mL 2.9% thioglycolate medium (Sigma, USA) was injected into the peritoneal cavity of the male SD rats (200 ± 20g). Four days later, the rat was sacrificed and the skin carefully removed, leaving the peritoneal membrane intact; 15−20 mL of ice cold tissue culture medium was injected into the peritoneal cavity and the abdomen gently pressed for about 30−60 s to bring the cells into suspension. Then, the center of the peritoneum was held up with forceps and medium aspirated out using a 20 gauge needle.

The washed cells were suspended in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin G (100 U/mL), and streptomycin (100 mg/mL), and then they were placed on 24-well culture plates at a density of 3×10^5 cells/cm^2. After incubation for 24 h at 37 °C, 5% CO_2, nonadherent cells were washed off with culture medium and cells were cultivated for another 48 h.

***In Vitro* Cytotoxicity Test for Man-Ad5-PTEN Using CCK-8 Kit.** The cytotoxicity of mannan-modified adenovirus was evaluated by the cell counting kit-8 (CCK-8, Beyotime Institute of Biotechnology, Jiangsu, China) assay according to the report.[36] Briefly, Chang normal hepatocyte cells were placed in 96-well plates at 1×10^4 per well. One day later, the cells were treated respectively with 100 μL of PBS, naked Ad5-PTEN, and Man-Ad5-PTEN at a variety multiplicity of infection (MOI) of 80, 40, and 20 pfu/cell. After 4 h incubation, 200 μL of DMEM complete media was added to each well, and cells were cultured for another 48 h. At the end point, 20 μL CCK-8 (5 g/L) was added for further 4 h of incubation at 37 °C. The cell numbers in triplicate wells were measured as the absorbance (450 nm) of reduced WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) using a microplate reader (model-550, Biorad USA). Cell viability was calculated according to the formula: $(A_{\text{treated}} - A_{\text{background}}) \times 100/(A_{\text{control}} - A_{\text{background}})$, in which cells without any treatment were used as controls and the background well had no cells in it.

Evaluation of Gene Transfectivity *in Vitro*. NIH/3T3 cells were seeded at 1×10^5 cells per well on 24-well plates with complete DMEM medium and incubated at 37 °C 5% CO_2 until confluence reached ∼70%. Meanwhile, the harvested macrophages mentioned above were cultured in completed RPMI 1640 medium and also incubated at 37 °C, 5% CO_2, until confluence reached ∼70%. For *in vitro* transfection, the culture medium was removed and the cells were washed twice with PBS. Then, 0.5 mL culture medium containing Ad5-LacZ or Man-Ad5-LacZ with or without free mannan (20 mM) were added dropwise to each well and incubated with the cells at 37 °C, 5% CO_2, in which a multiplicity of infection (MOI) of 40

was used in the adenovirus transductions. Four hours later, the infection solution was replaced again with fresh completed medium and incubated for an additional 40 h. Then, the cells were allowed to assess transduction efficiency by quantification of β-galactosidase expression which was determined by assay for total protein and activity of β-galactosidase in cells according to previous report.[37] The results were standardized for total protein concentrations in the samples using a BCA kit (Pierce, USA) according to manufacturer's instructions. Independent triplicate wells were analyzed for each sample.

***In Vitro* Anticancer Activity.** To test the *in vitro* anticancer activity, hepatocellular carcinoma cells H22 were harvested from *in vivo* passage. First, the stocked murine hepatoma H22 cells were subcultured in complete RPMI 1640 culture medium, and then 2×10^7 cells (200 μL) were injected into the abdominal cavity of mouse. Seven days later, the mouse with ascites tumor was sacrificed by N_2 inhalation and sanitized by immersing the body in 75% ethanol. The intraperitoneal tumor cells were collected, washed twice, and resuspended in complete medium, plated at a density of 6×10^4 cells/well in 96-well tissue culture plates and subsequently treated for 48 h with PBS, doxorubicin (2 μg/mL, Beijing Huafeng United Technology, Beijing, China), Man-Ad5-PTEN (MOI 100), naked Ad5-PTEN (MOI 100), the combined formulation of doxorubicin (2 μg/mL) and Man-Ad5-PTEN (MOI 100). After 48 h infection, phase-contrast photomicrography was carried out using a Nikon TE2000-S microscope (Japan) and the cell growth inhibition was determined by CCK-8 kit assay as mentioned above.

Invasion and Migration Assay. The invasiveness assay of hepatocellular carcinoma cells (H22) was carried out through transwell and it was quantified as the number of cells invading through Matrigel (BD Biosciences) coated transwell inserts (8 μm pore size, Corning Costar Corp) as described previously.[38]

First, to construct the basement barriers the inner surface of cell invasion chamber was coated with 40 μL matrigel. Then the transwell chambers were incubated under ultraviolet irradiation overnight and reconstituted in 100 μL PBS for 2 h at 37 °C. Before the invasion experiment, the H22 cells in 6-well tissue plates were treated with PBS, naked Ad5-PTEN (MOI 50, 100), Man-Ad5-LacZ (MOI 100), doxorubicin (1, 2 μg/mL), Man-Ad5-PTEN (MOI 100), and the combined treatment of doxorubicin with Man-Ad5-PTEN, respectively. Twenty-four hours later, the tested cells were suspended in 100 μL of serum-free medium and added to the upper chamber of the transwell insert. There were three parallel wells in each group. After 24 h of incubation, cells that had invaded to the lower surface of each Matrigel-coated membrane were fixed with 70% ethanol, stained with hematoxylin and eosin (H&E), and counted in five randomly selected fields under a light microscope.

The migration assay was also carried out with steps as same as those described above in the cell invasion test, except that the inner surface of the polycarbonate filters in the transwell chambers were not coated with matrigel.

Flow Cytometric Analysis of Cell Cycle and Apoptosis. Hepatocellular carcinoma cells (H22) were plated in 6-well tissue plates and treated respectively with PBS, naked Ad5-PTEN (MOI 100), Man-Ad5-LacZ (MOI 100), doxorubicin (2 μg/mL), Man-Ad5-PTEN (MOI 100), and the combined formulation of doxorubicin with Man-Ad5-PTEN.

For cell-cycle analysis, 48 h after infection the treated cells were collected, washed with ice-cold PBS, and fixed with 70% ice-cold ethanol overnight at −20 °C. The cell pellets were uniformly resuspended in a mix of 50 μg/mL propidium iodide (PI) and 20 μg/mL RNase A, and incubated at 37 °C for 30 min in the dark. Finally, flow cytometry was performed on a Becton Dickinson FACScan and analyzed by ModiFit software (Verity Software House, Inc., Topsham, ME). The percentages of nuclei in G0-G1, S, and G2-M phases of the cell cycle were determined from at least 20,000 ungated cells. The experiment was repeated three times.

To further investigate the efficacy of the performed Man-Ad5-PTEN on hepatocellular carcinoma cells H22, the induced cell apoptosis was quantitatively studied by Flow Cytometry with annexin V-FITC-labeled Apoptosis Detection Kit I (Nanjing KeyGen Biotech Co. Ltd., Nanjing, China). Annexin-V staining was performed to identify the cell apoptosis by using annexin-V-fluoresein isothiocyanate, which specifically binds phosphatidyI serine (PS) residues on the cell membrane. Propidium iodide (PI) was used to identify the cell apoptosis by binding to DNA once the cell membrane became permeable. In brief, after infection the treated cells (1×10^6 each well) were collected, washed with ice-cold PBS, and resuspended in 500 μL binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) with 5 μL annexin V-FITC and 5 μL propidium iodide (PI) added. After incubation for 10 min at room temperature in the dark, the stained cells were immediately analyzed using FACS flow cytometer (Becton Dickinson, USA). The data obtained were analyzed by Cell Quest software.

