行政院國家科學委員會專題研究計畫成果報告

利用 thrombospondin-1進行人類食道鱗狀上皮細胞癌基因治療研究

計畫類別：個別型計畫
計畫編號：NSC91-2314-B-006-086-
執行期間：91年08月01日至92年07月31日
執行單位：國立成功大學醫學系外科

計畫主持人：吳明和
計畫參與人員：林木源  蕭璦莉  吳昭良

報告類型：精簡報告
處理方式：本計畫可公開查詢

中華民國92年10月31日
行政院國家科學委員會補助專題研究計畫成果報告

利用攜帶抗血管新生因子 Thrombospondin-1 轉殖基因腺病毒進行人類食道鱗狀上皮細胞癌基因治療研究

計畫類別： □ 個別型計畫 □ 整合型計畫

計畫編號：NSC91-2314-B-006-086

執行期間：91 年 8 月 1 日至 92 年 7 月 30 日

計畫主持人：吳明和

計畫參與人員：林木源

蕭瓊莉

吳昭良

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執行單位：國立成功大學醫學院外科學科

中華民國 年 月 日
The Application of Antiangiogenic Factor (Thrombospondin-1) in the Treatment of Esophageal Squamous Cell Carcinoma Xenografts

中文摘要

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Thrombospondin-1 (TSP-1) was identified as the first natural inhibitor of angiogenesis. Recently, antiangiogenic gene therapy has been applied for clinical studies in lung cancer and head and neck cancer. However, no preclinical studies have yet demonstrated an anticancer effect of adenoviral-mediated TSP-1 gene therapy on esophageal cancer. We herein evaluated the effect of TSP-1 adenoviral gene therapy on human esophageal squamous cell carcinoma to test the ability of clinical application. Two human esophageal cancer cell lines (CE81T and CE146T) were used. The transduction efficiency, TSP-1 protein expression, and growth suppression were assessed by using the recombinant adenoviral vector Ad5CMV-TSP-1. The transduction efficiency was 30%–50% at multiplicity of infection of 100 plaque-forming units (PFU)/cell. A significant growth suppression following an Ad5CMV-TSP-1 infection was observed in both cancer cell lines. Western blot analysis confirmed the presence of exogenous TSP-1 protein expression. CE-81T xenografts in nude mice transduced with Ad5CMV-TSP-1 demonstrated significant growth suppression.

關鍵詞

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腺病毒載體，食道癌基因治療，抗血管新生因子

English Abstract

Thrombospondin-1 (TSP-1) was identified as the first natural inhibitor of angiogenesis. Recently, antiangiogenic gene therapy has been applied for clinical studies in lung cancer and head and neck cancer. However, no preclinical studies have yet demonstrated an anticancer effect of adenoviral-mediated TSP-1 gene therapy on esophageal cancer. We herein evaluated the effect of TSP-1 adenoviral gene therapy on human esophageal squamous cell carcinoma to test the ability of clinical application. Two human esophageal cancer cell lines (CE81T and CE146T) were used. The transduction efficiency, TSP-1 protein expression, and growth suppression were assessed by using the recombinant adenoviral vector Ad5CMV-TSP-1. The transduction efficiency was 30%–50% at multiplicity of infection of 100 plaque-forming units (PFU)/cell. A significant growth suppression following an Ad5CMV-TSP-1 infection was observed in both cancer cell lines. Western blot analysis confirmed the presence of exogenous TSP-1 protein expression. CE-81T xenografts in nude mice transduced with Ad5CMV-TSP-1 demonstrated significant growth suppression.
suppression. These data suggest that Ad5CMV-TSP-1 may thus be a potentially effective therapeutic agent for locally advanced esophageal cancer.

Introduction

The incidence of esophageal squamous cell cancer (ESCC) shows great geographic variation. In Taiwan, it is the six most common cause of cancer death in male[1]. Recently, a great deal of scientific interest has focused on the extratumoral processes associated with tumor progression in esophageal cancer, one of which is angiogenesis[2]. Angiogenesis is the mechanism utilized by the human body to produce new blood vessels. It occurs as a normal physiological process during prenatal development, the female reproductive cycle, and wound healing[3,4]. However, abnormal angiogenesis also occurs during the development of solid tumors and their metastases. Tumors require blood vessels to supply nutrients and oxygen and with access to sufficient blood supply, the cancer is free to grow and spread. Without an adequate blood supply, the tumor cannot grow larger than a pea and is thus nonlethal[5-8]. To allow for continuous growth, cancer cells release substances that induce angiogenesis resulting in the formation of new capillaries. Within a given microenvironment, the angiogenic response is determined in part by a balance between angiogenesis inducers (VEGF, bFGF, IL-8, etc.) and inhibitors (angiostatin, endostatin, thrombospondin, etc.)[8-12].

