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Suppression of Renal Cell Carcinoma Growth and Metastasis with Sustained Antiangiogenic Gene Therapy

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Abstract

Renal cell carcinoma (RCC) is the third most common urologic neoplasm. This aggressive malignancy has proven refractory to conventional treatment options. Antiangiogenic agents have shown early success in treating metastatic disease. The highly vascular nature of RCC appears particularly susceptible to this approach. This study investigates the potential of sustained expression of an endostatin-angiostatin fusion protein in an early-stage model of RCC to inhibit tumor growth and metastasis. Subcutaneous RCC-29 tumors were induced in athymic nude mice. Once tumors reached volumes of 10 and 25 mm³, subjects received intratumoral injections of a nonreplicating adenoviral vector every 20 days until the conclusion of the trial. The mice were randomly assigned to three treatment groups: saline control, viral Ad-GFP control, and Ad-EndoAngio. Tumor volumes were measured twice weekly for 80 days. During days 40-50 of the trial, subjects underwent dual-photon optical imaging of the tumor vasculature to ascertain angiogenic changes. All animals underwent postmortem histopathological analysis to assess for metastatic disease in the kidney, lung, liver, brain, and spleen. Results indicate that tumors treated with Ad-EndoAngio displayed 97% growth reduction compared with controls ($p < 0.001$). Further, *in vivo* tumor vascular imaging illustrated a reduction in blood vessel number and lumen diameter size. Kaplan-Meier analysis suggested dramatic survival advantage with Ad-EndoAngio treatment. Importantly, histopathological examination demonstrated marked lung and liver metastasis suppression in the treatment arms. These results suggest that sustained EndoAngio gene therapy has effective antiangiogenic action against human RCC tumors and possesses potential as a novel treatment for metastatic renal cell carcinoma.

INTRODUCTION

RENAL CELL CARCINOMA (RCC) is the third most common urologic neoplasm, accounting for 180,000 diagnoses in the sixth and seventh decades of life (Jemal *et al.*, 2007). Further, its age-adjusted incidence has been increasing annually for the past 30 years (Chow *et al.*, 1999). For those with metastases, 1- and 5-year survival rates are a dismal 50 and 10%, respectively (Motzer *et al.*, 2004). To date, radiochemotherapy has largely proven

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AUTHOR DISCLOSURE STATEMENT

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ineffective. Immunotherapy with interleukin (IL)-2 and interferon- α has shown modest results, with average overall response ranging between 10 and 15% and only a 2- to 3-month survival prolongation (Muss, 1987). These limitations have prompted investigation into new techniques for metastatic disease treatment.

Antiangiogenic therapy holds exceptional promise as an inhibitor of tumor neovasculature and potential agent for metastatic RCC. Growing evidence confirms that solid tumor growth is dependent on the formation of new blood vessels (Holash *et al.*, 1999). The manipulation and blockade of the proangiogenic cascade dominates the more recent RCC treatment developments. Current therapeutic options include antibodies that block vascular endothelial growth factor (VEGF) such as bevacizumab, small-molecule inhibitors of tyrosine kinase receptors specific to VEGF including sunitinib and sorafenib, and the mammalian target of rapamycin inhibitor temsirolimus, which downregulates hypoxia-driven factors (Patel *et al.*, 2006).

A number of endogenous molecules have antiangiogenic properties as well. Two factors in particular have previously demonstrated tumor growth inhibition and angiostatic effects. Endostatin, a domain of type XVIII collagen, disrupts microfilament integrity, leading to endothelial cell motility inhibition, apoptosis induction, and tumor growth suppression. Further, it blocks VEGF-induced tyrosine phosphorylation and downstream kinase signaling (O'Reilly *et al.*, 1997). This 20-kDa inhibitory protein is reported to be a potent inhibitor of primary tumor angiogenesis and micrometastasis development (Sauter *et al.*, 2000). Angiostatin, another naturally occurring inhibitor of angiogenesis, was discovered to block *in vivo* tumor growth by restricting the formation of new tumor blood vessels (O'Reilly *et al.*, 1994). The 38-kDa fragment of plasminogen causes direct apoptosis of endothelial cells and induces E-selectin upregulation, thus inhibiting cell migration (Wahl *et al.*, 2005).