***In Vivo* Antitumor Efficacy on Intraperitoneal Hepatocellular Carcinoma.** Hepatocellular carcinoma cells H22 were harvested from *in vivo* passage as mentioned above in the *in vitro* anticancer activity and adjusted to a concentration of 1.0×10^7 cells/mL. Then, about 0.5 mL of the preformed cell suspension (including 0.5×10^7 cells) was injected into the abdominal cavity of mouse. Two days later, all the mice were randomly divided into six groups ($n = 10$) as follows: group I, PBS-treated; group II, Naked Ad5-PTEN; group III, doxorubicin-treated; group IV, Man-Ad5-PTEN treated; group V, Man-Ad5-LacZ treated; group VI, the combined treatment of Man-Ad5-PTEN with doxorubicin, in which the particle number of Ad5 is 1×10^9 and the amount of doxorubicin is about 2 mg/kg of body weight. Every formulation with 200 μL volumes was intraperitoneally injected every three days for two weeks and the body weight was detected on days 0, 5, and 15. The average growth rate (AGR) of body weight in different group was calculated according to the formula: $(\text{Weight}_{\text{day 15}} - \text{Weight}_{\text{day 0}}) \times 100/\text{Weight}_{\text{day 0}}$.

Statistical Analysis. Data were expressed as the means ± standard deviation of at least three independent experiments. Significant differences between sample means were calculated using Stastica, version 6.0 (StatSoft, USA) by one-way ANOVA (analysis of variance) followed by posthoc testing with Dunnett's method. Data were considered to be statistically significant if $P < 0.05$.

RESULTS

Man-Ad5-PTEN Showed No Cytotoxicity to Normal Hepatocyte Cells. The cytotoxicity of Ad5-PTEN or preformed Man-Ad5-PTEN at different multiplicity of infection (MOI) of 80, 40, and 20 pfu/cell was assessed by CCK-8 kit assay upon the Chang normal hepatocyte. Figure 1 demonstrated that PBS, naked Ad5-PTEN, and Man-Ad5-PTEN showed similar effects on cell viability even at the highest MOI of 80 pfu/cell after 48 h of incubation. This result

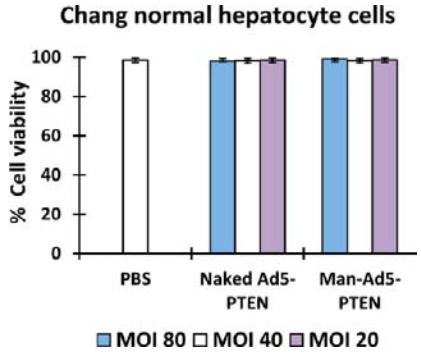


Figure 1. Viability of Chang normal hepatocyte cells determined by CCK-8 kit. Chang normal hepatocyte cells were treated with 100 μL of PBS, naked Ad5-PTEN, and Man-Ad5-PTEN at a variety multiplicity of infection (MOI) of 80, 40, and 20 pfu/cell, respectively. After 48 h incubation, 20 μL CCK-8 (5 g/L) was added for a further 4 h incubation at 37 °C. The absorbance of cells in triplicate wells was measured at wavelength of 450 nm. Results are presented as mean ± standard deviation (S.D.) (n = 3).

suggested that both naked Ad5-PTEN and Man-Ad5-PTEN have no cytotoxicity to Chang normal hepatocyte cells *in vitro*.

Mannan Conjugation Strategy Enhanced the Adenovirus-Mediated Gene Expression. To examine the gene transfection mediated by mannan-modified adenovirus, both NIH/3T3 cells lacking mannose receptor (MR) and macrophages expressing large numbers of MR were infected in triplicate with Ad5-LacZ or Man-Ad5-LacZ at a MOI of 40, and the transfectivity was quantitatively determined as LacZ gene expression (picogram) per microgram of total protein. Figure 2

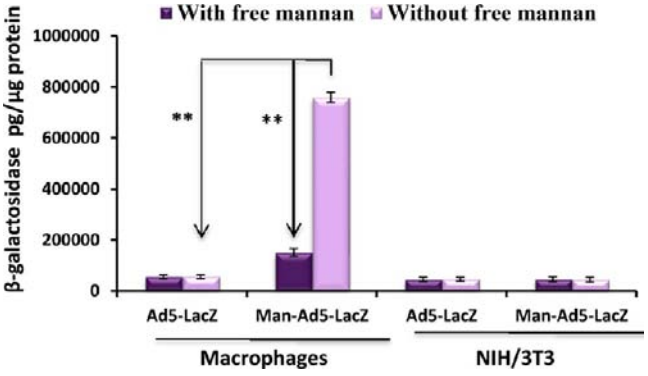


Figure 2. Transfection activity and effect of copresence of 20 mM mannan on the transfection activity of mannan modified adenovirus. Both macrophages and NIH/3T3 cells were treated, respectively, with Ad5-LacZ or Man-Ad5-LacZ with or without free mannan (20 mM), in which a multiplicity of infection (MOI) of 40 was used in the adenovirus transductions. The quantitative detection of β-galactosidase gene expressions was determined by using the β-galactosidase enzyme assay system. Each value represents the mean ± standard deviation (S.D.) (n = 3). Statistical analysis was performed by analysis of variance (** indicates $P < 0.01$).

describes the result of reporter gene expression of LacZ in both macrophages and NIH/3T3 cells transfected with Ad5-LacZ or Man-Ad5-LacZ. In macrophages, the transduction efficiency of Man-Ad5-LacZ increased about 12-fold to 758 751.8 pg β-gal/μg protein compared with that of naked Ad5-LacZ (56 522 pg β-gal/μg protein). The presence of 20 mM mannan significantly decreased the gene expression with Man-Ad5-LacZ from 758 751.8 pg β-gal/μg protein to 151 750 pg β-gal/μg protein ($P < 0.01$), but not that with naked Ad5-LacZ. Meanwhile, the transfection activity of Man-Ad5-LacZ was also investigated in NIH/3T3 cells for comparison, in which there was no significant difference ($P > 0.05$) in transfection activity between Man-Ad5-LacZ (with 45 239.8 pg β-gal/μg protein) and naked Ad5-LacZ (with 46 311.8 pg β-gal/μg protein). Unlike the case of macrophages, the presence of 20 mM mannan did not inhibit gene expression with Man-Ad5-LacZ. These results suggest that the mannan conjugation enhanced the adenovirus-mediated gene expression and Man-Ad5-LacZ was recognized by mannose receptors.

Anticancer Activity. The anticancer activity of Man-Ad5-PTEN upon hepatocellular carcinoma cells was evaluated by CCK-8 kit and the morphological consequences of different exposures were detected by microscopy. As shown in Figure 3, compared to the controlled group, both the size and number of these intracellular deposits increased in the treated groups including doxorubicin, Man-Ad5-PTEN, or the combined formulation. Meanwhile, the membranes of some cells were found to be ruptured as indicated by the solid arrow. From Figure 4, it could be seen that all treatments except PBS decreased the cell viability of hepatocellular carcinoma cell, and the mannan conjugation to Ad5-PTEN resulted in significantly lower cell viability as compared to naked Ad5-PTEN (67.2% cell viability induced by Man-Ad5-PTEN as compared with 87.2% cell viability induced by naked Ad5-PTEN). Further, as shown, the combined treatment of Man-Ad5-PTEN with doxorubicin inhibited hepatocellular carcinoma cell viability more effectively than the treatments with each single agent alone (41.8% vs 67.2%, 41.8% vs 66.4%). This may be due to co-action of both the enhanced PTEN gene therapy and doxorubicin chemical therapy.

Man-Ad5-PTEN Inhibits Invasion and Migration of Hepatocellular Carcinoma Cells. In early reports, it was demonstrated that Ad5-PTEN could regulate tumor cell spreading and migration, inhibit tumor cells, and has pro-apoptotic, anti-metastatic, and anti-angiogenic properties.

