Suppression of Lung Tumor Growth and Metastasis in Mice by Adeno-Associated Virus-Mediated Expression of Vasostatin

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Abstract

Purpose: Angiogenesis inhibitors have strong therapeutic potential as antitumor agents in suppressing tumor growth and metastatic progression. Vasostatin, the N-terminal domain of calreticulin, is a potent angiogenesis inhibitor. In this study, we determined the effectiveness of vasostatin delivered by recombinant pseudotype adeno-associated virus 2/5 (rAAV2/5-VAS) as a gene therapy approach for lung cancer treatment.

Experimental Design: We used rAAV2/5 to deliver vasostatin intratumorally or systemically in different mouse lung tumor models — subcutaneous, orthotopic xenograft, and spontaneous metastasis lung tumor models. The therapeutic efficacy of rAAV2/5-VAS was determined by monitoring tumor volume, survival rate, and degree of neovascularization after treatment in these models.

Results: Mice bearing subcutaneous tumor of rAAV2/5-VAS pretreated Lewis lung carcinoma cells showed >50% reduction in primary tumor volume and reduced spontaneous pulmonary metastases. The tumor-suppressive action of AAV2/5-VAS in subcutaneous human lung tumor A549 xenograft correlated with a reduced number of capillary vessels in tumors. In the orthotopic xenograft model, rAAV2/5-VAS suppressed metastasis of A549 tumors to mediastinal lymph nodes and contralateral lung. Furthermore, treatment of immunocompetent mice in the spontaneous lung metastases model with AAV2/5-VAS after primary tumor excision prolonged their median survival from 21 to 51.5 days.

Conclusion: Our results show the effectiveness of rAAV2/5-VAS as an angiogenesis inhibitor in suppressing tumor growth during different stages of tumor progression, validating the application of rAAV2/5-VAS gene therapy in treatment against lung cancer.
producing secretory proteins, allowing persistent expression of the transgene product. They have been successfully used for delivery of angiogenesis inhibitors for cancer therapy in different murine models (15–17). The most extensively studied AAV serotype 2 (AAV2) vector has a low transduction efficiency in polarized airway epithelia (18). However, cross-packaging AAV2 vector genome into AAV5 capsid, which results in pseudotyped AAV2/5 vector that share a similar safety profile as AAV2, can mediate efficient gene transfer to the lung (14, 19).

In this study, we evaluated the efficacy of vasostatin delivered by the rAAV2/5 viral vector (rAAV2/5-VAS) in subcutaneous, orthotopic xenograft, and spontaneous metastasis models of lung cancer. These mouse models serve to simulate different aspects of tumor growth, allowing assessment of vasostatin gene therapy during different stages of lung cancer development. In an ex vivo setting, we showed that rAAV2/5-mediated expression of human vasostatin from highly metastatic Lewis lung carcinoma could reduce neovascularization during tumor initiation, resulting in suppression of primary tumor growth and subsequent pulmonary metastasis. The antitumor effect of rAAV2/5-VAS was further confirmed by treating preexisting xenografts in nude mice at subcutaneous and orthotopic transplantation sites. During advanced tumor stages, long-term expression of vasostatin after intratracheal delivery was shown to prolong the survival of immunocompetent mice which have undergone surgery for primary tumor removal. This is the first report demonstrating the antimetastatic effects of vasostatin on orthotopic xenograft and metastatic tumor models. Our findings show that rAAV2/5-mediated vasostatin is an effective candidate for lung cancer gene therapy.

Materials and Methods

Cells and animals. A549 human lung adenocarcinoma cells, Lewis lung carcinoma (LLC) cells, MS1 mouse pancreatic endothelial cells, and HEK293 cells were obtained from American Type Culture Collection.

C57B6 and BALB/c nude mice were obtained from the Laboratory Animal Unit of University of Hong Kong. All animal procedures were approved by the Ethics Committee of University of Hong Kong and done according to institutional guidelines.

