PANCREAS

Treatment of pancreatic carcinoma by adenoviral mediated gene transfer of vasostatin in mice

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Gut 2006;55:259–265. doi: 10.1136/gut.2005.064980

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Revised version received 25 September 2005
Accepted for publication 28 September 2005

Background: Tumour growth is angiogenesis dependent and antiangiogenesis therapy may represent a promising therapeutic option.

Aims: To evaluate the inhibitory effect of vasostatin gene mediated by a replication deficient recombinant adenovirus (Ad) on human pancreatic cancer in vivo and to investigate the mechanism of action of vasostatin.

Methods: Human umbilical vein endothelium derived ECV304 cells were infected with Ad-vasostatin and Ad-lacZ, and compared with phosphate buffered saline (PBS). MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was used to estimate the proliferation of ECV304 cells; tube formation assay and chorioallantoic membrane assay were used to evaluate angiogenesis in vivo and in vitro. Xenografted nude mice with pancreatic cancer were established to observe in vivo tumour growth suppression. Microvessel density revealed by CD31 immunohistochemical staining was measured.

Results: Growth and tube formation of ECV304 cells infected with Ad-vasostatin were suppressed significantly compared with cells infected with Ad-lacZ or cells treated with PBS. Neovascularisation in the Ad-vasostatin group was less than that in the PBS and Ad-lacZ groups, based on chorioallantoic membrane results. Volumes of pancreatic tumours in the Ad-vasostatin group were significantly smaller than those in the PBS and Ad-lacZ groups at the end of the treatment period. Microvessel density in the Ad-vasostatin group was significantly lower than that in the Ad-lacZ and PBS groups.

Conclusion: The vasostatin gene mediated by adenovirus is efficient for gene therapy for pancreatic carcinoma. Suppression of vasostatin on proliferation of vascular endothelium cells and angiogenesis may account for its effect.

Pancreatic cancer is the fifth leading cause of cancer related mortality in the Western world. Pancreatic cancer is one of the most difficult cancers to treat, and the overall survival rate of 3% over five years has remained unchanged for the past three decades.¹ To date, tumour resection has remained the only curative therapy for pancreatic carcinoma. Irradiation and chemotherapy do not have a significant therapeutic effect on pancreatic cancer, so new therapeutic strategies are required.²

Tumour growth and metastasis formation are dependent on the existence of an adequate blood supply. As tumours grow larger, adequate blood supply to the tumour tissue is often ensured by new vessel formation, a process named angiogenesis.³ At present, it is generally accepted that tumour growth in cancers, including pancreatic cancer, is angiogenesis dependent.⁴ Antiangiogenic gene therapy represents an interesting alternative for the treatment for cancers, and a number of studies have demonstrated that a gene therapy based antiangiogenesis approach is an effective means of reducing tumour growth in animal models. Potent angiogenesis inhibitors such as endostatin,^{5–7} angiostatin,⁸ TNP-470,⁹ soluble flt-1,¹⁰ and NK4^{11,12} have recently been identified in various cancer cell lines, and their effects on tumour regression have been confirmed in experimental animal models.

Vasostatin, the N terminal domain of calreticulin inclusive of amino acids 1–180, is a potent angiogenesis inhibitor. Vasostatin directly inhibited in vitro the proliferation of endothelial cells, such as fetal bovine heart endothelial cells, human umbilical vein endothelial cells, and the mouse endothelial cell line SVEC4-10.^{13,14} Vasostatin also inhibited in vivo the growth of human colon carcinoma and Burkitt lymphoma in nude mice and prolonged survival of mice with

Meth A and LL/2c tumours.^{14,15} Pancreatic cancer is one of the most intractable malignancies; does the angiogenesis inhibitor vasostatin have an effect on the hypovascular tumour? To address this question, in the present study, we constructed a replication deficient recombinant adenovirus encoding the vasostatin gene and tested whether intratumoral administration of vasostatin gene shows antitumour activity in pancreatic cancer models in mice.