To clarify the direct effect of PTEN expression on the invasiveness of hepatocellular carcinoma cells, the highly invasive H22 cells were treated with Man-Ad5-PTEN or control Ad5-LacZ and then their invasive ability was assessed using Transwell assay. As shown in Figure 5, a significant reduction in the number of invasive cells was seen when the cells were treated with Man-Ad5-PTEN, as compared with the control PBS-treated cells (**$p < 0.0001$). No significant difference in invasiveness was observed when the cells were treated with Man-Ad5-LacZ. Doxorubicin induced the decreased invasion and migration by cancer cells in a dose-dependent manner. Moreover, we evaluated the invasiveness and migration of hepatocellular carcinoma cells which were pro-treated with the combined formulation of Man-Ad5-PTEN (MOI 100) and doxorubicin (2 μg/mL). The result showed more significant inhibitory effects on the invasion (***$P < 0.00001$) and migration ($^{\#\#\#}P < 0.00005$) than any other test group, which suggested that Man-Ad5-PTEN may further enhance the tumor inhibition effect with the assistance of chemotherapeutic agent of doxorubicin.

Cell Cycle Perturbations after Treatment. To test whether the antiproliferative effect of Man-Ad5-PTEN was related to cell cycle arrest, hepatocellular carcinoma cells were treated with the different formulations mentioned above and the cycle progression was examined by the flow cytometry (Figure 6). The cell cycle kinetics showed that the G2/M phase

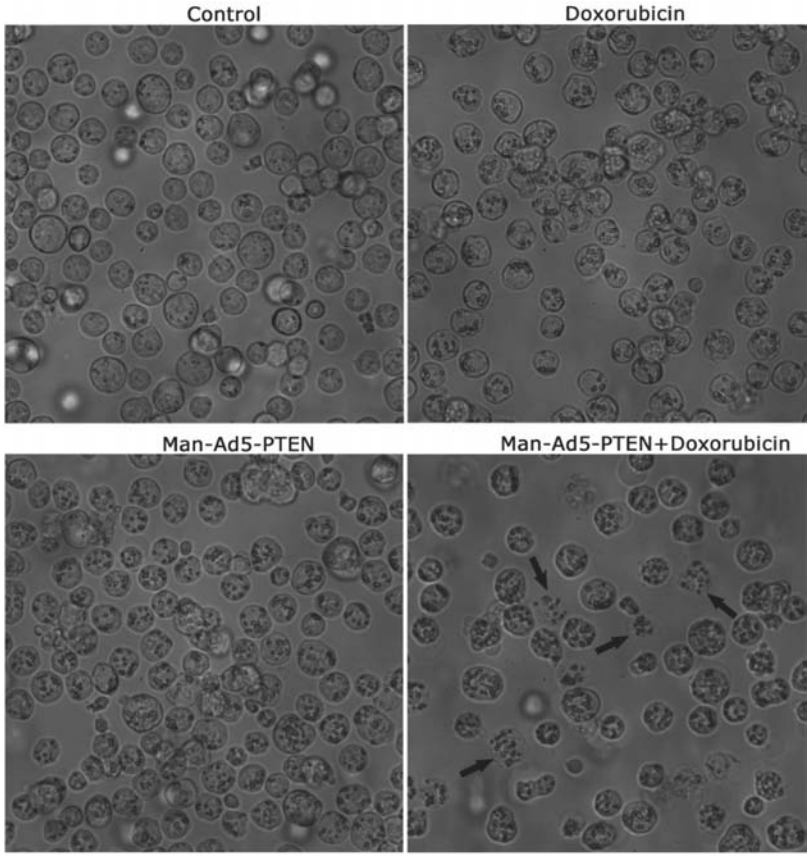


Figure 3. Morphology detection on hepatocellular carcinoma cells. Hepatocellular carcinoma cells were treated with PBS, doxorubicin (2 μg/mL), Man-Ad5-PTEN (MOI 100), naked Ad5-PTEN (MOI 100), and the combination of doxorubicin (2 μg/mL) and Man-Ad5-PTEN (MOI 100), respectively. After 48 h incubation, the cell morphological changes were detected by phase-contrast photomicrography using a Nikon TE2000-S microscope (200×). Solid arrow indicated the membrane ruptured cells.

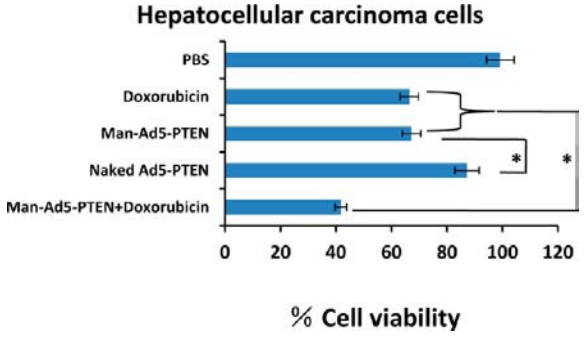


Figure 4. Growth inhibiton effect of mannan-modified adenovirus on hepatocellular carcinoma cells. Hepatocellular carcinoma cells were treated with PBS, doxorubicin (2 μg/mL), Man-Ad5-PTEN (MOI 100), naked Ad5-PTEN (MOI 100), and the combination of doxorubicin (2 μg/mL) and Man-Ad5-PTEN (MOI 100), respectively. After 48 h incubation, the cell viability was determined by CCK-8 kit. Results are presented as mean ± standard deviation (S.D.) (n = 3), $^{**}P < 0.01$.

fraction of H22 cells in control group was 31.38%, while the percentage of H22 cells treated with Man-Ad5-PTEN, doxorubicin, and the combined formulation increased significantly in G2/M phase, which were 61.19%, 70.29%, and 50.44%, respectively.

The percentage of S phase cell population in controlled cells was 65.91%, but decreased significantly in the treated groups, which were 35.07%, 27.47%, and 39.62%, respectively. This accumulation in G2/M phase was concomitant with a decrease in the percentage of cells in the S phase.

Man-Ad5-PTEN Causes Apoptosis in Hepatocellular Carcinoma Cells. To confirm that cell death found in the cell proliferation assay was caused by apoptosis, the double-staining flow cytometry assay with Annexin V-FITC and PI was used to quantitatively identify apoptotic cells after 48 h of infection with Man-Ad5-PTEN, doxorubicin, and the combined formulation of Man-Ad5-PTEN with doxorubicin, respectively (Figure 7). As shown in Figure 7, after treatment with PBS, the percentage of H22 cells positive for annexin V and PI were 1.2%, while the percentage of H22 cells positive for annexin V but negative for PI were 0.8%. After treatment with Ad5-PTEN, doxorubicin, or the combined formulation of Man-Ad5-PTEN and doxorubicin, the percentage of H22 cells positive for annexin V and PI were 16.95%, 21.50%, and 17.06%, respectively; while the percentage of H22 cells positive for annexin V but negative for PI were 16.13%, 9.00%, and 38.67%, respectively. These results suggested that both PTEN gene therapy and chemotherapy of doxorubicin triggered the onset of apoptosis and resulted in the significant growth inhibition of H22 cells.