Preparation of rAAV stocks. Vasostatin cDNA was cloned into pAAV-2 expression vector under the regulation of cytomegalovirus enhancer, chicken ¢-actin promoter, and a woodchuck hepatitis B virus posttranscriptional regulatory element to boost expression levels (20). Recombinant AAV1/2, AAV2/2, AAV2/5, and AAV2/8 stocks were generated by a three-plasmid helper virus-free packaging method (21) and purified on a CsCl gradient. The titer of rAAV vectors was generated by a three-plasmid, helper virus-free packaging method (21). Recombinant AAV1/2, AAV2/2, AAV2/5, and AAV2/8 stocks were generated by a three-plasmid helper virus-free packaging method (21) and purified on a CsCl gradient. The titer of rAAV vectors was determined by real-time PCR analysis as described previously (22). Control rAAV-eGFP vectors were prepared using identical method.

Transgene expression in vitro. LLC cells were infected with eGFP reporter virus (MOI, 10^5) rAAV2/1, rAAV2/2, rAAV2/5, rAAV2/8 for 72 h. eGFP expression was analyzed by flow cytometry (Beckman Coulter). Infected cells were determined by real-time PCR analysis as described previously (22). Control rAAV-eGFP vectors were prepared using identical method.

Subcutaneous A549 xenograft model. A549 human lung adenocarcinoma cells (5 x 10^6) resuspended in 100 μL PBS were injected into the dorsal flank of 6-week-old male BALB/c nude mice. When tumor size reached ~ 50 mm^3, mice were randomized into three groups (n = 6 per group). AAV2/5 encoding eGFP or vasostatin in PBS or PBS were injected into tumor under anesthesia. Tumor growth was monitored regularly for up to 30 days, and volume was calculated as described previously. Tumors were harvested for further analysis.

Orthotopic A549 xenograft model. A549 cells were resuspended in cold PBS with 1 mg/mL Matrigel (BD Biosciences) and concentrated to 10^6 cells/mL. 20 μL inoculum of cells were injected into the left lung parenchyma of 6-week-old male BALB/c nude mice (n = 8 per group) as described (23). Tumor-bearing mice were treated 1 week after inoculation with 2 x 10^11 vector genomes of virus of rAAV2/5-VAS or rAAV2/5-eGFP by intratracheal administration. Animals were sacrificed 50 days after tumor cell implantation. Lung samples were harvested for further examination, and the mass of enlarged lymph nodes was quantified.

Analysis of in vivo gene expression by reverse transcription-PCR. Total RNA from mice tissues was extracted by Trizol (Invitrogen), and first-strand cDNA was synthesized with Superscript II (Invitrogen) and tested for the expression of vasostatin and CD105 with the following primer pairs: Vas-F (5’-AACCTGACGCCC- CATTGCTCCTA-TCC), WPRE-R (5’-CTTCGGGCTGGACA-TAG) and CD105-F (5’-CGTCATCACTTCCTCCCT), CD105-R (5’-CAGTCGTCGCTGGT-GTG). PCR conditions were 45 s each at 94°C, 55°C, and 72°C for 32 cycles. 18S RNA was used as internal control.

Endothelial cell proliferation and tube formation assays. MS1 mouse pancreatic endothelial cells were used for assessing the biological LLC spontaneous pulmonary metastasis model. A highly metastatic subline of LLC (hm-LLC) were generated by three rounds of serial in vivo passage and recovered from spontaneous lung metastasis after subcutaneous inoculation of LLC cells. After in vitro expansion to sufficient numbers, cells were frozen until use for in vivo implantation.

For ex vivo treatment experiments, hm-LLC cells were infected with rAAV2/5-VAS or rAAV2/5-eGFP overnight before inoculation. Seven-week-old male C57B6 mice were anesthetized and received subcutaneous injection with 100 μL cell suspension (1 x 10^6 cells) of rAAV2/5-VAS, rAAV2/5-eGFP, or PBS. Animals were sacrificed 21 days after tumor cell inoculation. The tumors were excised together with adjacent skin from the underlying tissues, and feeding tumor blood vessels along the undersurface of the surrounding dermis were quantified by counting. The tumors were snap-frozen for RNA extraction or fixed in 4% paraformaldehyde for histologic and immunohistochemical analysis. In the second set of experiment, tumor growth was monitored regularly. Tumor volume was measured with a digital caliper and calculated using the formula 0.52 x a x b^2, wherein a and b are the largest and smallest diameters, respectively. Animals were sacrificed 21 days after tumor cell implantation. Lung samples were collected and analyzed in 3% paraformaldehyde, and tumor nodules on the lung were counted under a dissecting microscope.