METHODS

Recombinant adenovirus

A cDNA clone encoding vasostatin was isolated by amplifying the N terminal domain of calreticulin (amino acids 1–180) from the human skeletal muscle cDNA library (BD Biosciences, Franklin Lakes, New Jersey, USA) by polymerase chain reaction (PCR). The sense primer was 5'-gga aga tct atg gag ccc gcc gtc tac ttc-3' with the restriction site *Bgl*II and the initiation codon atg, and the antisense primer was 5'-ccc aag ctt tca ttc caa gga gcc gga ctc-3' with the restriction site *Hind*III and termination codon tga. The shuttle plasmid, pshuttle-CMV-vasostatin, in which the amplified fragment was inserted into the pshuttle-CMV (Stratagene, La Jolla, California, USA) downstream of the cytomegalovirus promoter, was established by ligation. After identification by digestion and sequencing, the shuttle plasmid was linearised with *Pme*I, and then the linearised shuttle plasmid was

Abbreviations: CAM, chorioallantoic membrane; CMV, cytomegalovirus; FBS, fetal bovine serum; MOI, multiplicity of infection; mRNA, messenger RNA; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; MVD, microvessel density; pfu, plaque forming units; RT-PCR, reverse transcription-polymerase chain reaction; PBS, phosphate buffered saline; EHS, Engelbreth Holm-Swarm

transformed into *Escherichia coli* BJ5183-AdEasy-1 (Stratagene) by electroporation. The plasmid was named pAd-vasostatin after homologous recombination and identification by kanamycin selection and restriction digest. The recombinant pAd-vasostatin was linearised with *PacI* and transfected into 293 cells to form Ad-vasostatin. The recombinant adenovirus was detected by examining the presence of the cytopathic effect and identified by PCR. Ad-vasostatin was amplified in 293 cells. The virus was purified with the adenovirus purification kit (Puresyn) according to the manufacturer's instructions. The titre of the virus was measured with a standard end point dilution assay using a method reported previously.^{16, 17} Ad-lacZ, the control virus, was similarly produced.

Cell lines

SW1990, a human pancreatic cancer cell line obtained from American Type Culture Collection (ATCC, Rockville, Maryland, USA) was maintained in RPMI 1640 (Gibco BRL Grand Island, New York, USA) supplemented with 10% fetal bovine serum (Gibco). PaTu8988, another human pancreatic cancer cell obtained from Dr Elsasser¹⁸ (Phillips University, Germany) and presented by Dr Pan,¹⁹ was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. ECV304, a cell line derived from human umbilical vein endothelium cells obtained from ATCC, was maintained in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total mRNA was prepared from PaTu8988/SW1990 cells infected with Ad-vasostatin and parental PaTu8988/SW1990 cells. Reverse transcription was performed starting with 2 µg of total mRNA, using oligo (dT) primer, other reagents, and procedures contained in the MmuLV RT-PCR kit (Promega, Madison, Wisconsin, USA) to form cDNA. cDNA (2 µl), 2 µl of 25 µM of each primer described above, 1 µl of dNTPs, and 1 µl of Taq DNA polymerase were used for PCR analysis. Amplification was performed for 35 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for one minute, and a final extension of 10 minutes at 72°C.

Western blot analysis

PaTu8988/SW1990 cells (1×10^6) infected with Ad-vasostatin and parental cells were washed twice with phosphate buffered saline (PBS) and lysed in a buffer containing 2% sodium dodecyl sulphate. The protein amount was estimated using a BCA protein assay kit (Pierce, Rockford, Illinois, USA), and 50 µg of total protein were loaded onto a 10% polyacrylamide slab gel and separated for two hours at a constant voltage (concentrated gel, 8 V/cm; separated gel, 15 V/cm). Proteins were transferred to nitrocellulose membranes using a Bio-Rad semidry transfer system. The membrane was incubated in 5% non-fat dry milk buffer to block non-specific binding sites. After washing three times in PBS plus 0.2% Tween 20, membranes were incubated in 5% non-fat dry milk in PBS plus 0.2% Tween 20 overnight at 4°C with goat polyclonal antibody to human Calregulin (Santa Cruz, Santa Cruz, California, USA) and horseradish peroxidase conjugated antibody to goat IgG. After several washes, bands were visualised with the ECL chemiluminescence detection system (Promega).