Man-Ad5-PTEN Prolongs the Survival Time of Tumor-Bearing Mice. The *in vitro* experiments have demonstrated that the mannan-modified Ad5-PTEN approach alone or combined with doxorubicin could efficiently inhibit proliferation of cancer cells, induce the G2/M cell cycle arrest, and prevent the invasion of hepatocellular carcinoma cells. Further,

dx.doi.org/10.1021/bc400215a | *Bioconjugate Chem.* 2013, 24, 1387−1397

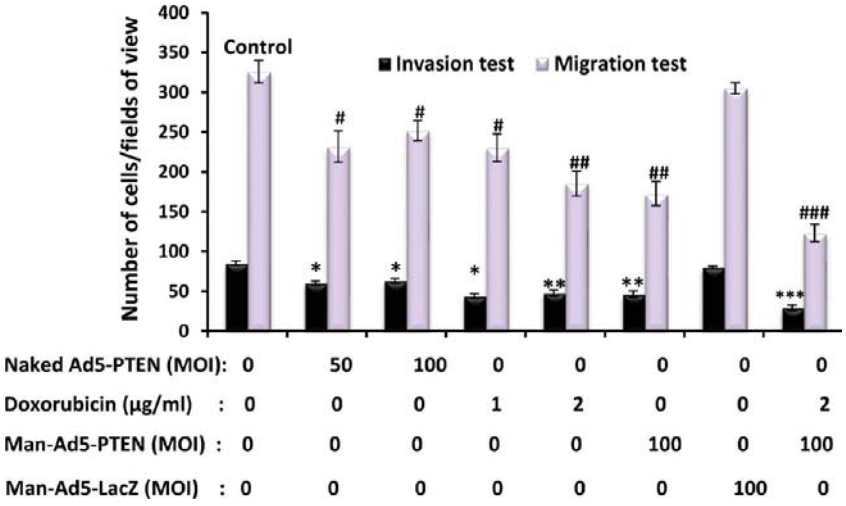


Figure 5. Inhibitory effects of PTEN and doxorubicin on invasion and migration of hepatocellular carcinoma cells. Hepatocellular carcinoma cells were cultured in medium with naked Ad5-PTEN (MOI 50, 100), Man-Ad5-LacZ (MOI 100), doxorubicin (1, 2 μg/mL), Man-Ad5-PTEN (MOI 100), or PBS, and their invasion through a reconstituted basement membrane barrier (Matrigel) was determined in a Boyden chamber assay as compared with the PBS-treated cells (control). The mean ± SD are shown. Statistical significance by Student's t test as compared with the control: Invasion, $^{*}P < 0.0005$, $^{**}P < 0.0001$, $^{***}P < 0.00001$ ($n = 3$); and Migration, $^{\#}P < 0.005$, $^{\#\#}P < 0.0005$, $^{\#\#\#}P < 0.00005$ ($n = 3$ in each group).

we evaluated the effect of such approaches on mice survival using intraperitoneal hepatocellular carcinoma models, in which six groups of mice were analyzed and Kaplan−Meier survival curves were plotted (Figure 8).

Concerning the survival time, Figure 8A and B showed that all groups injected with adenovirus or doxorubicin were significantly different from the PBS-injected group ($^{*}p < 0.05$ or $^{**}p < 0.0001$), except for the Man-Ad5-LacZ group with $p = 0.36$. In particular, the combined treatment of Man-Ad5-PTEN with doxorubicin greatly increased the mean survival time to 40 days and Man-Ad5-PTEN was significantly better than Naked Ad5-PTEN ($^{(\#\#)}p < 0.0001$). However, there was no difference between Man-Ad5-PTEN and doxorubicin ($p = 0.45$). When the Naked Ad5-PTEN, Man-Ad5-LacZ, Man-Ad5-PTEN, or doxorubicin groups were compared with the combination group, respectively, significant differences were also observed ($^{\Delta\Delta}p < 0.0001$, Figure 8B).

Meanwhile, we measured the weight of each mouse including the normal mice at days 0, 5, and 15, respectively, in which the normal mice were not injected with H22 tumor cells. As shown in Figure 8C, compared to the normal mouse group whose AGR was 24.73%, all of the mice treated, respectively, with PBS, naked Ad5-PTEN, doxorubicin, Man-Ad5-PTEN, Man-Ad5-LacZ, or the combined formulation of Man-Ad5-PTEN and doxorubicin, increased body weight significantly with a AGR of 56.28%, 51.86%, 52.65%, 53.26%, 54.16%, and 54.37%, respectively. Although AGR was different between the normal mice and the treated mice ($P < 0.05$), there was no significant difference between them among the treated mouse groups ($P > 0.05$).

■ DISCUSSION

Hepatocellular carcinoma (HCC) is one of the most common virus-associated cancers resulting in high mortality worldwide. For some patients for whom surgical treatments are not appropriate, one can only rely on chemotherapy. In conventional chemotherapy, for the desired therapeutic dose to reach the tumor, side effects usually occurred in most cases due to high toxicity levels. For example, doxorubicin is an antitumor drug commonly used against a wide spectrum of tumors. However, the clinical application of doxorubicin is restricted by its cardiotoxicity and the objective response rates from 13 published trials were <20%, the median survival was only 4 months.[40]

Moreover, the development of drug resistance toward chemotherapeutic agents often prevents the successful long-term use of chemotherapy for HCC.[41] Therefore, the development of more efficacious therapies for HCC remains urgent.

Gene therapy represents the exciting biotechnological advance that may revolutionize the conventional fashion of cancer treatment. The phosphate and tension homologue (PTEN) is a tumor suppressor gene that controls a variety of biological processes including cell proliferation, migration, and death. PTEN is only second to p53 in mutation frequency in human cancers, and it utilizes multiple mechanisms to control cellular growth, the most important of which is by inhibiting PI3K activation. It has been reported that PTEN is often deleted and mutated in a variety of human tumors. Specifically, several discoveries suggest that frequent genetic alterations and loss of expression of the PTEN gene were detected in HCC.[42] In the present study we investigated the therapeutic effect of PTEN on HCC.

To carry the gene of interest, an efficient gene delivery system is the key problem. At present, viral and nonviral methods of gene transfer have been used for clinical trials. The most widely used viral vectors are retroviruses, adeno-associated viruses, herpes viruses, and adenoviruses.[43] Among them, recombinant adenoviruses have been well studied as a model system in viral gene delivery and recently received much attention in cancer therapy for their practical advantages and application potentials. Among over fifty immunologically distinct serotypes of adenoviral family, the most frequently used vector is adenovirus type 5 (Ad5). The previous studies have developed some strategies using heterologous retargeting complexes such as chimeric fusion proteins and bispecific antibodies, or genetic capsid conjugations. In our work, we managed to couple the mannan ligand of mannose receptor to the surface of adenovirus and formed the mannan-modified