In intratracheal delivery experiments, 5 x 10^11 hm-LLC cells were injected s.c. into 7-week-old male C57B6 mice. Primary ectopic tumors reached a diameter of 5 mm in ~ 1 week. Animals were anesthetized, and the entire subcutaneous tumors, together with surrounding skin, were surgically excised. After wound closure, animals were randomized into three groups and intratracheally given 2 x 10^11 viral particles of rAAV2/5-VAS, rAAV2/5-eGFP, or PBS. Animals were humanely euthanized when they showed the inability to reach food or water, showed symptoms of emaciation, dehydration, or a 20% decrease in normal body weight. Four mice from each group were sacrificed 30 days after viral delivery or humanely euthanized, and lung tissues were harvested for histologic and immunohistochemical examination. The rest of the mice were sacrificed at the end of the experiment (10-week posttreatment), and their lungs were examined.

Cancer Therapy: Preclinical
activity of the secretory product mediated by rAAV2/5. Conditioned medium were harvested from LLC cells 60 h after infection with rAAV2/5-VAS or rAAV2/5-eGFP (MOI, 10^4). MS1 cells were seeded onto 96-well plates at 2 × 10^3 cells per well. After 24-h incubation, the medium was replaced by conditioned medium with the exception of control culture. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays were done at 24 h, 48 h, and 72 h, and cell viability was determined according to the formula relative cell viability = (A_{570-690} of conditioned medium–treated cells)/(A_{570-690} of control cells). Three independent experiments were done in triplicates for each treatment.

The tube-like network formation of MS1 cells was assessed on Matrigel after mixing cells with conditioned medium. MS1 cells (2 × 10^4) were seeded in triplicates into Matrigel-precoated 96-well plates for 8 h in the presence of CM/rAAV2/5-VAS or CM/rAAV2/5-eGFP. Formation of tubes was examined, and images were captured using an inverted microscope.

Histology, immunohistochemistry, and TUNEL assay. Lung and tumor samples were fixed, paraffin-embedded, and sectioned (5 μm) for histologic analysis. For immunohistochemical analysis, primary antibodies, including calreticulin (1:300, Stressgen Biotechnologies), CD34 (1:300, Santa Cruz), Ki-67 (1:300, Lab Vision), α-smooth muscle actin (α-SMA; 1:1,000, Sigma), and E-cadherin (1:500, Calbiochem) were used. Sections were subsequently incubated with horseradish peroxidase–conjugated secondary antibodies for 1 h. Staining was visualized using a 3,3′-diaminobenzidine kit (Zymed) and counterstained with hematoxylin. For double immunofluorescence of CD34 and α-SMA staining, FITC or Cy3-conjugated secondary antibodies (Sigma) were used.

TUNEL assay was done using In situ Cell Death Detection kit (Roche) according to manufacturer’s instructions. Cell nuclei were stained with propidium iodide.

Analysis of tumor microvessel density, Ki-67 proliferation index, and apoptotic index. Quantitative analysis of images was conducted using Image-Pro Plus analysis software (Media Cybernetics). Microvessel density (MVD) was assessed after CD34 staining by the method defined by Weidner and coworkers (24). Tumor sections were scanned by light microscopy at low power field (×40), and three fields with the most intense neovascularization (hotspots) were selected. Microvessel counts of hotspots were done at high power field (×400 hpf). The mean value of three hotspots was taken as the MVD, which was expressed as number of microvessels per hpf. For Ki-67 proliferation and apoptotic indices, positively stained cells and nuclei were counted on a minimum of 10 randomly selected ×400 hpf from representative tumor sections.
indices were calculated as number of (Ki-67 or TUNEL) positive cells divided by total cell count.

Statistical analysis. Student’s t test was used to compare the values or indices between different groups. Kaplan-Meier survival curves were compared using the log-rank test in GraphPad Prism.