Proliferation assay of ECV304 cells and tumour cells

For the proliferation assay, ECV304 cells were washed with PBS and dispersed in a 0.05% solution of trypsin/RPMI1640. A cell suspension was made with RPMI1640/10% fetal bovine serum (FBS) and adjusted to 1.0×10^4 cells/ml. Cells were

plated onto gelatinised 96 well culture plates (Corning Costar, Corning, New York, USA) (0.2 ml/well) and incubated at 37°C in 10% CO₂ for 24 hours. The media were then replaced with 0.2 ml of RPMI1640/10% FBS, and the test sample (Ad-vasostatin or Ad-lacZ) was applied. The numbers of ECV304 cells were quantified using a colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay with a minor modification, as previously described.²⁰ ECV304 proliferation assays were repeated at least three times. The proliferation assay of human pancreatic cancer cells SW1990 was performed in the same way.

Tube formation assay

A well established method was used for the process of in vitro angiogenesis assay^{6, 21, 22} with a kit from Chemicon (Temecula, California, USA). After matrix solution gelling in the 96 well tissue culture plate, ECV304 cells were premixed with RPMI 1640, RPMI 1640, and Ad-vasostatin (multiplicity of infection (MOI) = 50), RPMI 1640 and Ad-lacZ (MOI = 50), and then seeded at a concentration of 1×10^4 per well onto the surface of the polymerised gel. Cells were incubated at 37°C in 10% CO₂ for 18 hours. Images were recorded with a CCD camera (AxioCam) attached to an inverted light microscope (40× objective lens), and the length of the tubes was assessed using the KS400 imaging assay system. Each type of treated ECV304 cell was seeded into at least three wells, and the tube formation assays were repeated at least three times.

Chorioallantoic membrane (CAM) assay

The chicken embryo CAM assay is widely used for in vivo angiogenesis assay.²² Briefly, fertilised White Leghorn chicken eggs were incubated for seven days at 37°C and 60% humidity. A square window was opened in the shell and the membrane of the gas chamber was carefully removed to expose chorioallantoic membrane. Twenty four eggs were randomly divided into three groups, and were doped with 10^7 Ad-vasostatin, 10^7 Ad-lacZ, and PBS. The windows were sealed with sterile parafilm and the eggs were incubated for 72 hours under the same conditions. CAMs were photographed and the number of crosspoints of the blood vessels, which represents angiogenesis, was counted using the KS400 imaging assay system.

In vivo experiments

Xenografted nude mice with pancreatic cancer were established to observe the inhibitory effect of vasostatin on tumour growth. Balb/c nude mice were maintained under specific pathogen free condition in Shanghai Experimental Animals Centre of Chinese Academy of Sciences. SW1990 cells or PaTu8988 cells (grown logarithmically, 1.0×10^7 suspended in 100 µl of PBS) were subcutaneously injected into the hind flank of a five week old Balb/c nude mouse. As the tumour grew larger, it was divided into small even pieces and replanted into the hind flanks of five week old Balb/c nude mice. Tumours were grown in mice to more than passage three. When the tumours were palpable, animals were randomly divided into three groups: (a) experimental group (n = 6)—animals were injected directly into the tumours with 10^8 pfu adenovirus containing vasostatin gene (Ad-vasostatin) every third day; (b) control group (n = 6)—animals were injected directly into the tumours with 10^8 pfu adenovirus containing lacZ gene (Ad-lacZ) every third day; and (c) control group (n = 6)—animals were injected directly into the tumours with PBS every third day. Body weight and volume of xenografts were measured in a blinded fashion using callipers every third day during the three week treatment period, and tumour size was calculated according to the formula $AB^2/2$, where A is the longest diameter and B is the shortest diameter of the tumour measured in two

Figure 1 Construction of recombinant adenovirus carrying vasostatin gene. (A) The vasostatin gene was cloned from the human skeletal muscle cDNA library by polymerase chain reaction. (B) A 564 bp fragment was released from pshuttle-CMV-vasostatin and, compared with it, no fragment was released from pshuttle-CMV without vasostatin. (C) The pAd-vasostatin released a fragment of 4.5 kb after Pac I digestion. (D) A total of 293 cells transfected with pAd-vasostatin showed cytopathic effects.

different planes. Total mRNA was prepared from PaTu8988/SW1990 tumours and RT-PCR analyses were performed as described previously.