dx.doi.org/10.1021/bc400215a | Bioconjugate Chem. 2013, 24, 1387−1397

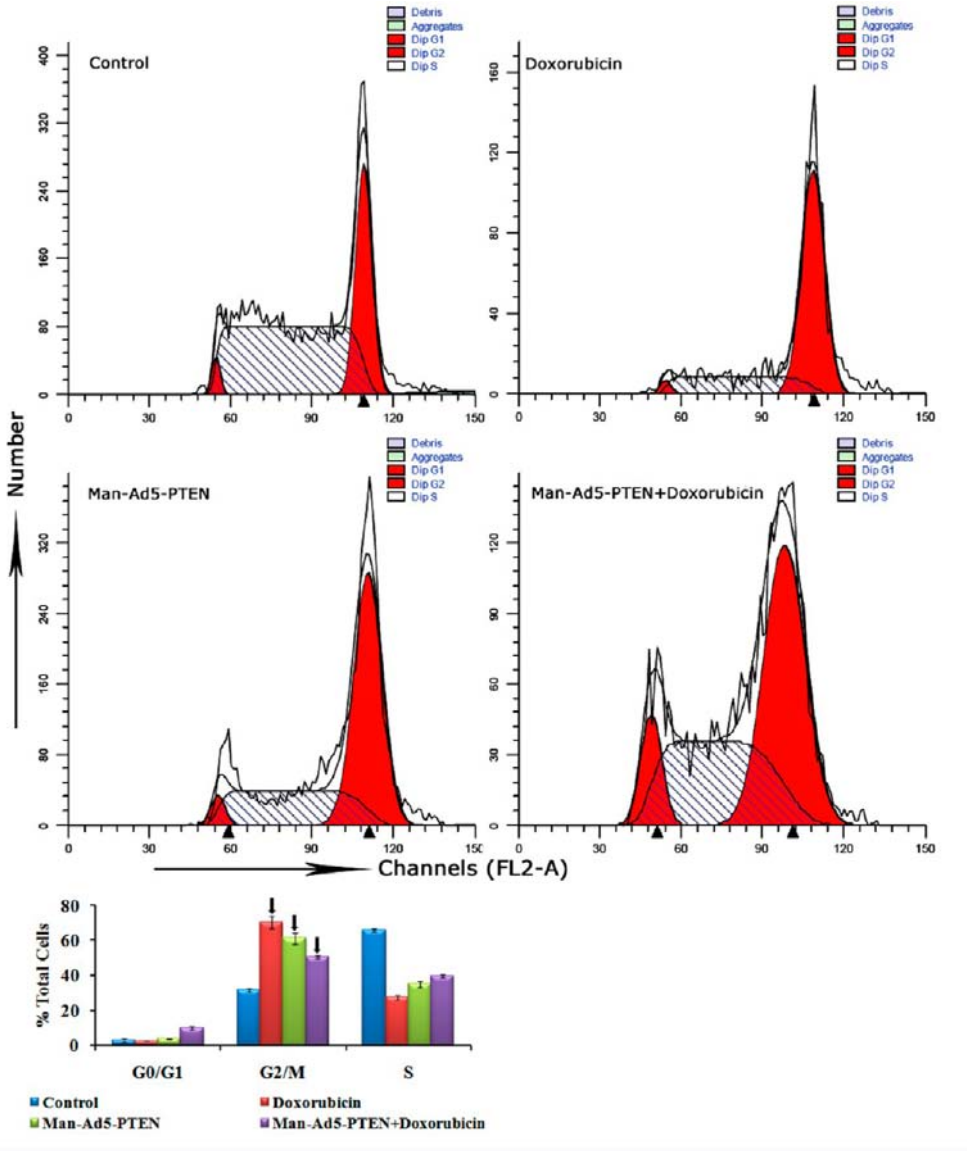


Figure 6. Hepatocellular carcinoma cells were arrested at G2/M phase by the treatment of Man-Ad5-PTEN. Cells were treated with PBS (as control), doxorubicin (2 μg/mL), Man-Ad5-PTEN (MOI 100), and Man-Ad5-PTEN (MOI 100) combined with doxorubicin (2 μg/mL) for 48 h, respectively. (A) The percentage of cells in each cell cycle phase (G1/G0, S, and G2/M) was determined by flow cytometry (n = 3). (B) The quantified data from experiment showed in (A). The treatment causes a G2/M block in tumor cells as indicated by arrows. Representative of three independent experiments.

adenovirus. Here, the mannose receptor (MR), also known as CD206, is a 180 kDa transmembrane C-type lectin that binds oligosaccharides terminating in mannose, fucose, or N-acetylglucosamine. As a member of the C-type lectin family of pattern recognition receptors, the MR may be integral in linking the innate and adaptive immune responses, as evidenced by the fact that MR-mediated uptake of certain exogenous glycoproteins by dendritic cells results in cross-presentation to CD8+ T cells.[44] Thus, it is generally accepted that MR plays an important role as a scavenging receptor and is an important component of the innate immune system.[45−47] Simmons and colleagues first demonstrated that ricin uptake into rat peritoneal macrophages was reduced by addition of exogenous lactose or mannan, and that maximal inhibition occurred when both lactose and mannan were present.[48,49]

Moreover, macrophages play an important role in host immune functions such as antigen presentation. Through transgene expression, attempts have been made to modulate the function and dysfunction of macrophages for the treatment of genetic metabolic diseases.[34] Meanwhile, macrophages are known to be very little susceptible to gene transfection by viral or nonviral vectors.[50] However, it is well-known that mannose receptors are expressed exclusively on macrophages, and so, introduction of mannan residues to vectors could provide an ideal gene delivery system.

In present study, the conjugation efficacy was assessed by periodic acid-Schiff (PAS) staining. In which, the PAS stain is a histochemical reaction commonly used to detect glycogen and other polysaccharides in biological specimen. The reaction of periodic acid oxidizes the diol functional groups in glucose and other sugars, creating aldehydes that react with the Schiff reagent to give purple−magenta color. Therefore, after the conjugation reaction, PAS stained positive for both the Man-Ad5 and the free mannan, whereas it is negative for the

dx.doi.org/10.1021/bc400215a | Bioconjugate Chem. 2013, 24, 1387−1397

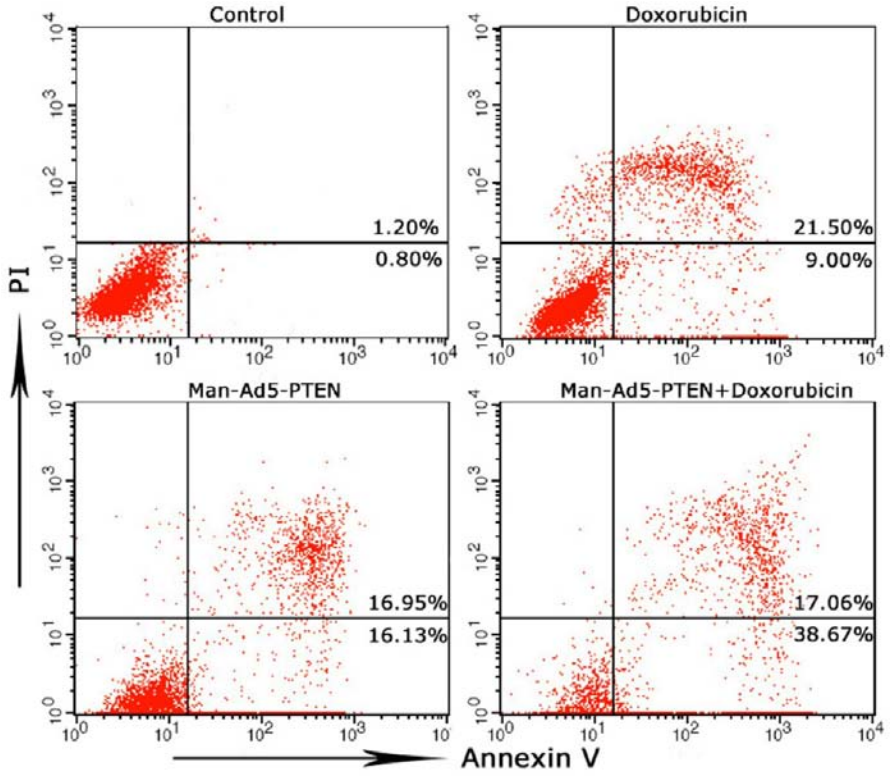


Figure 7. Man-Ad5-PTEN induced apoptosis in hepatocellular carcinoma cells. Cells were treated with PBS (as control), doxorubicin (2 μg/mL), Man-Ad5-PTEN (MOI 100), and Man-Ad5-PTEN (MOI 100) combined with doxorubicin (2 μg/mL) for 48 h, respectively. The apoptosis of HCC cells induced by Man-Ad5-PTEN and doxorubicin alone or together was detected by Annexin-V staining and flow cytometry. The percentage of apoptotic cells is indicated.

unconjugated control. In our experiment, after scanning the purple−magenta colored solution by ultraviolet spectrophotometer, we found it showed a max absorbance at wavelength of 544 nm. Therefore, by measuring the absorbance of sample at 544 nm, we could determine the amount of mannan according to the standard curve. In brief, following the reaction step in which the resulting oxidized mannan was mixed with Ad5-PTEN and incubated overnight at room temperature to perform the conjugation of mannan and adenovirus (Man-Ad5-PTEN), another dialysis process (molecular weight cut off, 50 000) was carried out to remove the extra oxidized mannan at 4 °C for 5 h against bicarbonate buffer (pH 9.0), and replaced with 100 mL of fresh buffer every other 30 min. Then, all the collected buffer was combined and 50 mL of it taken to enrich the oxidized mannan by Millipore ultrafiltration. After that, the concentrated oxidized mannan was subjected to PAS staining. After PAS staining, the absorbance was detected at 544 nm using a microplate reader to decide the amount of extra manan which did not react with Ad5. Because 1 mol substance has 6.02×10^{23} particles, the particle ratio between mannan and Ad5 was calculated as the formula: (Amount of total mannan − Amount of extra mannan) × (6.02×10^{23})/(particle number of Ad5), in which the amount is referred to as mole weight. At last, the result showed that the particle ratio of mannan per Ad5 is 20:1.