Studies using adenoviral vectors to express angiostatin and endostatin have produced significant antitumor effects in animal models and highlight the potential of antiangiogenic gene therapy for cancer treatment and metastasis suppression (Feldman *et al.*, 2000; Kuo *et al.*, 2001; Li *et al.*, 2006; Frau *et al.*, 2007). We have developed an adenovirus encoding a fusion protein between endostatin and angiostatin, Ad-EndoAngio, under the control of a universal promoter to produce a multifocal antiangiogenic factor. Previously, administration of this tailored vector produced marked antitumor effects on human renal cell carcinoma mouse xenografts (Mellon *et al.*, 2008). Importantly, antiangiogenic therapy inherently requires the sustained expression of suppressive factors for durable therapeutic effects. We hypothesized that prolonged delivery of Ad-EndoAngio to the tumor environment would provide sufficient production of inhibitory protein at the beginning stages of RCC tumor growth and subsequently alter its metastatic potential. The results of this study indicate that sustained expression of EndoAngio in early-stage disease delays disease progression, increases survival, and effectively suppresses metastases.

MATERIALS AND METHODS

Cell lines

RCC-29 cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin (Invitrogen, Grand Island, NY). Cells were maintained in culture at 37°C and 5% CO₂.

Adenoviral vectors

The adenoviruses Ad-EndoAngio and Ad-GFP used in this study were previously produced and described (Raikwar *et al.*, 2005). Ad-EndoAngio, a replication-defective adenovirus,

expresses human endostatin-angiostatin chimeric protein. This virus uses the composite promoter elongation factor (EF)-1 α , consisting of EF-1 α fused to the 5' untranslated region (UTR) of human T cell lymphotropic virus (HTLV). Ad-GFP, the control vector, is an adenovirus expressing green fluorescent protein (GFP) driven by the universal cytomegalovirus (CMV) promoter.

In vivo RCC-29 tumor model

All animal studies were performed with approval of the Indiana University Institutional Review Board. Subcutaneous tumors were induced by injecting 2×10^6 RCC-29 cells into the bilateral flanks of athymic nude mice. Tumors were measured biweekly with calipers. Volumes were calculated according to the following formula: volume = $0.52 \times \text{width}^2 \times \text{length}$. Mice were divided into two groups. Once tumors reached volumes of 10 and 25 mm³, respectively, subjects received intratumoral injections, into the right flank only, of an adenoviral vector every 20 days for a total of three injections. Three treatment groups were randomly assigned: phosphate-buffered saline (PBS) controls, Ad-GFP viral control, and Ad-EndoAngio. Besides the PBS-treated controls, each mouse received a total of 2×10^9 infective units (IFU) of adenovirus with each injection.

Serum EndoAngio assay

The serum presence of EndoAngio was confirmed and quantified by sandwich enzyme-linked immunosorbent assay (ELISA) directed against the endostatin portion of the chimeric protein. Blood samples were collected from six mice in each treatment group at two different time points during the trial and allowed to clot for 1 hr at room temperature (4°C). Samples were serially centrifuged for 15 min at $2000 \times g$. The serum was removed and further centrifuged for 15 min at $5000 \times g$ through Centricon 50-kDa filters (Millipore, Bedford, MA). Samples were stored at -80°C until assayed. The serum level was determined with a Quantikine human endostatin kit (R&D Systems, Minneapolis, MN). Briefly, a 96-well plate precoated with mouse monoclonal antibody against human endostatin was used for this assay. Serum samples were diluted 1:20 with PBS. One hundred microliters of assay diluent RD1W was added to each well. Fifty microliters of standard, control, or treatment sample was added to each well and incubated for 2 hr on an automated shaker at 4°C. The plate was subsequently washed four times with PBS containing 0.5% Tween 20. Antibody was added and the plate was again incubated for 2 hr on an automated shaker at 4°C. Contents were removed and the plate was again washed four times with PBS containing 0.5% Tween 20. Chromogenic substrate was added for color development. Absorbance was measured at 450 nm with an ELISA plate reader. The sensitivity of the assay was 0.023 ng/ml of endostatin.