Results

rAAV2/5 viral vector–mediated expression of biologically active vasostatin in vitro. Human vasostatin cDNA encoding the N-terminal fragment of human calreticulin (residues 1-180) was inserted into a rAAV2 expression vector cassette. To obtain optimal transgene expression for lung cancer treatment, we tested the transduction efficiency of different pseudoserotyped virion combinations in LLC cells. The vectors were packaged using the eGFP reporter gene, and transduction efficiencies of the serotypes rAAV2/2, rAAV2/1, rAAV2/5, and rAAV2/8 in LLC culture were compared. By flow cytometric analysis, rAAV2/5 showed the highest transduction rate of 63.5% (Fig. 1A). The ability of rAAV2/5 vector to mediate protein expression and secretion was tested by infecting HEK293 cells with rAAV2/5-VAS vector. Western blot analysis using anticalreticulin antibody showed the production and secretion of vasostatin into culture medium ~60 h after rAAV2/5-VAS infection (Fig. 1B). Protein expression was not detected in either cell lysates or conditioned medium from rAAV2/5-eGFP–transduced HEK293 cells.

We next examined the biological activity of secreted vasostatin mediated by rAAV2/5 viral vectors in vitro. The antiangiogenic activity of vasostatin was assessed by evaluating the viability of MS1 endothelial cells cultured in the presence of conditioned medium from rAAV2/5-VAS transduced LLC cells. There was significant inhibition of MS1 proliferation in rAAV2/5-VAS conditioned medium compared with control rAAV2/5-eGFP conditioned medium in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Fig. 1C). This inhibitory effect was specific for endothelial cells, as treatment with rAAV2/5-VAS conditioned medium on A549 lung adenocarcinoma cells did not reduce cell growth (data not shown). Using tube formation assay to test the effect of vasostatin on in vitro angiogenesis, rAAV2/5-VAS conditioned medium was shown to inhibit tube-like network formation of MS1 cells on Matrigel precoated plates, whereas tube formation was observed in MS1 cells incubated in rAAV2/5-eGFP conditioned medium (Fig. 1D). Thus, expression of rAAV2/5-encoded vasostatin was able to inhibit angiogenesis in vitro.

rAAV2/5-vasostatin expressed from hm-LLC cells suppressed subcutaneous tumor growth and metastasis. To test whether rAAV2/5-VAS can serve as an antiangiogenic factor and suppress tumor growth in vivo, we s.c. injected hm-LLC cells, which had been preincubated with rAAV2/5-VAS, rAAV2/5-eGFP, or PBS, into C57B6 mice. As an aggressive tumor model, the growth of tumor mass in control groups (PBS or rAAV2/5-eGFP–treated cells) was rapid, whereas tumor growth from rAAV2/5-VAS–treated hm-LLC cells was delayed and became palpable much later than control virus treatment. As a result, the mean tumor volume in mice with rAAV2/5-VAS–treated hm-LLC cells was significantly reduced to less than half of control tumors by 16 to 18 days after inoculation (Fig. 2A).

As hm-LLC cells can readily metastasize from the subcutaneous primary tumor site to the lung, we evaluated the ability of vasostatin-producing hm-LLC cells to modulate metastatic progression. The pulmonary metastatic burden of the treated mice was assessed by counting the number of tumor nodules on the surface of the lung at the conclusion of the experiment (day 21). Mice in control groups displayed numerous distinguishable pulmonary metastatic nodules (average of 20), whereas mice bearing rAAV2/5-VAS–treated hm-LLC cells showed fewer visible tumor nodules (average of 8). The reduction of surface nodules by >2-fold with rAAV2/5-VAS treatment (Fig. 2B) showed that vasostatin not only efficiently suppressed subcutaneous tumor growth but also reduced subsequent spontaneous lung metastasis.