To detect the transfer activity of mannan-modified adenovirus, both NIH/3T3 cells lacking mannose receptor (MR) and macrophages expressing large numbers of MR were infected in triplicate with Ad5-LacZ or Man-Ad5-LacZ at a MOI of 40. The results in the present study showed that in macrophages the gene expression of Man-Ad5-LacZ was significantly higher than that of naked Ad5-LacZ, and significantly inhibited by excess free mannan (Figure 2). In NIH/3T3 cells lacking mannose receptors, on the other hand, the transfection efficiency was not inhibited by excess free mannose (Figure 2) and has no significant difference ($P > 0.05$) between mannan-modified adenovirus and naked adenovirus. These results suggest that the complex of mannan and adenovirus was recognized by mannose receptors as expected and the mannan conjugation enhanced the adenovirus-mediated gene expression on mannose receptors extensive express cells. Additionally, we also detected the transfectivity of Man-Ad5-LacZ and Man-Ad5-LacZ-doxorubicin on the murine hepatoma H22 cells *in vitro,* taking naked Ad5-LacZ as control. The result showed that the average amounts of β-galactosidase in murine hepatoma H22 cells transduced by Man-Ad5-LacZ or Man-Ad5-LacZ-doxorubicin was $(3.18 \pm 0.11) \times 10^5$ and $(3.22 \pm 0.22) \times 10^5$ pg β-gal/μg protein, respectively. Their expression levels were about 8-fold higher than that of β-galactosidase in cells transduced by naked Ad5-LacZ, which is $(4.16 \pm 0.07) \times 10^4$ pg β-gal/μg protein. There is no difference in gene expression between Man-Ad5-LacZ and Man-Ad5-LacZ-doxorubicin, which indicated that doxorubicin (2 μg/mL) did not affect the gene expression when combined with Man-Ad5-LacZ. These results suggested that mannan-modified Ad5 could enhance the LacZ gene expression in murine hepatoma H22 cells. These results coming from *in vitro* experiment lay a good foundation for the *in vivo* experiments.

Our cell-cycle analyses demonstrate that Man-Ad5-PTEN treatment causes G2/M block in hepatocellular carcinoma cells (Figure 6). Stewart et al. also saw similar result with ours. They reported that Ad-PTEN treatment causes G2/M block in

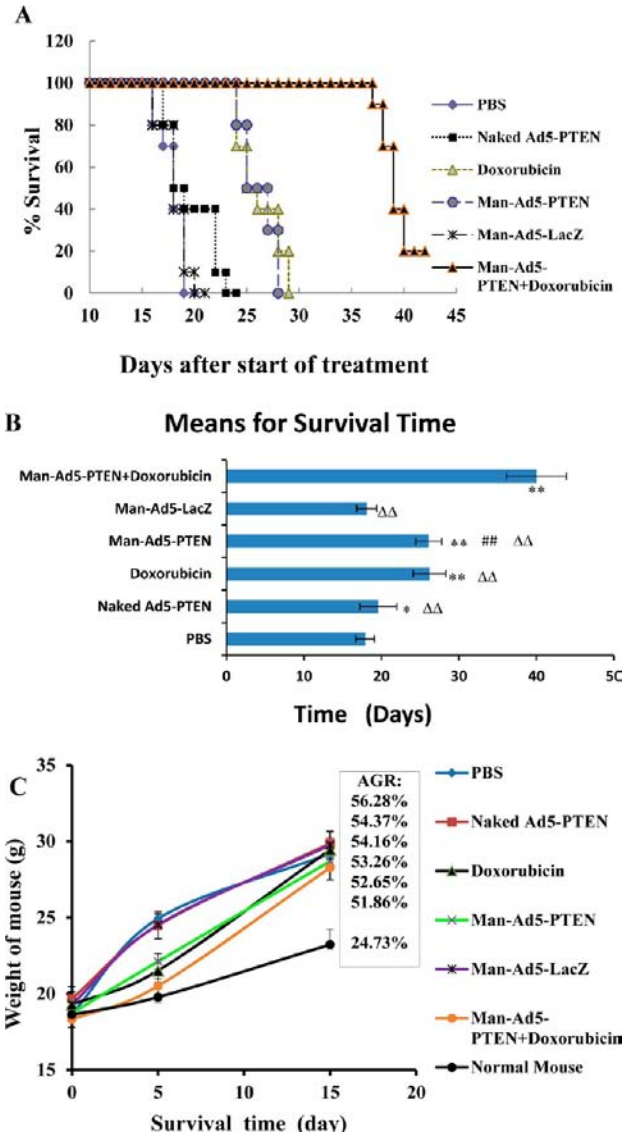


Figure 8. Man-Ad5-PTEN treatment prolonged the survival time of mice bearing intraperitoneal H22 tumor. (A) Survival curve of tumor-bearing mice given different treatments. (B) Means for survival time in each group. (C) Weight of mouse in different group. AGR representing the average growth rate of body mass was calculated according to the formula: $(\text{Weight}_{\text{day 15}} - \text{Weight}_{\text{day 0}}) \times 100/\text{Weight}_{\text{day 0}}$. Data are shown as mean ± SD $^{**}p < 0.0001$, PBS group vs the treated groups; (##) $p < 0.0001$, Man-Ad5-PTEN vs Naked Ad5-PTEN; $^{\Delta\Delta}p < 0.0001$, the combined treatment of Man-Ad5-PTEN with doxorubicin vs Man-Ad5-PTEN, Naked Ad5-PTEN, and Man-Ad5-LacZ, respectively. Similar results were obtained in three independent experiments. Each data point is represented as mean ± SD ($n = 10$).

melanoma cells.[35] This accumulation in G2/M phase was concomitant with a decrease in the percentage of cells in the S phase. These results suggest that the antiproliferation effects of Man-Ad5-PTEN result from reduced progression through the cell cycle, most likely due to a block or delay in the G2/M phase.

Tumor cell invasion and metastasis are fundamental and characteristic properties of carcinogenesis. Liver cancer possesses powerful abilities of migration and invasion, which is a formidable problem in clinical treatment of liver cancer and a key factor to prognosis of liver cancer.[51] Therefore, the inhibition of tumor cell invasion and metastasis are important mechanisms in the anticancer properties of anticancer drugs. Many recent studies have shown that chemopreventive and/or chemotherapeutic agents can inhibit tumor cell invasion and metastasis.[52] In our work, we investigated the effects of Man-Ad5-PTEN on migration and invasion of hepatocellular carcinoma cells using an H22 cell line. We found that Man-Ad5-PTEN induced a significant reduction in the number of invasive cells. Moreover, the combined treatment of Man-Ad5-PTEN (MOI 100) and doxorubicin (2 μg/mL) showed more significant inhibitory effects on the invasion and migration than the single treatment, which suggested that Man-Ad5-PTEN may further enhance the tumor inhibition effect with the assistance of chemotherapeutic agent of doxorubicin. Indeed, Man-Ad5-PTEN significantly prolonged the survival days of intraperitoneal H22 tumor-bearing mice.

