Combination therapy of androgen-independent prostate cancer using a prostate restricted replicative adenovirus and a replication-defective adenovirus encoding human endostatin-angiostatin fusion gene

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Abstract
Although prostate-restricted replicative adenovirus has exhibited significant antitumor efficacy in preclinical studies, it is necessary to develop more potent adenoviruses for prostate cancer gene therapy. We evaluated the synergistic killing effect of prostate-restricted replicative adenovirus and AdEndoAngio, a replication-defective adenovirus expressing the endostatin-angiostatin fusion protein (EndoAngio). When coadministered with AdEndoAngio, prostate-restricted replicative adenovirus significantly elevated EndoAngio expression, suggesting that AdEndoAngio coreplicates with prostate-restricted replicative adenovirus. Conditioned medium from prostate cancer cells infected by prostate-restricted replicative adenovirus plus AdEndoAngio inhibited the growth, tubular network formation, and migration of human umbilical vein endothelial cells better than conditioned medium from prostate cancer cells infected by AdEndoAngio alone. Furthermore, in vivo animal studies showed that the coadministration of prostate-restricted replicative adenovirus plus AdEndoAngio resulted in the complete regression of seven out of eight treated androgen-independent CWR22rv tumors, with a tumor nodule maintaining a small size for 14 weeks. The residual single tumor exhibited extreme pathologic features together with more endostatin-reactive antibody-labeled tumor cells and fewer CD31-reactive antibody-labeled capillaries than the AdEndoAngio-treated tumors. These results show that combination therapy using prostate-restricted replicative adenovirus together with antiangiogenic therapy has more potent antitumor effects and advantages than single prostate-restricted replicative adenovirus and deserves more extensive investigation. [Mol Cancer Ther 2006; 5(3):676 – 84]

Introduction
Prostate cancer is the second leading cause of male cancer-related deaths in the U.S. In 2005, it is estimated that new diagnoses of this disease may be second only to skin cancer, affecting 232,090 men, and that roughly 30,350 men will die from this disease in the U.S. Whereas early detection and several therapeutic approaches for locally confined prostate cancer offer excellent chances for long-term cure, 20% to 25% of patients will experience local recurrence and progress to advanced stage disease. Currently, the only treatment modality available for patients with advanced disease is hormone ablation therapy because prostate cancer proliferation is critically dependent on androgen. However, tumor regression is temporary and the disease inevitably progresses to androgen independent status.

Gene therapy offers a unique opportunity for androgen-independent prostate cancer treatment. A number of limitations, however, lead to the suboptimal efficacy of existing gene therapies. Over the past 10 years, gene therapy has not shown significant clinical success. These limitations include (a) low gene transfer efficiency by therapeutic vectors, (b) weak potency of therapeutic genes, (c) inadequate bystander effect, and (d) the molecular heterogeneity of prostate tumors (1). Because ONYX-015 virus has shown promise for cancer gene therapy, tumor/tissue-restricted replicative adenoviruses have drawn a lot of attention. The initial viral infection of the target cell can produce progeny virions that infect adjacent cancer cells, thereby improving in vivo infectivity, biodistribution, and bystander effects mediated by adenovirus (2, 3). However, tumor/tissue-restricted replicative adenoviruses exhibited only limited therapeutic efficacy in clinical trials when used as a monotherapy (4 – 6). To improve the efficacy of tumor/tissue-restricted replicative adenoviruses, they need to be combined with other therapeutic agents. Angiogenesis is controlled by a balance between angiogenic stimulators and inhibitors. This balance is perturbed in tumors by either overproduction of angiogenic inducers...
or underproduction of angiogenic inhibitors (7). Among many angiogenesis regulators, endostatin, which is a carboxyl-terminal proteolytic fragment of collagen XVIII, is the most potent angiogenesis inhibitor. It blocks endothelial cell proliferation, migration/invasion, and tubular network formation. Therapeutically, endostatin inhibits tumor growth and angiogenesis in a wide variety of animal tumor models with little toxicity, immunogenicity, and resistance (8, 9). Angiostatin, an amino-terminal fragment of plasminogen, also shows potent antiangiogenic and/or antitumor effects. Recombinant adenoviral vectors encoding angiostatin cDNA have elicited high antitumor and anti-metastatic effects (10, 11).