Thrombospondin-1 (TSP-1) was identified as the first natural inhibitor of angiogenesis. It is a multifunctional, Mr 450,000 trimeric glycoprotein that can prevent the induction of neovascularity in the rat cornea[13]. When expressed in cancer cells, it can block angiogenesis, tumor growth, and metastasis in various types of cancer cells[14-17]. Furthermore, it has been shown that TSP-1 can modulate endothelial cell adhesion, motility, and growth in addition to exerting an antiangiogenic effect on cord formation[18]. Moreover, human TSP-1 has been found to inhibit neovascularization by inducing the receptor-mediated microvascular endothelial cells[19].

Considerable controversies still remain as far as the prognostic value of angiogenesis in esophageal cancer is concerned. However, there is strong evidence that angiogenesis in esophageal cancer correlates significantly with the progress of esophageal cancer, including the tumor stage, grade, metastasis, and clinical outcome. However, with respect to the role of TSP-1 in esophageal cancer progression, much less is known. Recently, it was observed that TSP-1 expression played an important role in cancer cell growth and metastasis of human esophageal squamous cell carcinoma[20].

In this investigation, an
adenovirus-mediated TSP-1 for gene therapy in esophageal cancer was developed and it was determined whether the TSP-1 gene, expressed by adenoviral vector, could affect human esophageal cancer cell growth in the SCID mouse xenograft model.

**Materials and Methods**

**Cells and Recombinant TSP-1 Adenovirus (Ad5CMV-TSP-1)**

The tumor cell lines CE81T and CE146T were purchased from the CRCC, R.O.C. All tumor cell lines were maintained as a monolayer in culture in Dulbecco’s modified Eagle medium (DMEM) (Life Technologies, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum, 0.1% l-glutamine, and 0.05% Gentamicin. The media and sera were purchased from Life Technologies (Grand Island, NY, USA).

The recombinant adenoviral vectors used in this study are replication-deficient (del E1, del E3) adenovirus type 5 which are substituted by *Escherichia coli* LacZ (Ad5CMV-LacZ) or human TSP-1 cDNA and placed under control of the cytomegalovirus (CMV) promoter at the E1-deleted region.[21] Adenoviral vectors containing no cDNA (Ad5CMV/293) or β-galactosidase cDNA (Ad5CMV-LacZ) were used as a negative control. These viruses were obtained by the lysis of infected 293 cells (type 5 adenovirus transformed human embryonic kidney cell line). Titers of the viral stocks were determined by a plaque-forming assay [22] using 293 cells. These recombinant adenovirus vectors were stored at -80°C until use. The titer of this viral stock was 2.4 × 10^9 plaque-forming units (PFU)/ml in Ad5CMV-TSP-1 and 4.8 × 10^8 PFU/ml in others.

**In Vitro Transduction Efficiency and X-gal Staining**

The efficiency of gene transfer into tumor cells was analyzed with Ad5CMV-LacZ at different viral concentrations. Tumor cells in monolayer culture were infected with the virus at different multiplicities of infection (MOI) including 1, 10, and 100 at 37°C for 120 min, which were gently shaken every 15 min. The medium was then changed and the cells were reincubated at 37°C in a CO2 incubator. Gene transduction efficiency was analyzed by the detection of the LacZ gene expression by X-gal staining as described elsewhere.[23] Ad5CMV-LacZ-infected cells were fixed in 2% formalin and 0.2% glutaeraldehyde for 5 min at 4°C, washed twice in phosphate-buffered saline (PBS) and then stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), which produces a blue color in β-galactosidase-expressing cells.
Western Blot Analysis

The cells were infected with Ad5CMV-TSP-1 vector and then further cultured for 16 h. The cells were then washed with PBS three times and lysed in 0.5% Nonidet P-40, 20mM Tris-HCl (pH 7.5), 1 mM EDTA, 1mM phenylmethylsulfonyl fluoride, 50 µM leupeptin, 50 µM antipain, 50µM pepstatin A, and 50µM calpain inhibitor-I for 10 min at 4°C. The cell lysate was centrifuged at 13 000 g for 10 min and the supernatant was then used as a cell extract. An immunoblot analysis was performed using anti-TSP-1 monoclonal antibody (Transduction Laboratories, Lexington, KY, USA) as described previously.[24] Subsequent protein detection was performed using the enhanced chemiluminescence (ECL) detection system (Amersham, Arlington Heights, IL, USA).