In vivo tumor imaging

Tumor vascular imaging was performed by dual-photon microscopy (MRC1024 laser confocal/multiphoton microscope; Bio-Rad, Hercules, CA). Mice were injected intraperitoneally with 100 μ l (5 mg/ml) of rhodamine-conjugated bovine serum albumin (BSA) (Invitrogen Molecular Probes, Eugene, OR) to visualize tumor vasculature. Before imaging, the mice were anesthetized with ketamine-xylocaine (77 and 4.5 mg/ml, respectively) via intraperitoneal injection at 1 ml/kg. Tumor exposure was obtained via a skin-flap window. The exposed tumor was covered with surgical lubricant and imaged. Mice were maintained during imaging at 37°C with a heating pad. A $\times 60$ water immersion lens was used. Images were collected and processed with the Metamorph (Universal Imaging, Downingtown, PA) and ImageJ (version 1.37; National Institutes of Health, Bethesda, MA) programs.

General toxicity observation

All animals in the trial were observed biweekly regarding body appearance, weight, behavior, sensory organs, food tolerance, and general motor skills. No evidence of skin lesions and gross eye abnormalities was noted.

Tissue analysis

Subject animals were killed according to Institutional Animal Care and Use Committee-approved protocols when they showed signs of distress or tumor volumes measured greater than 1000 mm³. The date of euthanasia was recorded as the end day of survival and autopsy was subsequently performed. All animals were examined for gross organ abnormalities. In particular, normal vasculature was observed in the kidneys, eyes, and skin. The lung, heart, liver, adrenal, kidneys, primary tumor, brain, and spleen were excised and evaluated for each animal. No evidence of normal angiogenesis interruption was noted in these organs. All tissues were formalin fixed and embedded in paraffin. Tissue blocks were then sliced with a microtome into 5- μ m sections and stained with hematoxylin and eosin (H&E). The sections were examined at $\times 50$ magnification with an Olympus BX41TF microscope (Olympus America, Center Valley, PA) for metastasis detection. Relevant fields were imaged with a color Olympus digital camera (Olympus America).

Statistical analysis

All statistical determination was performed with GraphPad Prism, version 4.0 (GraphPad, San Diego, CA). Results were analyzed by two-tailed Student *t* test to assess statistical significance. The χ^2 test was used to compare categorical differences. A *p* value less than 0.05 was considered statistically significant.

RESULTS

In vivo tumor inhibition

One aim of this study was to produce localized RCC tumor growth suppression by sustained antiangiogenic gene therapy. The RCC-29 line was selected for its aggressive nature and high metastatic potential (Ebert *et al.*, 1990). We injected mice bilaterally with RCC-29 cells and subsequently unilaterally administered adenoviral vectors intratumorally when lesions reached 10 and 25 mm³, respectively. Repeated injections were performed every 20 days until the end of the trial. A total of three injections was administered to each subject. Mice were observed biweekly and tumors were measured bilaterally. Figure 1 represents the treated flank data. For the 10-mm³ trial arm, PBS controls had tumor volumes of 257.39 ± 29.61 mm³ by day 30 of the trial. Likewise, the negative viral control group Ad-GFP had a volume of 229.53 ± 37.08 mm³, which was within standard error of the PBS controls. In contrast, tumors treated with Ad-EndoAngio had average volumes of 10.32 ± 1.14 mm³ on day 30, indicating statistically significant 96.0% tumor growth inhibition ($p < 0.001$) as compared with PBS controls. This inhibition of local tumor growth increased to 98.3% ($p < 0.001$) by day 50. The 25-mm³ tumor arm also displayed remarkable growth restriction. The saline and GFP control arms did not demonstrate significant growth differences by day 30 of the trial. The Ad-EndoAngio treatment group demonstrated an 85.2% reduction ($p < 0.001$), with the average volume being 31.00 ± 3.43 mm³ by day 30. This inhibition increased to 93.6% ($p < 0.001$) by day 50 of treatment, as compared with saline controls.