Recently, an endostatin and angiostatin fusion protein, EndoAngio, was developed and exhibited prolonged half-life and greater antiangiogenic effects (12). It has been reported that its replication-deficient therapeutic adenovirus can coamplify with tumor/tissue-restricted replicative adenoviruses (1, 13). The resulting selective replication adenovirus combined with a replication-defective adenovirus encoding EndoAngio cDNA have elicited high antitumor and anti-metastatic effects (10, 11).

Materials and Methods

Cells and Cell Culture

Human umbilical vein endothelial cells (HUVEC) were obtained from Cambrex Bio Science (East Rutherford, NJ) and were maintained in endothelial-specific medium EGM-2 (Cambrex) according to the manufacturer’s instructions. Human embryonic retinoblast (HER911, a gift from Leiden University and Crucell, Leiden, the Netherlands) was cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. HER911E4, which is a HER911 derivative with adenoviral E4 gene under the control of tetO (14), was maintained in HER911 culture medium additionally supplemented with 0.1 mg/mL hygromycin B (Calbiochem, San Diego, CA) and 2 μg/mL doxycycline (Sigma, St. Louis, MO). HEK293 was a transformed human embryonic kidney cell line that expresses complementing adenoviral E1 proteins supporting the replication of E1-deleted recombinant adenoviruses. HEK293 was maintained in MEM (Invitrogen, Carlsbad, CA) containing 10% FBS, 1% penicillin/streptomycin, and 1% MEM non-essential amino acids. Prostate cancer cell lines C4-2, CWR22rv, PC-3, and DU145 were all maintained in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin.

Construction of Recombinant Adenoviruses

The construction of the AdE4PSESE1a prostate-restricted replicative adenovirus was described in detail in an earlier publication (3). To construct a recombinant adenovirus (AdEndoAngio) expressing EndoAngio, the entire expression cassette including EF1α-human T-cell lymphotrophic virus promoter, human endostatin-angiostatin fusion gene, and polyadenylic acid signal was excised from pBlas-hEndo-angio expression vector (Invivogen, San Diego, CA) and subcloned into the adenoviral transfer vector pDElsp1A (Microbix Biosystems, Ontario, Canada), resulting in the plasmid pDElsp1A-hEndo-angio. These adenoviral transfer vectors were cotransfected with adenoviral vector pMJ17 into HEK293 cells and generated the desired replication-defective recombinant adenovirus, AdEndoAngio.

Preparation of Conditioned Medium

CWR22rv cells (4 × 10⁶) were plated in 100-mm culture dishes 24 hours before virus infection. The cells were infected by 100 virus particles (v.p.) per cell of AdE4PSESE1a or AdEndoAngio, or both 50 v.p. per cell of AdE4PSESE1a and 50 v.p. per cell of AdEndoAngio. The media were changed 8 hours post-viral infection. The conditioned medium was harvested 1 or 3 days after viral infection and concentrated by Centricon YM10 (Millipore, Billerica, MA). The conditioned medium collected were used for testing EndoAngio expression by Western blotting, and the conditioned media collected at 3 days were used for evaluating antiangiogenic activity on HUVEC in vitro by growth assay, tubular formation, and cell migration assay.

Western Blotting

Proteins (20 μg) from conditioned medium were subjected to SDS-PAGE separation and transferred to a PDS membrane (Millipore) using a NOVEX gel system (Invitrogen). The transferred membrane was probed with an anti-endostatin antibody (Abcam, Cambridge, MA), followed by a horseradish peroxidase–conjugated anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Antibodies on membrane were visualized by chemiluminescence (Pierce, Rockford, IL).