In Vitro Transduction of Recombinant Adenoviral Vector

Esophageal cancer cells were inoculated at densities of 1 x 10^4 96-well culture plate 24 h before infection. The cells were infected with Ad5CMV-TSP-1 at different MOI ranging from 100 to 1000. After 48 h, 10 µl/well PreMix WST-1 was added to each well. The cells were incubated for 2 h, then the absorbance of the sample were measured against a background control as blank using a microtiter plate (ELISA) reader. Hexaplet cultures of each cell line with different MOI treatments were counted.

In Vivo Transduction of Recombinant Adenoviral Vector

Balb/c SCID female mice (6–8 weeks old) were used. The animal experiments were performed at the Laboratory Animal Center in National Cheng Kung University College of Medicine, closely following the guidelines of the Guide for Animal Experimentation, College of Medicine, National Cheng Kung University (1993) edited by the Laboratory Animal Center, National Cheng Kung University College of Medicine. The inhibition of in vivo tumorigenicity of CE 81T cells by Ad5CMV-TSP-1 was evaluated after the inoculation of 2 x 10^6 cells of CE 81T (five mice/ group) into the flank. Tumor development and growth were checked three times per week. When the tumor size reached approximately 200 mm^3, then, the therapeutic ability of Ad5CMV-TSP-1 was evaluated by the intratumoral injection to an established tumor. The tumor volume was calculated according to the (length x width^2) x 0.45 and presented as the mean (± standard error of the mean) mm^3.

Results

Transduction Efficiency of Recombinant Adenovirus on Esophageal Cancer Cell
The condition for optimal adenoviral transduction of CE 81T and CE 146T cells was determined by transfection with AdCMVLacZ. The relationship between the number of infected cells and the number of adenoviral particles used appeared to be a linear shape. The transduction efficiencies of these two cell lines were similar; the cells inoculated with a single dose of AdCMVLacZ at MOI 100 exhibited around 30-50% blue cells (Fig. 1).

**Fig. 1.** Transduction efficiency of recombinant adenovirus on esophageal carcinoma cell lines CE81T (closed circles), CE146T (open circles) and J82 (reverse triangles). AdCMV β-galactosidase (LacZ) was used to infect the cells at different multiplicity of infection (MOI) ranging from 1 to 100.

The percentage of X-gal-positive cells was obtained from scoring 500 cells each on replicate dish

The Cell proliferating capacity of CE81T and CE146T at various MOIs of AdCMV-Tsp-1 ranging from 100 to 1000 are shown in Fig. 2. AdCMV-TSP-1-infected cells revealed similar proliferating capacity to those of noninfected cells at MOIs 100. But AdCMV-TSP-1-infected cells at MOIs 1000 showed prominent proliferating inhibition.

**Fig. 2.** In vitro proliferating capacity of Ad CMV-TSP-1-infected human esophageal carcinoma cell lines. The cells were inoculated at densities of 1 x 10^4 in 96-well culture plate 24 h before infection. The cells were infected with Ad5CMV-TSP-1 at different MOI ranging from 100 to 1000. After 48 h, 10 μl/well PreMix WST-1 was added to each well. The cells were incubated for 2 h, then the absorbance of the sample were measured against a background control
Expression of exogenous TSP-1 protein in Ad5CMV-TSP-1-Infected CE81T and
CE146T Cells

A western blot analysis was performed to examine the protein expression level of
TSP-1 in CE81T cells after infection of Ad5CMV-TSP-1 at MOI 100 (Fig. 3). A
small amount of TSP-1 was expressed before infection (Fig. 3, first lane). A
high level of TSP-1 protein expressed after infection (Fig. 3, second lane).