Vasostatin from transduced hm-LLC cells suppressed tumor-induced neovascularization. To determine whether suppression of primary and metastatic tumor growth in mice was a direct result of the antiangiogenic activity of viral-encoded vasostatin, the formation of new blood vessels induced by tumor was assessed after subcutaneous inoculation of tumor cells pretreated with rAAV2/5-VAS or rAAV2/5-eGFP. Tumors were excised with the adjacent dermis at day 7 after tumor cell inoculation. Tumors
from animals treated with rAAV2/5-VAS–transduced hm-LLC cells seemed pale and flat compared with tumors obtained from control animals (Fig. 3A). The tumor-feeding vessels along the undersurface of the surrounding dermis were significantly reduced in tumors from rAAV2/5-VAS–pretreated hm-LLC cells (Fig. 3B), indicating a reduction in angiogenic potential. The PBS or rAAV2/5-eGFP pretreated hm-LLC cells formed tumors with multiple large feeding blood vessels (7.8 ± 0.9 and 8 ± 0.8 vessels, respectively), whereas tumors from rAAV2/5-VAS pretreated hm-LLC cells displayed significantly fewer (3.2 ± 0.8 vessels) and narrower blood vessels. Histologic analysis showed multiple lumen-like formations containing RBC present in the tumors of control groups (Fig. 3D, a and b), demonstrating mature blood vessel formation. Tumor vascularity was observed in almost the entire tumor with peripheral vessels penetrating throughout control tumors. However, in rAAV2/5-VAS–pretreated tumors, vascularity was observed only at the tumor periphery. Expression of CD105, an activated endothelial cell marker, was shown to be significantly reduced in rAAV2/5-VAS pretreated tumors compared with control tumors (Fig. 3C). Fewer host endothelial cells migrated into rAAV2/5-VAS–pretreated tumors as indicated by the relative level of CD105 transcripts in tumors at this stage, thus confirming rAAV2/5-VAS suppression of tumor growth through inhibition of angiogenesis. In addition, we examined the expression of E-cadherin, a cell adhesion molecule which negatively correlates with the invasive potential of tumor cells (25). rAAV2/5-VAS–pretreated tumors showed strong positive signals (Fig. 3D, f), which may be associated with lower metastatic potential, whereas weak signals were observed in control tumors (Fig. 3D, d and e), suggesting an inherent ability of these cells to metastasize.

![Diagram](image_url)
Intratumoral injection of rAAV2/5-VAS inhibited subcutaneous lung cancer xenograft growth. We further assessed the therapeutic potential of rAAV2/5-VAS on a subcutaneous lung cancer xenograft model by intratumoral delivery of rAAV2/5-VAS, rAAV2/5-eGFP, or PBS. The subcutaneous model was established by implantation of A549 cells (5 × 10⁶) subcutaneous into the dorsal flank of nude mice, and tumor growth was monitored for up to 1 month after treatment. Vasostatin gene expression was confirmed by reverse transcription–PCR in tumors receiving rAAV2/5-VAS (Fig. 4B). Treatment with 2 × 10¹¹ vector genomes of rAAV2/5-VAS retarded subcutaneous xenograft progression as mean tumor volumes were significantly reduced in mice receiving rAAV2/5-VAS compared with those receiving PBS or rAAV2/5-eGFP (Fig. 4A). Histologic analysis of tumor samples revealed massive apoptosis in large extended areas in tumor sections obtained from rAAV2/5-VAS group compared with those from control groups (rAAV2/5-eGFP or PBS; data not shown). The proliferation index (proportion of Ki67-positive cells) of tumors treated with rAAV2/5-VAS was reduced compared with control group (mean index of 50.2 ± 10.7% versus 37.5 ± 4.3%; Fig. 4C, a). In contrast, ~2-fold increase in apoptosis (proportion of TUNEL-positive cells) was detected (41.7 ± 7.9% versus 18.4 ± 2.0%; Fig. 4C, b) in rAAV2/5-VAS–treated tumors compared with control mice (Fig. 4C, b). To determine whether the poor growth of rAAV2/5-VAS–treated subcutaneous xenografts was...
due to poor blood vessel supply, we stained the tumor sections with antibodies against CD34 and $\alpha$-SMA and measured MVD. A significant decrease in MVD (61.2 ± 8.3 versus 170.8 ± 26.9 vessels per hpf) in mice treated with rAAV2/5-VAS was observed compared with tumors in control mice (Fig. 4C, c). In addition, a decrease in SMA-positive mural cells associated with endothelial cells was detected in rAAV2/5-VAS–treated tumors (Fig. 4C, c). These results suggested that rAAV2/5-VAS treatment was effective in reducing the number of capillary vessels, as well as inducing apoptosis, resulting in the repression of vascular tumor mass expansion.