Cell Proliferation Assay

HUVEC were plated in 96-well plates (1 × 10⁴ cells per well) and exposed to 10 μg/mL of conditioned medium. Eight wells were used for each virus. The growth media were changed every other day and HUVEC proliferation was assayed 7 days after administration of conditioned medium by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The data were expressed as the percentage of live cells versus mock-infected cells and SD of three independent experiments. Comparisons were made between each single treatment and the combination treatment using one-way ANOVA.
Tubular Network Formation Assay

Tubular network formation on Matrigel was assayed according to a modified protocol described in a previous publication (15). Briefly, HUVEC cells were labeled with a red-fluorescent lipid dye 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), according to the manufacturer’s instructions (Invitrogen). Twenty-four-well plates were coated with 250 μL of Matrigel at 4°C and incubated at 37°C for 30 minutes. HUVECs (2.5 × 10⁴) in 100 μL of EGM-2 medium were labeled by DiI and mixed with 10 μg/mL of conditioned medium harvested from virus-infected cells as described above. The mixtures of HUVECs and conditioned medium were dispensed in each well and incubated for 8 hours. The cells were photographed under a fluorescent phase-contrast microscope at ×40 magnification. The tubular network formation was quantified by averaging the number of connecting branches in 10 randomly chosen fields. A two-factor analysis of AdE4PSESE1a and AdEndoAngio was done to test interaction using the control group as the zero level of each treatment, AdEndoAngio and AdE4PSESE1a. Comparisons were made between each single treatment and the combination treatment using one-way ANOVA. In addition, two-way ANOVA was used to test the interaction between AdEndoAngio and AdE4PSESE1a.

HUVEC Cell Migration Assay

The HUVEC cell migration assay was done as described by Schleef and Birdwell (16). Briefly, a confluent monolayer of red HUVEC cells stained by DiI on 24-well plates was scratched using a sterile 200 μL plastic pipette tip. Displaced cells were removed with three washes of PBS, and fresh EGM-2 medium containing CWR22rv conditioned medium (10 μg/mL) or PBS was added. Cells and cell gaps were observed by fluorescent phase-contrast microscopy at 0, 12, and 24 hours after scratching. The position of the scratched edge was noted and the migrated distance was compared. We quantified the gap distances with the SPOT software 4.1 (Diagnostic Instruments, Inc., Sterling Heights, MI). One-way ANOVA was done to compare the difference between each single treatment and the combination treatment. In addition, two-way ANOVA was used to test the interaction between AdEndoAngio and AdE4PSESE1a.

Animal Experiments

CWR22rv tumor models were established by injecting 2 × 10⁶ cells s.c. in the right flank of athymic nude mice (6-week-old males). Mice were castrated 3 days after cell injection. Mice were randomly grouped when tumor size reached ~30 mm³ at around 2 to 3 weeks after cell injections (10 tumors in the AdCMVGFP-treated group, 15 tumors in the AdE4PSESE1a-treated group, 16 tumors in the AdEndoAngio-treated group, and 8 tumors in the combination therapy group) and received intratumoral injections of 2 × 10⁹ v.p. of AdCMVGFP, AdE4PSESE1a, AdEndoAngio, or 1 × 10⁸ v.p. of AdE4PSESE1a and 1 × 10⁹ v.p. of AdEndoAngio in combination in 50 μL 1× PBS by using a syringe with a 27-gauge needle. Tumor appearance and tumor sizes were monitored once every week and the tumor volumes were calculated by using the formula (length × width² × 0.5236; ref. 17). Mice were sacrificed when tumor size exceeded 500 mm³. Statistical analysis was done by SAS Version 9 (SAS Institute Inc., Cary, NC). One-way ANOVA was used to compare the tumor growth ratios between the combination treatment group versus each treatment alone. A Kaplan-Meier survival analysis was used to compare the combination treatment versus each treatment alone with an event defined at the first week of nonrecurrrent tumor disappearance and with tumor progression as censored. A log-rank test was used to analyze differences in time to disappearance between treatments. To confirm the log-rank result, logistic regression was done to compare tumor disappearance versus nonregression between treatments. The model fit was verified using the Hosmer-Lemeshow goodness-of-fit test.