Uninjected tumors on the contralateral flank also displayed striking results (Fig. 2). Compared with PBS controls (208.92 ± 34.32 mm³) the Ad-GFP viral control group was not significantly different, with an average tumor volume at 30 days of 198.42 ± 31.65 mm³ for the 10-mm³ arm. Likewise, the 25-mm³ saline and viral controls were not significantly different, with

average tumor volumes on day 30 of 254.87 ± 39.22 and 194.20 ± 20.02 mm³, respectively. The mice treated contralaterally with Ad-EndoAngio again showed dramatic tumor inhibition. By day 30, the 10-mm³ treatment arm displayed an average volume of 10.21 ± 1.28 mm³, a 95.2% reduction ($p < 0.001$) compared with controls. The inhibition increased to 96.7% by day 50. Similarly, the Ad-EndoAngio group of the 25-mm³ arm demonstrated 89.0% inhibition with an average volume of 28.19 ± 3.91 mm³ ($p < 0.001$) on day 30. This also increased to 92.3% inhibition by day 50 of the trial.

Serum EndoAngio confirmation

To evaluate the systemic expression of our angiogenesis inhibitors, serum samples were collected from mice in each treatment group at two time points during the trial: on about day 20 (10-mm³ treatment group) and day 30 (25-mm³ treatment group), when a significant local tumor effect of therapy was observed, and on approximately day 45, when a significant survival advantage was witnessed. These sampling dates roughly coincided with 10 days after the second and third viral injections, respectively. The presence of EndoAngio was monitored by ELISA as described in Materials and Methods. The mean serum EndoAngio concentration in the 10-mm³ treatment group was 5.91 ± 2.89 ng/ml on day 20 and 6.17 ± 1.44 ng/ml by day 40 (Fig. 3A). Similarly, EndoAngio at 6.01 ± 1.53 ng/ml was detected in the 25-mm³ treatment group on day 30 and the serum level remained sustained by day 45 at 7.68 ± 1.19 ng/ml (Fig. 3B). In contrast, no EndoAngio was detected in the sera of mice treated with saline or Ad-GFP intratumoral injections in either tumor volume arm. Although the sera went through multiple filtrations and the concentration values may not be true representations, this assay does confirm that EndoAngio was produced systemically and remained stable in serum past 40 days.

Tumor vasculature imaging

Previous assessment of antiangiogenic effects on tumor specimens was largely performed postmortem. An alternative technique, dual-photon fluorescent microscopy, provides distinct advantages over previous histopathological analyses. Using this real-time imaging modality, dynamic blood flow, vessel functionality, vascular permeability, and vessel diameter can be assessed. Importantly, dual-photon imaging allows for monitoring of antiangiogenic therapy progress while the animal is alive. This approach uses a fluorescent dye, rhodamine-conjugated BSA, to provide vascular contrast. Six mice from each treatment group were analyzed by this technique. Injected flank images (Fig. 4A) were compared on the basis of vessel characteristics. Tumors in the PBS and Ad-GFP control groups all displayed mature vasculature. Vessels demonstrated laminar, high-velocity flow. In addition, the lumen diameter was normal in caliber. In comparison, those mice treated with Ad-EndoAngio had significantly smaller diameter vessels, slower blood flow, and pronounced tortuosity. Using the ImageJ program (National Institutes of Health), each vessel diameter was measured and average diameters were calculated. The results are depicted in Fig. 4B. The mean vessel diameter for the 10-mm³ saline and GFP control groups was 53.40 ± 9.53 and 68.08 ± 9.85 mm³, respectively. The mean diameter for the 10-mm³ Ad-EndoAngio treatment arm was 12.13 ± 1.74 mm³, significantly different from the controls ($p < 0.001$). Further, the 25-mm³ arm displayed similar results. The mean diameters of the saline control, Ad-GFP control, and Ad-EndoAngio groups were 56.73 ± 13.18 , 54.71 ± 8.75 , and 12.76 ± 1.87 mm³, respectively. These results confirm that Ad-EndoAngio treatment dramatically alters tumor vasculature function and size, leading to subsequent tumor growth inhibition.