Immunohistochemistry

To assess microvessel density (MVD) in tumours, goat CD31 polyclonal antibody (Santa Cruz) was used in immunohistochemistry, as previously described.^{23, 24} Cryostat sections of tumours were fixed with 4% paraformaldehyde for 30 minutes. Endogenous peroxidase was blocked with 3% hydrogen peroxide in ion free water for 15 minutes. After blocking non-specific reactions with 10% normal goat serum for 10 minutes, sections were incubated with a 1:100 dilution of goat CD31 polyclonal antibody overnight at 4°C. Sections were rinsed with PBS and incubated with a 1:200 dilution of horseradish peroxidase conjugated horse anti-goat immunoglobulin G antibody (Dako Cytomation, Glostrup, Denmark) for 30 minutes. Sections were rinsed with PBS and developed with DAB solution for 10 minutes. Then they were counterstained with haematoxylin.

MVD was assessed using a method reported previously.^{23, 25} Images were recorded with a CCD camera (AxioCam) attached to a microscope (40× objective lens), and microvessels were counted in three fields using the KS400 imaging assay system. The average density of three fields in each section was determined.

Statistical analysis

Quantitative results are expressed as mean (SEM). Statistical analysis was performed using one way analysis of the variance (ANOVA) with computer software SAS 6.12 (SPSS, Chicago, Illinois, USA). A p value of <0.05 was accepted as statistically significant.

Figure 2 Expression of the vasostatin gene measured by reverse transcription-polymerase chain reaction. Lane M, DNA marker (2000, 1000, 750, 500, 250, 100 bp); lane 1, PaTu8988 + Ad-vasostatin; lane 2, SW1990 + Ad-vasostatin; lane 3, PaTu8988; lane 4, SW1990.

RESULTS

Clone of gene of interest

An 564 bp full length vasostatin cDNA was obtained by amplifying the N terminal domain of calreticulin (amino acids 1–180) by PCR (fig 1A). The shuttle plasmid, in which the amplified fragment was inserted, downstream of the cytomegalovirus promoter, was established by ligation and then confirmed by digestion with *Bgl* II and *Hind* III. A 564 bp fragment was released from pshuttle-CMV ligated with vasostatin and, compared with it, no fragment was released from pshuttle-CMV without vasostatin (fig 1B). Identified by sequencing (data not show), the correct plasmid was named pshuttle-CMV-vasostatin.

Construction of recombinant adenovirus vectors

After being linearised with *Pme* I, the pshuttle-CMV-vasostatin was transformed into *E coli* BJ5183-AdEasy-1 by electroporation. After homologous recombination and identification by kanamycin selection and restriction digest, the correct plasmid, which released a fragment of 4.5 kb, was named pAd-vasostatin (fig 1C). The recombinant adenovirus was detected by looking for the presence of cytopathic effects (fig 1D). Ad-vasostatin was purified and tittered as 2.51×10^{10} pfu/ml using a standard end point dilution assay.

Expression in pancreatic cancer cells infected with Ad-vasostatin

Expression of vasostatin was detected by reverse transcription (RT)-PCR and western blot analysis. RT-PCR analysis showed that a 564 bp segment could be detected in Ad-vasostatin infected pancreatic carcinoma cells but not in parental PaTu8988/SW1990 cells (fig 2). Western blot analysis using antibodies to vasostatin was performed. As shown in fig 3, a polyclonal antibody to vasostatin detected a single protein band at 22 kDa in both PaTu8988/Ad-vasostatin+ and SW1990/Ad-vasostatin+.