Histology and Immunohistochemistry

Tumors were removed, immediately fixed in buffered formalin, processed, embedded in paraffin, and cut into histologic sections. Tumor sections were stained with H&E according to the standard protocol. For the EndoAngio detection, a polyclonal rabbit antibody reactive to endostatin (Abcam) was used at a 1:200 dilution. For microvessel density analysis, rat monoclonal antibodies reactive to mouse CD31 (BD Biosciences, San Diego, CA) were used at a 1:200 dilution. The slides were reacted with primary antibodies overnight in a humidified chamber at 4°C. After being rinsed once with PBS, a biotinylated polyclonal anti-rabbit or anti-rat second antibody (BioGenex, San Ramon, CA) was applied to slides at a dilution of 1:500 and incubated for 1 hour. After washing with PBS, slides were incubated with avidin-peroxidase complex (Vector Laboratories, Burlingame, CA) for 1 hour, washed once with PBS, stained with freshly prepared diaminobenzidine solution for 15 minutes and counterstained with hematoxylin. The stained capillaries were quantified by averaging the counting of capillaries in 10 randomly chosen fields. One-way ANOVA was done to compare the difference between each single treatment and the combination treatment.

Results

Prostate-Restricted Replicative Adenovirus and AdE4PSESE1a Enhanced the Transgene Expression of AdEndoAngio by Coinfection

There have been several reports that transgene expression by a replication-defective adenovirus could be enhanced by coinfection with a tumor/tissue-restricted replicative adenovirus, presumably by coamplification (1, 13). To determine whether the EndoAngio fusion protein expression delivered by AdEndoAngio could be enhanced by coinfection of a prostate-restricted replicative adenovirus, AdE4PSESE1a, we analyzed the EndoAngio fusion protein expression following the infection of CWR22rv cells with AdE4PSESE1a, AdEndoAngio, or AdEndoAngio/AdE4PSESE1a (both viruses at half-dose). As shown in Fig. 1, conditioned medium harvested from the CWR22rv cells infected with AdEndoAngio (CM-AdEndoAngio) contained more proteins than conditioned medium harvested from the CWR22rv cells infected with AdEndoAngio/AdE4PSESE1a.
The expression of the fusion protein increased with time in CM-AdEndoAngio/prostate-restricted replicative adenovirus on day 1 after virus infection because we infected the cells with only a half-dose of AdEndoAngio in the combination group compared with AdEndoAngio alone. The expression of the fusion protein increased with time in CM-AdEndoAngio/prostate-restricted replicative adenovirus with more EndoAngio protein than in CM-AdEndoAngio alone on day 3. No fusion protein was detected in conditioned medium obtained from AdE4PSESE1a-infected cells (CM-prostate-restricted replicative adenovirus), as a negative control. These results suggest that coinfection with AdEndoAngio and AdE4PSESE1a could enhance the transgene expression of endostatin-angiostatin.

**EndoAngio Inhibited the Biological Activities of HUVECs**

To test whether the antiangiogenic activity of AdEndoAngio could be augmented by coinfection with AdE4PSESE1a, we did a HUVEC cell proliferation assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay], cell migration assay, and tubular network formation assay (Matrigel assay) using the conditioned medium described above. Both CM-AdEndoAngio and CM-AdEndoAngio/prostate-restricted replicative adenovirus inhibited HUVEC proliferation (Fig. 2A). As expected from the amount of expressed fusion proteins in Fig. 1, CM-AdEndoAngio/prostate-restricted replicative adenovirus exerted much stronger growth-inhibitory effects on HUVECs compared with CM-AdEndoAngio (P = 0.0002) or CM-prostate-restricted replicative adenovirus (P = 0.0005) alone. In contrast, CM-prostate-restricted replicative adenovirus did not have any effect at all on the proliferation of HUVEC as well as conditioned medium harvested from PBS-treated CWR22r v cells (CM-PBS), implying that the inhibition of HUVEC proliferation was exclusively due to fusion proteins synthesized from the EndoAngio gene in AdEndoAngio adenovirus.

**Assays of in vitro tubular network formation using HUVECs** also confirmed that CM-AdEndoAngio/prostate-restricted replicative adenovirus elicited a stronger inhibitory effect on tubular network formation by HUVEC cells than CM-AdEndoAngio (P < 0.0001). The significant interaction between AdEndoAngio and AdE4PSESE1a in the two-way ANOVA analysis supports a synergistic effect between AdEndoAngio and AdE4PSESE1a (P < 0.0001). As expected, CM-prostate-restricted replicative adenovirus did not affect tubular network formation, compared with CM-PBS (Fig. 2B).