Metastasis suppression and survival prolongation

Results of long-term survival are depicted in Fig. 5. After Ad-EndoAngio administration every 20 days, significant survival advantage was observed. Of the mice in the 10-mm³ treatment arm, 100% were alive after 60 days ($p < 0.001$). In striking contrast, 100% of the PBS and GFP

controls were dead by day 60. Similarly, 100% of the Ad-EndoAngio-treated mice in the 25-mm³ arm were alive by day 60 and all control animals were dead ($p < 0.001$). The survival increase after Ad-EndoAngio treatment also correlated with the absence of solid organ metastasis. In the 10- and 25-mm³ arms, control mice treated with PBS or Ad-GFP had visible RCC metastases in their lungs (Fig. 6A and B) whereas no lung metastases were found in Ad-EndoAngio-treated mice ($p = 0.0002$ and 0.0007 , respectively). Similar results were observed with liver metastases (Fig. 7). With both treatment arms, those mice administered Ad-EndoAngio displayed no liver metastases. In contrast, PBS and saline controls had significant numbers of metastatic RCC lesions ($p = 0.0002$ and 0.0004 , respectively).

DISCUSSION

Angiogenesis is tightly regulated by a wide variety of positive and negative signals, which provides opportunities to disturb the net balance between proangiogenic and antiangiogenic factors, thus interrupting the angiogenic process (Folkman *et al.*, 1989; Hanahan and Folkman, 1996). Unique to antiangiogenic therapy, endothelial cells rather than cancer cells are targeted. The resulting loss of tumor vasculature limits the nutrient supply to the tumor and inhibits growth or induces apoptosis in surrounding cancer cells. Numerous antiangiogenic strategies are currently being used for the treatment of advanced renal cell carcinoma. Therapeutic options include the delivery of antibodies that block VEGF, such as bevacizumab; small-molecule inhibitors of tyrosine kinase receptors specific to VEGF, including sunitinib and sorafenib; and mammalian target of rapamycin inhibitors, such as temsirolimus, that downregulate hypoxia-driven factors, among others (Patel *et al.*, 2006). Sorafenib and sunitinib are now approved by the U.S. Food and Drug Administration for use in the treatment of advanced RCC. Early results are promising, although their exact place in the treatment spectrum is yet to be established. Importantly, their use illustrates the vital role that antiangiogenic therapy plays in kidney cancer therapy.

Gene therapy presents an attractive alternative to the administration of antiangiogenic agents. This approach allows the accumulation of high concentrations of the agent locally for better treatment effect and less nonspecific systemic toxicity. The highly vascular renal cell carcinoma has long been postulated to be a potential target of antiangiogenic gene therapy. Our approach to block the angiogenic cascade is to use adenoviruses to express angiostatic proteins. We observed a dramatic effect by targeting the tumor vasculature at multiple levels, using two antiangiogenic proteins: endostatin and angiostatin. Endostatin is the NC1 domain of the α_1 chain of type XVIII collagen. Endostatin inhibits endothelial cell proliferation, migration/invasion, and tube formation. The inhibitory action of endostatin has been proposed to involve binding to receptor $\alpha_5\beta_1$ (Sudhakar *et al.*, 2003), possibly with low-affinity binding to glypican-1 and glypican-4 or through high-affinity binding to an unidentified molecule on endothelial cells (Karumanchi *et al.*, 2001). Angiostatin is an internal fragment of plasminogen. It induces endothelial cell apoptosis (Claesson-Welsh *et al.*, 1998) and blocks the migration of endothelial cells (Ji *et al.*, 1998). These activities may be mediated by perturbing the $\alpha_v\beta_3$ signaling pathway (Tarui *et al.*, 2002). A combination of endostatin and angiostatin has been reported to have an improved therapeutic effect in B16F10 melanoma, L1210 leukemic cells (Scappaticci *et al.*, 2001), and ovarian cancer (Ponnazhagan *et al.*, 2004).