In summary, the strategy of mannan conjugation could enhance adenovirus-mediated PTEN gene therapy effects on murine hepatocellular carcinoma cells *in vitro* and *in vivo*. It may be used to target gene therapy in other kinds of tumors by intratumoral administration or by intravenous administration. It may be concluded that mannan-modified Ad5-PTEN with the help of doxorubicin may be a powerful and effective tool for future HCC clinical exploration and therapy.

AUTHOR INFORMATION

Corresponding Author

*Tel +86 0830 3162291. Fax +86 0830 3162291. E-mail zhongzhirong@126.com.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are thankful for the financial supports of the National Natural and Science Foundation of China (No. 81202479), the Key Project of Chinese Ministry of Education (No. 212148), and the Key Project of Natural Science Foundation of Luzhou Medical College.

REFERENCES

(1) Liu, H., Liang, Y., Wang, L., Tian, L., Song, R., Han, T., Pan, S., and Liu, L. (2012) In vivo and in vitro suppression of hepatocellular carcinoma by EF24, a curcumin analog. *PLoS One 7*, e48075.

(2) Umemura, T., Ichijo, T., Yoshizawa, K., Tanaka, E., and Kiyosawa, K. (2009) Epidemiology of hepatocellular carcinoma in Japan. *J. Gastroenterol. 44* (Suppl 19), 102−7.

(3) Sakabe, T., Tsuchiya, H., Kanki, K., Azumi, J., Gonda, K., Mizuta, Y., Yamada, D., Wada, H., Shomori, K., Nagano, H., and Shiota, G. (2013) Identification of the genes chemosensitizing hepatocellular carcinoma cells to interferon-alpha/5-fluorouracil and their clinical significance. *PLoS One 8*, e56197.

(4) Wang, J., Zhang, Z., Wang, X., Wu, W., and Jiang, X. (2013) Size- and pathotropism-driven targeting and washout-resistant effects of boronic acid-rich protein nanoparticles for liver cancer regression. *J. Controlled Release 168*, 1−9.

(5) Wang, J., Ma, Y., Jiang, H., Zhu, H., Liu, L., Sun, B., Pan, S., Krissansen, G. W., and Sun, X. (2011) Overexpression of von Hippel-Lindau protein synergizes with doxorubicin to suppress hepatocellular carcinoma in mice. *J. Hepatol. 55*, 359−68.

(6) Zhang, J., Wang, Y., Zhen, P., Luo, X., Zhang, C., Zhou, L., Lu, Y., Yang, Y., Zhang, W., and Wan, J. (2013) Genome-wide analysis of miRNA signature differentially expressed in doxorubicin-resistant and parental human hepatocellular carcinoma cell lines. *PLoS One 8*, e54111.

(7) Carr, B. I. (2004) Hepatocellular carcinoma: current management and future trends. *Gastroenterology 127*, S218−24.
(8) Gish, R. G., Porta, C., Lazar, L., Ruff, P., Feld, R., Croitoru, A., Feun, L., Jeziorski, K., Leighton, J., Gallo, J., and Kennealey, G. T. (2007) Phase III randomized controlled trial comparing the survival of patients with unresectable hepatocellular carcinoma treated with nolatrexed or doxorubicin. *J. Clin. Oncol. 25*, 3069−75.
(9) Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S. I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., Bigner, S. H., Giovanella, B. C., Ittmann, M., Tycko, B., Hibshoosh, H., Wigler, M. H., and Parsons, R. (1997) PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science 275*, 1943−7.
(10) Steck, P. A., Pershouse, M. A., Jasser, S. A., Yung, W. K., Lin, H., Ligon, A. H., Langford, L. A., Baumgard, M. L., Hattier, T., Davis, T., Frye, C., Hu, R., Swedlund, B., Teng, D. H., and Tavtigian, S. V. (1997) Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat. Genet. 15*, 356−62.
(11) Rasheed, B. K., Stenzel, T. T., McLendon, R. E., Parsons, R., Friedman, A. H., Friedman, H. S., Bigner, D. D., and Bigner, S. H. (1997) PTEN gene mutations are seen in high-grade but not in low-grade gliomas. *Cancer Res. 57*, 4187−90.
(12) Wang, S. I., Puc, J., Li, J., Bruce, J. N., Cairns, P., Sidransky, D., and Parsons, R. (1997) Somatic mutations of PTEN in glioblastoma multiforme. *Cancer Res. 57*, 4183−6.
(13) Tashiro, H., Blazes, M. S., Wu, R., Cho, K. R., Bose, S., Wang, S. I., Li, J., Parsons, R., and Ellenson, L. H. (1997) Mutations in PTEN are frequent in endometrial carcinoma but rare in other common gynecological malignancies. *Cancer Res. 57*, 3935−40.
(14) Cairns, P., Okami, K., Halachmi, S., Halachmi, N., Esteller, M., Herman, J. G., Jen, J., Isaacs, W. B., Bova, G. S., and Sidransky, D. (1997) Frequent inactivation of PTEN/MMAC1 in primary prostate cancer. *Cancer Res. 57*, 4997−5000.
(15) Yokomizo, A., Tindall, D. J., Drabkin, H., Gemmill, R., Franklin, W., Yang, P., Sugio, K., Smith, D. I., and Liu, W. (1998) PTEN/MMAC1 mutations identified in small cell, but not in non-small cell lung cancers. *Oncogene 17*, 475−9.
(16) Lu, W., Zhou, X., Hong, B., Liu, J., and Yue, Z. (2004) Suppression of invasion in human U87 glioma cells by adenovirus-mediated co-transfer of TIMP-2 and PTEN gene. *Cancer Lett. 214*, 205−13.
(17) Xiong, X., Ren, H. Z., Li, M. H., Mei, J. H., Wen, J. F., and Zheng, C. L. (2011) Down-regulated miRNA-214 induces a cell cycle G1 arrest in gastric cancer cells by up-regulating the PTEN protein. *Pathol. Oncol. Res. 17*, 931−7.
(18) Arafa el, S. A., Zhu, Q., Shah, Z. I., Wani, G., Barakat, B. M., Racoma, I., El-Mahdy, M. A., and Wani, A. A. (2011) Thymoquinone up-regulates PTEN expression and induces apoptosis in doxorubicin-resistant human breast cancer cells. *Mutat. Res. 706*, 28−35.
(19) Zhang, Y., Chen, L. H., Wang, L., Wang, H. M., Zhang, Y. W., and Shi, Y. S. (2010) Radiation-inducible PTEN expression radiosensitises hepatocellular carcinoma cells. *Int. J. Radiat. Biol. 86*, 964−74.
(20) van Duijn, P. W., Ziel-van der Made, A. C., van der Korput, J. A., and Trapman, J. (2010) PTEN-mediated G1 cell-cycle arrest in LNCaP prostate cancer cells is associated with altered expression of cell-cycle regulators. *The Prostate 70*, 135−46.
(21) Monk, R. J., Malik, F. G., Stokesberry, D., and Evans, L. H. (1992) Direct determination of the point mutation rate of a murine retrovirus. *J. Virol. 66*, 3683−9.
(22) Noureddini, S. C., and Curiel, D. T. (2005) Genetic targeting strategies for adenovirus. *Mol. Pharm. 2*, 341−7.
(23) Ross, S. A., Song, X., Burney, M. W., Kasai, Y., and Orlicky, D. J. (2003) Efficient adenovirus transduction of 3T3-L1 adipocytes stably expressing coxsackie-adenovirus receptor. *Biochem. Biophys. Res. Commun. 302*, 354−8.
(24) Zhong, Z., Han, J., Wan, Y., Zhang, Z., and Sun, X. (2011) Anionic liposomes enhance and prolong adenovirus-mediated gene expression in airway epithelia in vitro and in vivo. *Mol. Pharm. 8*, 673−82.
(25) Zhong, Z., Wan, Y., Shi, S., Han, J., Zhang, Z., and Sun, X. (2012) Co-delivery of adenovirus and carmustine by anionic liposomes with synergistic anti-tumor effects. *Pharm. Res. 29*, 145−57.
(26) Zhong, Z., Wan, Y., Han, J., Shi, S., Zhang, Z., and Sun, X. (2011) Improvement of adenoviral vector-mediated gene transfer to airway epithelia by folate-modified anionic liposomes. *Int. J. Nanomed. 6*, 1083−93.
(27) Luten, J., van Steenbergen, M. J., Lok, M. C., de Graaff, A. M., van Nostrum, C. F., Talsma, H., and Hennink, W. E. (2008) Degradable PEG-folate coated poly(DMAEA-co-BA)phosphazene-based polyplexes exhibit receptor-specific gene expression. *Eur. J. Pharm. Sci. 33*, 241−51.
(28) Hashimoto, M., Morimoto, M., Saimoto, H., Shigemasa, Y., and Sato, T. (2006) Lactosylated chitosan for DNA delivery into hepatocytes: the effect of lactosylation on the physicochemical properties and intracellular trafficking of pDNA/chitosan complexes. *Bioconjugate Chem. 17*, 309−16.
(29) Kim, Y. K., Choi, J. Y., Yoo, M. K., Jiang, H. L., Arote, R., Je, Y. H., Cho, M. H., and Cho, C. S. (2007) Receptor-mediated gene delivery by folate-PEG-baculovirus in vitro. *J. Biotechnol. 131*, 353−61.
(30) Magnusson, S., and Berg, T. (1989) Extremely rapid endocytosis mediated by the mannose receptor of sinusoidal endothelial rat liver cells. *Biochem. J. 257*, 651−6.
(31) Yeeprae, W., Kawakami, S., Yamashita, F., and Hashida, M. (2006) Effect of mannose density on mannose receptor-mediated cellular uptake of mannosylated O/W emulsions by macrophages. *J. Controlled Release 114*, 193−201.
(32) Magnusson, S., and Berg, T. (1993) Endocytosis of ricin by rat liver cells in vivo and in vitro is mainly mediated by mannose receptors on sinusoidal endothelial cells. *Biochem. J. 291* (Pt 3), 749−55.
(33) Hebert, E., and Monsigny, M. (1994) Galectin-3 mRNA level depends on transformation phenotype in ras-transformed NIH 3T3 cells. *Biology of the cell/under the auspices of the European Cell Biology Organization 81*, 73−6.
(34) Kawakami, S., Sato, A., Nishikawa, M., Yamashita, F., and Hashida, M. (2000) Mannose receptor-mediated gene transfer into macrophages using novel mannosylated cationic liposomes. *Gene Ther. 7*, 292−9.
(35) Stewart, A. L., Mhashilkar, A. M., Yang, X. H., Ekmekcioglu, S., Saito, Y., Sieger, K., Schrock, R., Onishi, E., Swanson, X., Mumm, J. B., Zumstein, L., Watson, G. J., Snary, D., Roth, J. A., Grimm, E. A., Ramesh, R., and Chada, S. (2002) PI3 kinase blockade by Ad-PTEN inhibits invasion and induces apoptosis in RGP and metastatic melanoma cells. *Mol. Med. 8*, 451−61.
(36) Cao, Y., Yu, S. L., Wang, Y., Guo, G. Y., Ding, Q., and An, R. H. (2011) MicroRNA-dependent regulation of PTEN after arsenic trioxide treatment in bladder cancer cell line T24. *Tumour Biol. 32*, 179−88.
(37) Zhong, Z. R., Shi, S. J., Han, J. f., Zhang, Z. R., and Sun, X. (2010) Anionic liposomes increase the efficiency of adenovirus-mediated gene transfer to Coxsackie-adenovirus receptor deficient cells. *Mol. Pharmaceutics 1*, 105−115.
(38) Han, L., Zhang, A. L., Xu, P., Yue, X., Yang, Y., Wang, G. X., Jia, Z. F., Pu, P. Y., and Kang, C. S. (2010) Combination gene therapy with PTEN and EGFR siRNA suppresses U251 malignant glioma cell growth in vitro and in vivo. *Med. Oncol. 27*, 843−52.
(39) Xie, Y., Zhang, H., Sheng, W., Xiang, J., Ye, Z., and Yang, J. (2008) Adenovirus-mediated ING4 expression suppresses lung carcinoma cell growth via induction of cell cycle alteration and apoptosis and inhibition of tumor invasion and angiogenesis. *Cancer Lett 271*, 105−16.
(40) Nerenstone, S. R., Ihde, D. C., and Friedman, M. A. (1988) Clinical trials in primary hepatocellular carcinoma: current status and future directions. *Cancer Treat. Rev. 15*, 1−31.
(41) Bruix, J., Boix, L., Sala, M., and Llovet, J. M. (2004) Focus on hepatocellular carcinoma. *Cancer Cell 5*, 215−9.