Fig. 3. Western blotting analysis
demonstrated the TSP-1 expression in
CE81T cells after Ad5CMV-TSP-1
infection. The protein was extracted 24
hours after infection. A small amount of
TSP-1 was expressed before infection
(first lane). A high level of TSP-1 protein
expressed after infection (second lane).

In Vivo Antitumor Effect of
Ad5CMV-TSP-1 Infection

The in vivo therapeutic effect of
intratumoral Ad5CMV-TSP-1 injection is
shown in Fig. 4. On days 21 and 28,
either Ad5CMV-TSP-1 or PBS were
injected into tumor. The mean tumor
volumes per five mice following
injection were plotted against the number
of days since s.c. inoculation; bars
indicate standard error of the mean. In
this experiment, the tumors injected with
PBS showed no growth suppression. The
intratumoral injections of
Ad5CMV-TSP-1 significantly inhibited
tumor growth after treatment. Two
injections of Ad5CMV-TSP-1
suppressed tumor growth by 30%–40%,
compared with PBS, when comparing the
average tumor volume of these two
groups.

Fig. 4. The effect of
Ad5CMV-TSP-1 and Ad5CMV-TSP-1
infection on in vivo tumor growth of
CE81T cells. Established tumors were
treated after reaching a size of 200 mm
3 with injections delivered on days 21 and
28 of PBS or the virus (total dose 2.2 x $10^{11}$ plaque-forming units). The mean tumor volumes per five mice following injection were plotted against the number of days since subcutaneous transplantation; bars, standard deviation. Closed squares, Ad5CMVTSP-1; open squares, PBS

Discussion

Esophageal cancer is often unresectable and frequently resistant to chemoradiotherapy. Recent advances in molecular genetics have now made it possible to perform targeted gene transfer into tumor cells. In the present study, it has been demonstrated that adenovirus-mediated TSP1 gene transduction efficiently suppressed tumor growth of human esophageal cancer cell lines both in vitro and in vivo.

First, the transduction efficiency of Ad5CMV-LacZ in esophageal carcinoma cells was evaluated, and was found to be lower than that of other cell lines examined, which include J82, and showed from 90% to 100% infection efficiencies after incubation at 100 MOI of Ad5CMV-LacZ. This relatively low efficiency could be due to a cell factor, including receptor variations and differences in membrane characteristics among the cell lines. Furthermore, Ad5CMV-TSP-1 was observed to successfully deliver and express TSP-1 protein into esophageal cancer. These results imply that the exogenous TSP-1 gene introduced by Ad5CMV-TSP-1 is efficiently translated into TSP-1 protein demonstrated with western blot analysis.

Second, this investigation have established a TSP-1 expression adenoviral vector. Compared to the controls, although there was almost no effect on proliferation of esophageal cancer cells in vitro, TSP-1, expressed by the viral vector, inhibited the growth of esophageal cancer xenograft. These results suggest that in the SCID mouse model, TSP-1 efficiently inhibits the growth of esophageal cancer xenografts. In addition, the antitumorat effect of TSP-1 was not due to direct inhibition of tumor cell proliferation, but was associated with significant inhibition of tumor angiogenesis.

A cure of cancer means that all malignant cells should be eradicated. Until now, many kinds of anticancer strategies have been attempted including gene therapy. Especially in the area of gene therapy, most research has focused on cytokine, tumor suppressor, and suicide genes as the therapeutic genes. To cure cancer, these therapeutic genes should be delivered to almost all target cells, although there is a "bystander effect" that might exist for some genes. However, it is difficult or almost impossible to deliver the therapeutic gene to all target cells. The antiangiogenic strategy like the one reported in this study is different than other strategies, as it does not directly target the tumor cells. The antiangiogenic gene can induce the necrosis of tumor cells, even if the gene
is delivered to only some of the target
cells, just enough to adjust the balance of
angiogenic stimulators and inhibitors in
the nearby microenvironment, which
blocks the blood supply of the tumoral
tissues by antiangiogenesis. Therefore,
although complete inhibition of tumor
growth was not accomplished in this
study, it is believed that it still might be
possible to thoroughly control tumor
growth by developing a better delivery
system and way of administration.

Therefore, our results suggest that viral
vector expressed the TSP-1 gene
efficiently in esophageal cancer cells and
greatly reduced esophageal cancer tumor
growth and angiogenesis in the mouse
xenograft model. Thus, TSP-1 may be a
potentially useful gene for esophageal
cancer gene therapy.

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