The migration ability of HUVECs was evaluated by an in vitro scratch wound assay. A confluent monolayer of HUVEC cells was artificially wounded by a 200 µL micropipette tip and incubated with conditioned medium harvested above. The migrating HUVEC cells could fill up the gaps 24 hours after incubation with CM-PBS and CM-prostate-restricted replicative adenovirus. In contrast, CM-AdEndoAngio markedly decreased HUVEC movement whereas CM-AdEndoAngio/prostate-restricted replicative adenovirus almost completely halted HUVEC movement at 24 hours after incubation. We quantified the gap distances with SPOT software 4.1. The CM-AdEndoAngio/prostate-restricted replicative adenovirus elicited a stronger inhibitory effect on the migration of HUVEC cells than CM-AdEndoAngio (P < 0.0001). The significant interaction between AdEndoAngio and AdE4PSESE1a in the two-way ANOVA analysis supports a synergistic effect between AdEndoAngio and AdE4PSESE1a (P < 0.0001; Fig. 2C).

Collectively, these results clearly showed that coinfection had a stronger effect on HUVEC proliferation, migration and tubular network formation, and that AdE4PSESE1a and AdEndoAngio showed a synergistic effect.

**Cotransduction Enhanced Antitumor Efficacy**

We evaluated the antitumor efficacy of AdEndoAngio either alone or in combination with AdE4PSESE1a on the growth of androgen-independent CWR22rv s.c. tumors in athymic mice. As illustrated in Fig. 3A, the animal survival plot showed that only 20% of the mice survived in the AdE4PSESE1a intratumoral injection group and 37.5% of the mice survived in the AdEndoAngio group at 14 weeks. AdE4PSESE1a or AdEndoAngio alone inhibited tumor growth initially, compared with the AdCMV-GFP-treated group, but most of the treated tumors eventually grew exponentially (Fig. 3B-D). On the other hand, coinjection of AdE4PSESE1a and AdEndoAngio resulted in complete regression of seven out of eight androgen-independent CWR22rv tumors in castrated nude mice hosts. One slow-growing nodule was kept to a small size for 14 weeks (Fig. 3E). Kaplan-Meier survival analysis was done using data through week 7 in order to include all tumor disappearance events and to minimize informative censoring (censoring related to tumor growth). Out of 23 total censored observations among the three treatment groups, 17 were censored at ≥7 weeks and 6 were censored due to animal sacrifice at weeks 5 or 6 (3 each). In statistical analysis, the combination of AdE4PSESE1a and AdEndoAngio was superior to either AdE4PSESE1a or AdEndoAngio alone as tested by the log-rank test (P = 0.0003 and 0.046, respectively) and by logistic regression (P = 0.008 and 0.039, respectively). Goodness-of-fit of the logistic regression model was confirmed (P > 0.999). Median time to...
disappearance in the combination treatment was 4 weeks (95% confidence intervals, 3–7 weeks). The results revealed enhanced therapeutic efficacy following oncolytic and antiangiogenic combination therapy in this androgen-independent prostate cancer animal model.

A Tumor Treated by Combinational Therapy Presents Distinct Pathologic Features

When a tumor from the intratumoral injection groups was harvested, we observed an interesting pathologic phenomenon in the single residual tumor mass treated by the combinational therapy. The tumor cells were arranged loosely in small patches surrounded by necrosis, and some of the tumor cells showed nuclei condensation and cytoplasmic acidophilia (the cytoplasm showed increased eosinophilia), suggesting that these tumor cells were undergoing necrosis (ref. 18; Fig. 4A). Randomly located patches and large foci of irregular necrosis were detected in AdE4PSESE1a-treated tumors, but the remaining tumor cells