To determine the therapeutic efficacy of extended antiangiogenic gene therapy, we explored the repeated administration of Ad-EndoAngio to treat established renal cell carcinoma (RCC) tumors. A human RCC-29 tumor subcutaneous model was established in nude mice. Mice were randomized into two treatment arms when tumor volume reached either 10 or 25 mm³, respectively (approximately 2 weeks after cell inoculation) and treated with 2×10^9 IFU of either Ad-EndoAngio ($n = 9$), Ad-GFP ($n = 9$), or saline control ($n = 9$) via intratumoral injections. Tumor volume was monitored twice weekly. Viral injections were repeated every

20 days for a total of three injections. Ad-EndoAngio produced significant tumor growth inhibition after 30 days of treatment. Ad-EndoAngio produced 97% growth inhibition ($p < 0.001$) of 10-mm³ RCC tumors and 92% inhibition ($p < 0.001$) for 25-mm³ tumors as compared with saline and viral controls.

Another important finding from this trial of sustained antiangiogenesis is the inhibition of both RCC lung and liver metastases in our mouse xenograft model. When compared with viral and saline-treated controls, EndoAngio-administered animals demonstrated no lung and liver metastases when examined postmortem. Although in this model metastasis may be directly related to primary tumor size, these results warrant further investigation and validation. These results suggest that repeated administration of Ad-EndoAngio generates an environment grossly unfavorable for tumor growth and indirectly inhibits metastatic lesions. Also, our *in vivo* imaging provides strong confirmation of the efficacy of our antiangiogenic therapy. EndoAngio is known to directly induce endothelial apoptosis, inhibit cellular migration, and disrupt microfilament integrity, thus impairing the ability of tumors to recruit new angiogenesis and increase blood supply. This is displayed in the treatment groups as diminished vessel diameter, decreased blood flow, and reduced vascular branching. These images also provide direct diameter comparison between treatment groups. EndoAngio produced statistically significant ($p < 0.001$) vessel lumen reduction. These results illustrate the potent antiangiogenic effects of EndoAngio.

The most significant result from our data is the occurrence of bilateral tumor regression regardless of injection side. We believe this contralateral effect is mediated by the secretion of therapeutic amounts of circulating EndoAngio to systemically alter the uninjected right side. By injecting the adenoviral vector subcutaneously into the left tumor, the tumor cells themselves and the surrounding epithelial cells may serve as a “protein factory” to generate and secrete enough circulating EndoAngio protein not only to alter the local microenvironment but also to systemically inhibit distant lesions. This systemic expression was confirmed by ELISA at two different time points during the animal trial. Further, adenoviruses are known to accumulate in the liver and the contralateral effect could be mediated by the reticuloendothelial system (Wood *et al.*, 1999). This result suggests that prolonged expression of angiostatic protein has potential systemic inhibition. It also serves as proof of principle that distant administration can affect metastatic sites and provide clinical efficacy.

Gene therapy has limitations, such as limited viral spreading within tumors, limited direct cytotoxicity, and viral immunity (Folkman, 1998). To effectively target tumor vasculature, vectors must provide long-term expression without eliciting vector-associated toxicity or immunity. Importantly, repeated adenovirus-mediated gene transfer of antiangiogenic agents in our study did not show toxicity or interruption of normal vascular processes. Studies in both human and animal trials have confirmed the safety and efficacy of long-term endostatin/angiostatin administration (Shichinohe *et al.*, 2001; Folkman, 2002; Huang *et al.*, 2007). Another important note is that assessment of the efficacy of EndoAngio was performed in an immunocompromised model. Furthermore, the replication-defective vector used in this study would require chronic administration and could be limited by the induction of neutralizing antibodies if used in an immune-intact model (Rosenberg *et al.*, 1998). Although a number of studies demonstrated similar results with antiangiogenic vectors in immune-competent animals (Ichikura *et al.*, 2006; Cai *et al.*, 2008), alternative vector systems such as lentivirus, gutless adenovirus, or adeno-associated virus (AAV) should be investigated in future.