Proliferation Assay of ECV304 cells and tumour cells

At the end of the treatment period of 72 hours, proliferation of ECV304 cells infected with Ad-vasostatin was significantly suppressed at MOIs of 25 and 50 compared with PBS treated

Figure 3 Detection of vasostatin protein. Lane 1, PaTu8988 + Ad-vasostatin; lane 2, SW1990 + Ad-vasostatin; lane 3, PaTu8988; lane 4, SW1990.

Figure 4 Effect of infection with Ad-vasostatin on growth of ECV304 cells (A, B) and SW1990 cells (C, D) in vitro. ECV304 cells (2×10^3 cells/well) were planted in 96 well culture plates and infected with Ad-vasostatin or Ad-lacZ at a multiplicity of infection (MOI) of 25 (A, C) and 50 (B, D), or treated with phosphate buffered saline (PBS). Proliferation of cells was quantified at 12, 24, 48, and 72 hours using the modified MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Data are presented as the mean (SD) of six wells. Proliferation of ECV304 cells infected with Ad-vasostatin was significantly suppressed at MOIs of 25 and 50 at 72 hours compared with PBS treated ECV304 cells and Ad-lacZ infected ECV304 cells ($p < 0.05$). Proliferation of SW1990 cells infected with adenovirus vectors was slightly suppressed but there were no significant differences.

ECV304 cells and Ad-lacZ infected ECV304 cells ($p < 0.05$). Proliferation of ECV304 cells infected with Ad-lacZ was slightly suppressed at MOIs of 25 and 50 compared with PBS treated cancer cells, but there was no difference between proliferation of PBS treated ECV304 cells and Ad-lacZ infected cells at any MOI (25 or 50) (fig 4). The data indicate that vasostatin induced by adenovirus vectors has a significant inhibitory effect in vitro on proliferation of ECV304 cells.

Proliferation of SW1990 cells infected with adenovirus vectors was slightly suppressed, according to the graded MOI, compared with that of PBS treated cancer cells. However,

there was no difference between proliferation of Ad-vasostatin infected cells and Ad-lacZ infected cells at any MOI (25 or 50) (fig 4); that is, vasostatin induced by adenovirus had no inhibitory effect in vitro on proliferation of SW1990 cells.

In vitro angiogenesis

Tube formation is a multistep process involving cell proliferation, adhesion, migration, differentiation, and growth. Assays of in vitro tube formation using ECV304 cells, a type of human umbilical vein endothelium cell line, confirmed the adenovirus mediated expression of biologically active

Figure 5 Antiangiogenic activity of Ad-vasostatin in tube formation assay. (A) When cultured on ECMatrix, ECV304 cells rapidly aligned and formed hollow tube-like structures. (B) Transduction with a control Ad-lacZ vector did not affect tube formation. (C, D) However, the length of tubes in cells transduced with Ad-vasostatin was significantly reduced ($p < 0.05$). PBS, phosphate buffered saline.

Figure 6 Inhibition of vascularisation in the chorioallantoic membrane (CAM) assay. The chicken CAM assay was performed as described in materials and methods. (A) CAM angiogenesis treated with phosphate buffered saline (PBS), (B) Ad-lacZ, (C) or Ad-vasostatin. (D) Number of crosspoints of the blood vessels, which represent angiogenesis. ** $p < 0.01$.

vasostatin. ECMatrix is a solid gel of basement proteins prepared from the Engelbreth Holm-Swarm (EHS) mouse tumour and consists of laminin, collagen type IV, heparan sulphate proteoglycans, entactin, and nidogen. It also contains various growth factors (transforming growth factor β , fibroblast growth factor) and proteolytic enzymes (plasminogen, tissue plasminogen activator, matrix metalloproteinases) that occur normally in EHS tumours. When cultured on ECMatrix, ECV304 cells rapidly aligned and formed hollow tube-like structures (fig 5A). Transduction with a control Ad-lacZ vector did not affect tube formation (fig 5B). However, the length of tubes in cells transduced with Ad-vasostatin was significantly reduced (fig 5C,D).