Figure 2. Biological activities of conditioned medium from virus-infected CWR22rv cells. A, conditioned medium from the combination therapy inhibits HUVEC proliferation. HUVEC cells (1 × 10⁴/well) were seeded and subjected to 10 μg/mL of conditioned medium from AdE4PSESE1a-, AdEndoAngio-, combined virus-infected, or PBS-treated CWR22rv. Eight wells were used for each virus. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was done 7 d after conditioned medium treatment. Columns, the percentage of live cells versus mock-infected cells of three independent experiments; bars, SD; ++, P = 0.0002; +++, P = 0.0005. B, conditioned medium inhibits HUVEC tubular network formation. HUVEC cells (2.5 × 10⁴) labeled by DiI were suspended in 100 μL EGM-2 medium and mixed with 10 μg/mL conditioned medium. The mixtures were dispensed in each well in 24-well plates coated with 250 μL Matrigel and incubated for 8 h. The cells were photographed and tubular network formation was quantified by counting the number of connecting branches between discrete endothelial cells. Columns, the percentage of live cells versus mock-infected cells of three independent experiments; bars, SD; ++, P = 0.0002; +++, P = 0.0005. C, conditioned medium inhibits HUVEC migration. A confluent monolayer of red HUVEC cells labeled by DiI on 24-well plates was scratched using a sterile 200 μL plastic pipette tip. Displaced cells were removed with three washes, and fresh EGM-2 containing conditioned medium was added. The cell gaps were observed at 0, 12, and 24 h after scratching (see above). The cell gap was quantified by SPOT software and data are presented as above (++ P < 0.0001). These results indicated that AdEndoAngio exerted a stronger antiangiogenic effect when coadministered with a prostate-restricted replicative adenovirus in vitro.
showed round/oval-shaped nuclei with fine granular chromatin, which indicated an active growing condition (Fig. 4B). The tumor cells in the AdEndoAngio-treated group showed no significant abnormal morphologic changes and no necrotic tissues could be detected inside the tumor mass (Fig. 4C). Again, the combination therapy showed a superior therapeutic effect to either AdE4PSESE1a or AdEndoAngio monotherapy. Immunohistochemical analysis revealed rich EndoAngio protein expression towards the periphery of the tumor mass in the cotransduction group (Fig. 4D), no EndoAngio protein expression in the AdE4PSESE1a-treated group (Fig. 4E), and only limited EndoAngio protein expression in the AdEndoAngio-treated group (Fig. 4F). Compared with the AdCMVGFP- (Fig. 5A), AdE4PSESE1a- (Fig. 5B), or AdEndoAngio-treated tumors (Fig. 5C), quantification of CD31 antibody-labeled capillaries revealed a significant decrease in capillary numbers within the residual tumor \( (P < 0.0001) \); Fig. 5D), although the labeled capillaries in the AdEndoAngio group also decreased significantly compared with AdCMVGFP-treated tumors \( (P < 0.0001) \). The labeled capillaries in the AdE4PSESE1a group showed a higher decrease compared with those in the AdEndoAngio group, mainly because of some big island-like necrotic areas inside the tumor masses.

Discussion

Our and other’s previous investigations revealed that tumor/tissue-restricted replicative adenoviruses only partially suppress tumor growth in most solid tumors as a monotherapy. Limited viral spreading inside the tumor/
of EndoAngio fusion protein in the targeted CWR22rv prostate cancer cell line and further augmented the biological activity of EndoAngio fusion protein to inhibit the proliferation, tubular network formation, and cell migration of human endothelial cells in vitro. These results suggest that this combination therapy significantly enhances the antiangiogenic effects of AdEndoAngio.

In animal studies, we observed a stronger therapeutic effect for the androgen-independent CWR22rv s.c. tumor model. AdEndoAngio and AdE4PSESE1a combination therapy was able to eliminate seven out of eight treated tumors. We believe that the potent antitumor effect resulted from a collaborative effort between AdEndoAngio and AdE4PSESE1a. Our previous study observed inefficient viral replication in AdE4PSESE1a-treated tumors that resulted in a tumor growth rate somewhat in advance of the tumor cell death rate, ultimately resulting in a failed therapy (3). In this study, AdEndoAngio coamplified with AdE4PSESE1a expressed a large amount of antiangiogenic factor, EndoAngio, to stop the growth of tumor cells, thus allowing AdE4PSESE1a enough time to eliminate the whole tumor mass by direct cell killing. Histologic results suggest that the residual tumor cells were in a necrotic condition. Cells showed increased eosinophilia, which was partly due to the loss of normal basophilia imparted by the RNA in the cytoplasm, and was partly due to the increased binding of eosin to denatured intracytoplasmic proteins (18). This result suggests that viral replication and the antiangiogenic factor together generated an environment highly unfavorable for tumor growth even when the therapy did not eliminate the tumor mass initially. A similar strategy was recently reported to treat androgen-independent C4-2 tumors (25). Jin et al. administered a tumor/tissue-restricted replicative adenovirus, Ad-hOC-E1, via tail vein injection and an antiangiogenic virus, Ad-Flik1-Fc, via intratumor injection. The combination modality using different viral administration methods probably did not give Ad-Flik1-Fc an adequate chance to amplify for better therapeutic effects.