We believe the nature of our gene therapy is optimal for a complementary approach to existing treatment options and could provide therapeutic synergism. In fact, previous studies have displayed an additive effect when antiangiogenic agents are combined with standard chemotherapy or radiation (Mauceri *et al.*, 1998; Sweeney *et al.*, 2001). Alternatively, Ad-

EndoAngio could be combined with cytotoxic or apoptosis-inducing gene therapy to create a “choke and kill” phenomenon. This combination gene therapy approach, using both oncolytic adenovirus with angiogenesis inhibitors or apoptosis inducers, has improved antitumor effects compared with single agents alone in previous studies (Calvo *et al.*, 2002; Zhang *et al.*, 2004). Further, our gene therapy could be coupled with current angiogenesis inhibitors such as sunitinib, sorafenib, and bevacizumab.

In conclusion, we have shown that sustained Ad-EndoAngio expression produces potent, reproducible inhibition of RCC tumors. Further, this therapy provided a significant survival advantage over untreated animals. We confirmed that circulating levels of our chimeric protein produce distant tumor suppression as well. *In vivo* imaging confirmed antiangiogenic effects on tumor vasculature. These results indicate the strong potential of Ad-EndoAngio gene therapy in the treatment of metastatic renal cell carcinoma. Further work is needed to define its efficacy and role in future therapeutic approaches.

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FIG. 1.

In vivo tumor inhibition, injected flank. These graphs represent intratumoral infection with antiangiogenic adenoviral vectors in the 10-mm³ (A) and 25-mm³ (B) treatment arms. Data from individual groups were pooled and plotted. Statistical significance was determined by two-way analysis of variance (ANOVA). Error bars indicate the standard error of the mean. The treatment group, Ad-EndoAngio, demonstrated significant tumor growth inhibition after 30 days as compared with controls. * $p < 0.001$. PBS, phosphate-buffered saline; Tx, treatment.

FIG. 2.

In vivo tumor inhibition, contralateral uninjected flank. These graphs represent growth suppression of contralateral uninjected tumors. Mice treated with Ad-EndoAngio demonstrated significant inhibition of growth by day 30. Data were pooled and plotted. Statistical determination was performed by two-way ANOVA. * $p < 0.001$.

FIG. 3.

Serum antiangiogenic factor, EndoAngio, levels. Sera were collected at two time points from mice treated with saline, Ad-GFP, and Ad-EndoAngio. An ELISA for human endostatin was performed with a commercial kit as described in Materials and Methods. Points represent the mean serum EndoAngio level of six animals for each group at each time point. Error bars indicate the standard error of the mean. EA, EndoAngio.

FIG. 4.

In vivo tumor vascular imaging. Mice were administered rhodamine-conjugated BSA and tumors were exposed for imaging via a skin flap. Subsequent imaging was performed with a Bio-Rad MRC1024 multiphoton microscope, using a $\times 60$ water immersion objective. Ad-EndoAngio-treated tumors in both 10-mm³ and 25-mm³ treatment arms demonstrate significant blood vessel diameter reduction as compared with controls (**A**). Using the ImageJ program (National Institutes of Health), each vessel diameter was measured and average diameters were calculated (**B**). * $p < 0.001$.

FIG. 5.

Survival of mice treated with adenovirus encoding an antiangiogenic factor. Survival was recorded from all mice in each group. Statistical significance was determined by Kaplan-Meier test. * $p < 0.001$.

FIG. 6. Lung metastasis suppression after Ad-EndoAngio treatment. (A) The presence of metastases was identified by H&E staining of samples from mice treated with saline, Ad-GFP, and Ad-EndoAngio. (B) Metastatic lesions for each animal were recorded and statistical analysis was performed by χ^2 test.

FIG. 7. Liver metastasis suppression after Ad-EndoAngio treatment. **(A)** The presence of metastases was identified by H&E staining of samples from mice treated with saline, Ad-GFP, and Ad-EndoAngio. **(B)** Metastatic lesions for each animal were recorded and statistical analysis was performed by χ^2 test.