CAM assay

As new blood vessels are budded from pre-existing blood vessels, we can evaluate angiogenesis from the number of crosspoints of the vessels. The number of crosspoints of the vessels was reduced significantly after treatment with Ad-vasostatin (fig 6C, 6D) compared with Ad-lacZ (fig 6B) or PBS (fig 6A) alone ($p < 0.01$).

In vivo pancreatic cancer model

Heterotopic murine pancreatic carcinoma was successfully established in the flank of Balb/c nude mice. Administration of Ad-vasostatin to SW1990 tumours and PaTu8988 tumours in nude mice resulted in suppression of tumour growth compared with the control groups. As shown in fig 7, the volumes of SW1990 tumours and PaTu8988 tumours in the Ad-vasostatin group were significantly smaller than those in the PBS and Ad-lacZ groups after six days of treatment ($p < 0.05$) and at the end of the treatment period; differences were significant ($p < 0.01$).

Expression of vasostatin in PaTu8988/SW1990 tumours was detected by RT-PCR. RT-PCR revealed that a 564 bp segment was detected; this was similar to the data for Ad-vasostatin infected pancreatic carcinoma cells (data not shown).

Vessel staining and counting

MVD in SW1990 tumours was assessed by immunostaining with CD31 antibody. Representative images of the three groups are shown in fig 8. MVD of Ad-vasostatin treated

Figure 7 Inhibition of tumour growth on established pancreatic cancers in nude mice. Xenografted pancreatic cancers were established by injection of SW1990 cells (A) or PaTu8988 cells (B) into five week old Balb/c nude mice. Treatment consisted of injection directly into the tumours with 10^8 pfu Ad-vasostatin, Ad-lacZ, or phosphate buffered saline (PBS) every third day. Tumour sizes were measured and are presented as means.

Figure 8 Immunohistochemical staining of tumours with goat CD31 polyclonal antibody. Representative images of phosphate buffered saline (PBS) treated SW1990 tumours (A), Ad-lacZ treated SW1990 tumours (B), and Ad-vasostatin treated SW1990 tumours (C). (D) Microvessel density of Ad-vasostatin treated tumours was significantly lower (76.2 (16.8) vessels/mm²) than that of Ad-lacZ treated tumours (144.0 (16.6)) and PBS treated tumours (147.0 (22.7)) ($p < 0.05$). Data are presented as mean (SD) ($n = 6$ in each group).

tumours (76.2 (16.8) vessels/mm²) was significantly lower than that of Ad-lacZ treated tumours (144.0 (16.6)) and PBS treated tumours (147.0 (22.7)).

DISCUSSION

In the present study, we demonstrated the tumour suppressor effect of vasostatin mediated by adenovirus targeted at angiogenesis and vascular endothelial cells in human pancreatic cancer xenograft models. In general, there are two types of targets in cancer therapy. The most widely accepted target is tumour cells. A number of therapy have tumour cells as their target, such as chemotherapy, irradiation, immunotherapy, etc. After Folkman²⁶ proposed that neoplasms may remain dormant without angiogenesis and growth is critically dependent on angiogenesis if tumours are larger than 2 mm³, increasing evidence has suggested that solid tumours and their metastases are angiogenesis dependent. Angiogenesis, the formation of new microvessels generated by vascular endothelial cells, provides essential nutrition for the rapid growth of malignant cells. Antiangiogenesis therapies, which block the blood supply of a tumour, provide a new strategy for the treatment of solid tumours.²⁷⁻²⁸ Antiangiogenic therapies for cancer targeted at vascular endothelial cells have received widespread attention in recent years because of the potential effectiveness against multiple types of tumours and less drug resistance.²⁹ Pancreatic cancer is one of the most intractable malignancies, and a number of studies of antiangiogenesis therapy for pancreatic cancer have been reported.¹⁰⁻³⁰

Vasostatin is the N terminal domain of calreticulin inclusive of amino acids 1–180 and has been reported as a potent angiogenesis inhibitor used for the treatment of multiple cancers.¹⁴⁻¹⁵ In this study, in pancreatic cancer models in mice, we investigated the inhibitory effect of vasostatin mediated by adenovirus. Adenovirus can infect dividing and non-dividing cells and gain high efficiency for transgene expression,³¹ which is widely used in cancer gene therapy. In the present study, to investigate the possibility of vasostatin gene therapy for pancreatic cancer, we cloned the human vasostatin gene and constructed a replication deficient recombinant adenovirus encoding vasostatin gene with human adenovirus serotype 5 vector which is rendered

replication defective by deletion of the E1 and E3 genes. After pancreatic cancer cells SW1990/PaTu8988 were infected with Ad-vasostatin for 72 hours, vasostatin mRNA expression was clearly detected by RT-PCR and vasostatin protein expression was demonstrated by western blot assay.