Although coinjection of AdE4PSESE1a and AdEndoAngio can be used to treat locally advanced or recurrent prostate cancer, it is difficult to use this modality to treat metastatic prostate cancer. We are currently integrating the EndoAngio expression cassette into AdE4PSESE1a to make an antiangiogenic prostate-restricted replicative adenovirus for treating metastatic prostate cancer. Zhang et al. (20) recently reported the creation of an antiangiogenic oncolytic virus, ZD55-sflt-1, by inserting a soluble human vascular endothelial growth factor receptor, sflt-1(1-3), into an E1B-55-deleted oncolytic adenovirus vector. In that report, four of eight tumors were completely eradicated by incorporating ZD55-sflt-1 therapy with 5-fluorouracil therapy. However, that is only a 50% success rate. One possible reason for the lower therapeutic effect in that study compared with ours might be the relative potency of the antiangiogenic factors used. EndoAngio might have stronger antiangiogenic effects than sflt-1. Another possibility is that E1B-55-deleted adenovirus has a lower tumor-killing efficacy than AdE4PSESE1a. The E1B-55-deleted adenovirus, also known as mutant dl1520 or ONX-015, is reported to selectively replicate in and kill cancer cells with mutations in the p53 gene or dysfunctional p53 gene product (26), but ONX-015 has an attenuated oncolytic capacity compared with wild-type adenovirus as a consequence of E1B-55 kDa deletion (4). In addition, a recent report showed that the replication of AdE4PSESE1a and AdEndoAngio could be enhanced by a synergistic effect on tumor cell death.

**Figure 4.** Histopathology of virus-treated tumors. Tumor masses were collected, formalin fixed, and paraffin embedded. Sections were subjected to H&E or immunohistochemical staining by anti-endostatin antibody. The tumor cells in the only residual tumor treated by combination therapy (A) was apparently undergoing necrosis, but the tumor cells in the remaining tumor were in an active growing condition in AdE4PSESE1a-treated tumors (B). The tumor cells showed normal morphology and no necrosis in the AdEndoAngio-treated tumors (C). Rich EndoAngio protein expression can be detected at the periphery of the tumor mass in the combination therapy group by immunohistochemistry (D), whereas no EndoAngio protein expressing cells were seen in the AdE4PSESE1a-treated tumors (E) and only limited positive-staining cells were observed in AdEndoAngio-treated tumors (F).
ONYX-015 does not depend on the p53 status of the target cell; instead, the capability of tumor cells to export viral late mRNAs is the main determinant of viral replication of ONYX-015 (27). It was also reported that not all tumor cells support the replication of ONYX-015 (28).

Although we observed significant therapeutic efficacy with the combination of oncolytic adenovirus plus replication-deficient adenovirus for advanced prostate cancer s.c. models in immunocompromised mice, the high prevalence of preexisting immunity to adenovirus 5 in patients may substantially limit its future clinical utility, despite the effect of both advanced prostate cancer and chemotherapy on immunocompetence. However, it should be possible to temporarily suppress the patients’ immune system if the therapy is really effective in eliminating cancers. Alternatively, we can incorporate immune modulators such as Fas ligand into the gene therapy vectors to locally suppress the immune system.

In conclusion, we have developed a combination therapeutic modality for androgen-independent prostate cancer that was able to eliminate seven out of eight treated tumors by combining an antiangiogenic therapy and a prostate-restricted replicative adenovirus. The in vivo therapeutic efficacy suggests that using the autoangiogenic prostate-restricted replicative adenovirus is a promising strategy to treat androgen-independent prostate cancer and deserves more extensive investigation. Further developmental work is warranted for an antiangiogenic prostate-restricted replicative adenovirus strategy for treating metastatic prostate cancer.

References


Molecular Cancer Therapeutics

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