**Suppression of orthotopic lung tumor growth and spread after intratracheal administration of rAAV2/5-VAS.** To simulate the growth and spread of human lung carcinoma, we used an orthotopic implantation model. In this model, primary tumor derived from A549 cells first developed at the inoculated site, which would then invade the visceral pleura and mediastinal lymph nodes and metastasize to the contralateral lung. To evaluate the therapeutic effects of vasostatin, viral vectors were intratracheally given 1 week after tumor cell inoculation into the left lung. When the experimental animals were examined at 50 days after initial cell inoculation, orthotopic A549 xenograft was found to develop in all grafted mice at the inoculated site. Mice receiving intratracheal $2 \times 10^{11}$ vector genomes of rAAV2/5-VAS treatment produced substantially fewer tumors in the lungs and mediastinal lymph nodes when compared with controls at 50th day of postinoculation (Fig. 5A, a and b). Although rAAV2/5-VAS–treated mice showed similar local tumor volume as control mice (Fig. 5A, c), they exhibited over 50% decrease in mediastinal lymph node weight (Fig. 5A, d). Gross macroscopic examination of rAAV2/5-VAS–treated mice showed fewer nodules in their contralateral lungs than control mice, suggesting a decrease in severity of metastases in treated mice. The intratracheal delivery of rAAV2/5-VAS did not produce major changes in cell proliferation of primary orthotopic tumors (Fig. 5B). In contrast, primary tumors in
mice receiving rAAV2/5-VAS had a 1.6-fold fewer number of vessels per surface unit than those of control group (Fig. 5C). These results showed that rAAV2/5-VAS treatment via tracheal administration could retard angiogenesis in the primary tumor, delay the spread of tumor cells, and achieve an antimetastatic effect.

Increased survival rate of mice and diminished expansion of lung metastases by intratracheal delivery of rAAV2/5-VAS. To evaluate the antimetastasis effect and survival enhancement of long-term rAAV2/5-VAS expression in vivo, we used a spontaneous pulmonary metastasis model wherein hm-LLC cells were inoculated s.c. to initiate primary tumor and subsequent pulmonary metastases. When the primary subcutaneous tumor reached ~0.5 cm in the longest diameter at 1 week postinoculation, the primary tumor was surgically removed, a procedure that resulted in accelerated metastasis development in the lung. When mice were sacrificed 30 days after rAAV2/5-VAS or rAAV2/5-eGFP treatment or were humanely euthanized during this period, the lungs were harvested for examination of metastatic incidence and severity. All the mice in control groups exhibited massive hemothorax and developed metastatic nodules in their lungs. In control animals, large, vascularized tumors with hemorrhages were found in majority of the lungs (Fig. 6A, a). In contrast, the lung surface of one in four mice receiving rAAV2/5-VAS was tumor-free, whereas the others had several small metastasis nodules (Fig. 6A, a). Moreover, rAAV2/5-VAS treatment reduced the mean weight of lungs with metastatic tumors by >40% (Fig. 6A, b). Histologic analysis...
showed that large tumors from the control group were composed of more heterogeneous cell populations and hemorrhage in the central lung section (Fig. 6B, a and b). On the other hand, minimal vascularity was observed in tumor nodules in lungs which received rAAV2/5-VAS (Fig. 6B, c and d). Upon termination of the experiment at 10 weeks after treatment, significant prolonged survival was observed in rAAV2/5-VAS–treated tumor-bearing mice (Fig. 6D).