Adenovirus mediated vasostatin gene suppressed pancreatic tumours in mice. In the present study, in order to observe the inhibitory effect of vasostatin on tumour growth, a pancreatic cancer model had to be established. In general, there are two types of models for pancreatic cancer³²⁻³³: one is induced with drugs such as N-methyl-N'-nitrosourea, N-nitrosobis(2-oxopropyl)amine, which is orthotopic but unstable and is not used very often; and the other is xenograft models. The subcutaneous xenograft model may have less orthotopic growth characteristics than the orthotopic xenograft model, but it is easier to investigate the growth of tumour. Therefore, we employed two different types of pancreatic cancer cell lines to establish the same subcutaneous xenograft model and make the results more repeatable and valuable. Adenovirus mediated vasostatin gene significantly inhibited the growth of SW1990 and PaTu8988 xenografted pancreatic tumours. After seven rounds of treatment, the growth of xenografted tumours treated with Ad-vasostatin was significantly inhibited compared with the control groups. The results were consistent with those of Pike and colleagues¹⁵ who reported that vasostatin significantly reduced the growth of established human colon carcinomas and Burkitt, and suppression of angiogenesis in vivo may be responsible.

To identify the target of vasostatin gene therapy in pancreatic cancer, the effects of vasostatin on both pancreatic cancer cells and vascular endothelial cells were studied. In vitro, pancreatic cancer cells were not growth inhibited by vasostatin, suggesting that vasostatin acts indirectly on pancreatic cancer cells. In contrast, proliferation of ECV304 cells infected with Ad-vasostatin was significantly suppressed at MOIs of 25 and 50 compared with PBS treated and Ad-lacZ infected cells, suggesting that vasostatin has significant inhibitory effects in vitro on vascular endothelial cells.

In the present study, we used the MVD assay, CAM assay, and tube formation assay to investigate the effects of vasostatin on angiogenesis both in vivo and in vitro.²² CD31

is a specific marker of endothelial cells and is usually stained to indicate MVD in tumours.^{34–38} Immunohistochemistry data showed that MVD of vasostatin treated tumours was significantly lower than that of Ad-lacZ and PBS treated tumours. The choriallantoic membrane assay is also a useful tool for in vivo angiogenesis analysis. In this study, vasostatin significantly reduced the number of crosspoints of the vessels compared with Ad-lacZ and PBS controls. In the tube formation assay which represents in vitro angiogenesis, the length of the tubes in cells transduced with Ad-vasostatin was significantly reduced compared with Ad-lacZ and PBS controls. Inhibition of cell proliferation by vasostatin may also contribute to inhibition of angiogenesis. All of these data demonstrate that vasostatin mediated by adenovirus has a high level of bioactivity and could significantly inhibit angiogenesis both in vivo and in vitro.

In conclusion, adenovirus mediated vasostatin gene is efficient for gene therapy in pancreatic carcinoma. Suppression of proliferation of vascular endothelial cells and angiogenesis in vitro and in vivo may account for the effects of vasostatin. Although further investigation is required to assess systemic versus regional issues, systemic adverse effects, and immunological response problems, adenovirus mediated vasostatin gene therapy may be a potent strategy to treat many malignant tumours, including pancreatic cancer, and represents a promising therapeutic option for malignancy with a poor prognosis.

ACKNOWLEDGEMENTS

The authors thank the biotechnicians working at the Shanghai Experimental Animal Centre of Chinese Academy of Sciences for expert biotechnical assistance.

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Conflict of interest: None declared.

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