(42) Ding, J., Gao, Y., Liu, R., Xu, F., and Liu, H. (2011) Association of PTEN polymorphisms with susceptibility to hepatocellular carcinoma in a Han Chinese population. *DNA Cell Biol. 30*, 229−34.
(43) Hatefi, A., Cappello, J., and Ghandehari, H. (2007) Adenoviral gene delivery to solid tumors by recombinant silk-elastinlike protein polymers. *Pharm. Res. 24*, 773−9.
(44) Burgdorf, S., Kautz, A., Bohnert, V., Knolle, P. A., and Kurts, C. (2007) Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation. *Science 316*, 612−6.
(45) East, L., and Isacke, C. M. (2002) The mannose receptor family. *Biochim. Biophys. Acta 1572*, 364−86.
(46) Taylor, P. R., Gordon, S., and Martinez-Pomares, L. (2005) The mannose receptor: linking homeostasis and immunity through sugar recognition. *Trends Immunol. 26*, 104−10.
(47) Lee, S. J., Evers, S., Roeder, D., Parlow, A. F., Risteli, J., Risteli, L., Lee, Y. C., Feizi, T., Langen, H., and Nussenzweig, M. C. (2002) Mannose receptor-mediated regulation of serum glycoprotein homeostasis. *Science 295*, 1898−901.
(48) Simmons, B. M., Stahl, P. D., and Russell, J. H. (1986) Mannose receptor-mediated uptake of ricin toxin and ricin A chain by macrophages. Multiple intracellular pathways for a chain translocation. *J. Biol. Chem. 261*, 7912−20.
(49) Gage, E., Hernandez, M. O., O'Hara, J. M., McCarthy, E. A., and Mantis, N. J. (2011) Role of the mannose receptor (CD206) in innate immunity to ricin toxin. *Toxins 3*, 1131−45.
(50) Fujita, T., Furitsu, H., Nishikawa, M., Takakura, Y., Sezaki, H., and Hashida, M. (1992) Therapeutic effects of superoxide dismutase derivatives modified with mono- or polysaccharides on hepatic injury induced by ischemia/reperfusion. *Biochem. Biophys. Res. Commun. 189*, 191−6.
(51) Wu, J. B., Fu, H. Q., Huang, L. Z., Liu, A. W., and Zhang, J. X. (2011) Effects of siRNA-targeting BMP-2 on the abilities of migration and invasion of human liver cancer SMMC7721 cells and its mechanism. *Cancer Gene Ther. 18*, 20−5.
(52) Choi, Y. H., Choi, W. Y., Hong, S. H., Kim, S. O., Kim, G. Y., Lee, W. H., and Yoo, Y. H. (2009) Anti-invasive activity of sanguinarine through modulation of tight junctions and matrix metalloproteinase activities in MDA-MB-231 human breast carcinoma cells. *Chem.-Biol. Interact. 179*, 185−91.