To show long-term expression of vasostatin by rAAV2/5 vectors, we did immunohistochemistry analysis on lung sections. Vasostatin expression was detected in the airway epithelium of lungs receiving rAAV2/5-VAS for 10 weeks, but not in lungs receiving rAAV2/5-eGFP (Fig. 6C). Long-term survivors did not reveal any changes in overall health except that dormant micrometastases of hm-LLC cells beside the large vessel in some lungs were observed (Fig. 6B, d). No abnormalities were noted in gross and histologic examination of lung, liver, intestine, spleen, kidneys, and heart from rAAV2/5-VAS–treated mice. In an independent set of experiment, when we investigated animals 30 days after receiving intratracheal delivery of rAAV2/5-mediated angiogenesis inhibitors, we had confirmed, by reverse transcription–PCR, the absence of transgene expression in any of these organs except the lungs (data not shown). As shown in Fig. 6D, the median survival time was 21 days in control groups and was significantly improved in rAAV2/5-VAS–treated mice, with a median survival of 51.5 days. These results strongly support the finding that lung-targeted administration of rAAV2/5-VAS leads to long-term expression of vasostatin and is effective and safe for lung cancer treatment.

Discussion

We show here, for the first time, that rAAV2/5-mediated delivery of the angiogenesis inhibitor vasostatin is an effective approach for lung cancer treatment. Vasostatin inhibited the growth of human lung adenocarcinoma cells in a subcutaneous tumor model and reduced spontaneous metastasis in nude mice bearing orthotopic xenografts. In addition, vasostatin delayed metastatic spread in immunocompetent mice that had undergone primary tumor resection after a single intratracheal administration of rAAV2/5-VAS. When combined with surgery, rAAV2/5-VAS gene therapy could potentially offer long-term survival benefit.

Success in treatment targeting angiogenesis is highly dependent on the delivery method used and the ability to sustain persistent expression of the therapeutic molecule in vivo (26). In this study, we evaluated the transduction efficiency of different combinations of vector on mouse LLC cells. We showed that the highest transduction efficiency was achieved with rAAV2/5, which is in line with our previous comparison of type 5 serotype on human A549 lung adenocarcinoma cells (27). Here, we determined that intratumoral and intratracheal delivery of rAAV2/5 vector resulted in the synthesis of bioactive vasostatin from transduced tumor cells and mouse airway epithelium in vivo. rAAV-mediated pulmonary transduction for systemic administration of secretory proteins was previously reported to result in functional protein secretion and persistence in the bloodstream for over 150 days after a single dose (19). We observed vasostatin expression in the lung for at least 10 weeks as evaluated upon termination of experiment. Despite previous reports wherein reduced subcutaneous tumor growth was achieved only through repeated administration of vasostatin cDNA or adenoviral vectors (7, 8), we observed tumor suppression after a single dose of i.t. injected rAAV2/5-VAS. Due to its efficiency in lung cell transduction, rAAV2/5 can be delivered via noninvasive routes and is therefore an attractive approach for future application in lung cancer therapy.

Preclinical studies of antiangiogenesis inhibitors suggest that treatment at early stages of tumor progression is more effective than intervention at later stages due to the complexity of cellular interactions in malignant tumors (16, 28). This stage-dependent effect was also observed in our study of vasostatin treatment in the different tumor models. We observed a delay in the initiation of angiogenesis produced by rAAV2/5-VAS–transduced hm-LLC cells in vivo. The down-regulation of tumor neovascularization by rAAV2/5-mediated vasostatin resulted in reduced spontaneous metastatic incidence and expansion. This was reflected in higher levels of E-cadherin expression in rAAV2/5-VAS–treated tumors, suggesting a suppression of invasive ability. As rAAV vectors are known to persist in an episomal state or concatameric form with low frequency of integration, the proportion of rAAV2/5-VAS–transduced tumor cells would be diluted in an expanding population of tumor cells, which would explain the resulting pulmonary metastasis, though much reduced, observed in mice bearing rAAV2/5-VAS–treated hm-LLC tumors. We also observed a reduction of CD105-positive endothelial cells in rAAV2/5-VAS–treated tumors. Vasostatin-mediated suppression of endothelial cell proliferation in tumor may result in reduced recruitment of mural cells, which is normally facilitated by endothelial cell secreted factors (29) and is required to stabilize nascent vessels (30). In our model, vasostatin gene delivery to preestablished subcutaneous A549 xenograft in nude mice led to a significant shrinkage of the tumor volume associated with lower MVD and a low degree of vessel maturation in tumors, indicating vasostatin treatment to be an effective strategy in tumor growth inhibition and metastasis.

Tumor invasion and metastasis are the major causes of treatment failure and death in lung cancer patients. Lymph node invasion and spread into contralateral lung through lymphatic routes presented in this model represent common clinical features of lung cancer progression (31, 32). The orthotopic A549 transplantation nude mice model used in this study, wherein tumor cells are directly injected into normal lung parenchyma resulting in solitary tumor in situ and metastases in lung and/or mediastinal lymph nodes, provides a stringent test of vasostatin gene therapy in a pathologic environment. Apart from reproducing human tumor metastasis pattern, the A549 orthotopic xenograft have been shown to be chemoresistant, a major problem in non–small cell lung carcinoma chemotherapy (33). Our results showed that a single intratracheal administration of rAAV2/5-VAS achieved systemic protein delivery, resulting in an obvious reduction of metastatic nodules. Together with the data on rAAV2/5-VAS–treated hm-LLC cells, our results suggest that vasostatin acts during the onset of metastasis to inhibit the angiogenic switch, resulting in the delay of tumor progression and metastasis. Primary tumor volume, however, was not reduced in the A549 orthotopic xenograft model. Similar results had been shown using endothelin gene therapy treatment on an orthotopic breast cancer.
model, wherein endostatin secreted from muscle retarded metastasis but not primary tumor growth (34). Angiostatin, which is induced in mice bearing LLC primary tumor, also has inhibitory effects on metastasis (35). Combined treatment with angiostatin and endostatin was shown to be more effective in the treatment of brain metastases than with either endostatin or angiostatin alone, demonstrating synergistic effects in combination therapy (17). Because the spectrum of proangiogenic factors differs at different tumor stages (36), combined use of different angiogenesis inhibitors may overcome the limitations associated with single molecule treatment.

Angiogenesis provides a principal route for tumor cells to exit the primary site to a distant site (37). In instances wherein cancer has spread into the bloodstream, surgical removal of visible tumors is often not beneficial. Surgical excision of lung cancer raises the risk of relapse from micrometastasis due to insufficient activity of endogenous angiogenesis inhibitors (38). For instance, an increase in systemic vascular endothelial growth factor levels found after pulmonary surgery and local radiation therapy results in the acceleration of the micro-metastases growth in lung cancer patients or animal models (39, 40). Administration of angiostatin in combination with radiation therapy was shown to reduce proliferation of lung metastases from LLC primary tumors (41, 42). These endogenous angiogenesis inhibitors, including vasostatin, have been shown not to perturb wound healing when maintained in the bloodstream at sustained levels (9, 43, 44). Our intratracheal administration of rAAV2/5-VAS effectively prevented the occurrence of spontaneous metastases and micrometastasis growth after surgical removal of the primary tumor, prolonging the survival of treated animals. This may be due to long-term sustained expression of angiogenic inhibitors, which could suppress the growth of micrometastases (45). In lung-directed viral administration, elevated transgene expression could be observed in the bronchial alveolar lavages and bloodstream (46). Vasostatin in both the airway lumen and bloodstream may establish an angiogenic defense to tumor growth, especially in the lung, which is a common site of primary tumor and metastases. Our results show that rAAV2/5-mediated vasostatin was effective in inhibiting the expansion of early colonies via intratracheal administration and can offer survival benefit in mice after primary tumor resection. The results from this preclinical therapy has contributed to defining a practical and effective approach for using rAAV2/5-VAS in combination with surgery in future clinical applications for human lung cancer therapy.

In summary, we have provided positive data for the safety, feasibility, and efficacy of using tumor-directed and lung-directed rAAV2/5 gene transfer of vasostatin as a potential antiangiogenic strategy against lung cancer. The effectiveness of vasostatin in disrupting lung tumor growth and metastasis during various phases of tumor development provides a promising basis toward further studies and clinical application of rAAV2/5-VAS for lung cancer therapy.
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