Antiangiogenic Gene Therapy of Cancer Utilizing a Recombinant Adenovirus to Elevate Systemic Endostatin Levels in Mice


Abstract

Gene therapy represents a possible alternative to the chronic delivery of recombinant antiangiogenic proteins to cancer patients. Inducing normal host tissues to produce high circulating levels of these proteins may be more effective than targeting antiangiogenic genes to tumor tissue specifically. Previously reported gene therapy approaches in mice have achieved peak circulating endostatin levels of 8–33 ng/ml. Here we report plasma endostatin levels of 1770 ng/ml after administration of a recombinant adenovirus. Growth of MC38 adenocarcinoma, which is relatively resistant to adenoviral infection, was inhibited by 40%. These findings encourage gene delivery approaches that use the host as a “factory” to produce high circulating levels of antiangiogenic agents.

Introduction

Tumors are dependent on angiogenesis for sustained growth (1), and therefore the treatment of cancer patients with inhibitors of angiogenesis is an attractive new strategy. Many of these biological agents are unstable in vitro and difficult to produce in large quantities (2). In addition, the dosing of these agents may be required on a chronic basis. For these reasons, antiangiogenic gene therapy has been proposed as an alternative to the delivery of recombinant protein (3, 4). Gene therapy of cancer has traditionally attempted to target tumor tissue with toxic or immunogenic transgenes. The apparent lack of toxicity of antiangiogenic agents suggests that maximizing circulating levels of these endogenous agents may be a more appropriate gene therapy strategy (3). The goal of this therapy would be to create a situation where the body becomes an endogenous “factory,” producing high circulating levels of the gene product. Because high levels of transgene products have been reported using adenoviral vectors (5), we investigated the ability of an adenoviral vector to elevate endostatin levels in mice as an approach to cancer treatment.

Materials and Methods

Cloning of the Murine endostatin Gene. Murine cDNA was obtained by isolating RNA (RNasey Mini kit; Qiagen, Valencia, CA) from snap-frozen 2-week-old C57BL/6 mouse (Charles River Laboratories, Wilmington, MA) liver and by treating with Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD). The murine endostatin gene was cloned into the TA cloning vector (Invitrogen, Carlsbad, CA) by PCR using the primers sense (GATCTCTAGACCCCATATG- GAGGATCATGTTTTAGCTGCTGCCCTTGCGGCACTGTCGACG-C GCGGGCCTACTCTACATGAGAAGGCTTCTCAG) and antisense (ACTGGAAGAGGTT-TTCTAGCTACTAG) (6). The 18-amino acid E3/19K signal sequence (MRYMLGLLAALAVCSAA) was inserted upstream from the endostatin sequence by PCR using the primers sense (GATCTCTAGACCCCATATG- GAGGATCATGTTTTAGCTGCTGCCCTTGCGGCACTGTCGACG-C GCGGGCCTACTCTACATGAGAAGGCTTCTCAG) and antisense (as above). Plasmid DNA was amplified in DH5α cells (Life Technologies), and the signal sequence-murine endostatin (ss-mEndo) sequence was confirmed (ABI Prism 310 autosequencer; PE Applied Biosystems, Foster City, CA).

Synthesis of Adenoviral Vectors. The ss-mEndo construct was digested with EcoRI and cloned by blunt-end ligation into the multiple cloning site of the adenoviral shuttle plasmid pAd/CMV.1. The resulting plasmid was recombined with type 5 E1A/B-deleted Ad2 as described previously (6, 7) and used to infect 293 cells (American Type Culture Collection, Manassas, VA). Plaque DNA was extracted using proteinase K digestion, phenol extraction, and ethanol precipitation and screened for ss-mEndo by PCR. The resulting virus, Ad-ss-mEndo, was amplified in 293 cells. A similar strategy was used to create control recombinant viruses containing the genes for β-gal (Ad-β-gal) and firefly luc (Ad-luc). Viruses were titered using a standard plaque-forming assay in 293 cells.

In Vitro Infection with Recombinant Ad. Cells were grown in complete medium consisting of DMEM with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, 0.5 μg/ml Fungizone, and 4 μM glutamine (Biofluids, Rockville, MD). Cells were infected at MOIs ranging from 0.1 to 100 (103 to 108 pfu per 106 cells in 1.0 ml of complete media) with Ad-ss-mEndo, Ad-β-gal, or no virus and incubated at 37°C for 24 h. Supernatants were centrifuged at 2 × 103 g for 5 min and assayed for endostatin using a competitive ELISA (Cytimmune Sciences, College Park, MD), according to the manufacturer’s instructions. 293 cell supernatants were concentrated 10-fold in cell culture columns (Centricon YM-10; Millipore, Bedford, MA). After overnight incubation at 37°C, the medium was aspirated and replaced with 2 ml of modified complete medium containing 5% FCS and 1 ng/ml basic fibroblast growth factor (R&D Systems, Minneapolis, MN) were added. After 72 h incubation at 37°C, proliferation was analyzed by WST-1 assay (Boehringer Mannheim, Indianapolis, IN).

Functional Assay of Virally Generated Endostatin. The human melanoma cell line 501 Mel (Z-H. Wang, National Cancer Institute) to adenoviral infection was tested by infecting cells with Ad-β-gal as described above and assaying for β-gal 24 h later using a staining kit (Boehringer Mannheim, Indianapolis, IN). Susceptibility of the murine hepatocyte line NMuLi (American Type Culture Collection) to Ad-β-gal infection was used as a positive control. The susceptibility of the murine colon adenocarcinoma cell line MC38 (developed in the Surgery Branch, National Cancer Institute) to adenoviral infection was tested by infecting cells with Ad-β-gal as described above and assaying for β-gal 24 h later using a staining kit (Boehringer Mannheim, Indianapolis, IN).}

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Mannheim), according to the manufacturer’s instructions. Inhibition of proliferation in each sample was calculated according to the formula:

\[
\text{Inhibition (\%)} = \left( \frac{A_{450\text{control}} - A_{450\text{sample}}}{A_{450\text{control}}} \right) \times 100
\]

where \(A_{450}\) is the absorbance at 450 nm measured in a Multiskan MCC/340 plate reader (Titertek, Huntsville, AL), and \(\text{control}\) represents uninfected cell supernatant.

**In Vivo Production of Endostatin.** Animal experiments were conducted according to protocols approved by the NIH Animal Care and Use Committee. Eight-week-old female nude mice (Charles River Laboratories) received injections i.v. by lateral tail vein with \(10^9\) or \(10^{10}\) pfu Ad-ss-mEndo or no virus in 100 \(\mu\)l of PBS (\(n = 5\) animals/group) to assess dose response and toxicity. Animal well-being was monitored for 6 days, and surviving mice were euthanized and autopsied. To assess the duration and amount of endostatin expression, mice were injected with \(10^9\) pfu of Ad-ss-mEndo or Ad-luc, or no virus, as described above (\(n = 10\) animals/treatment group). Tumors were measured in two dimensions using calipers on days 0, 2, 5, 7, 9, 11, and 13 by an investigator blinded to the treatment groups. Tumor volume was calculated according to the formula: \(\text{volume} = \text{width} \times \text{length} \times 0.52\).

**Statistical Analysis.** Data are presented as the mean ± SE. Comparisons between groups were performed using the Mann-Whitney U test (Instat 2.01, GraphPad Software), and two-tailed \(P < 0.05\) was considered significant.

**Results and Discussion**

Antiangiogenic treatment of cancer has been a topic of increasing interest with the recognition that tumors larger than 2–3 mm\(^3\) depend on angiogenesis for growth (1) and with the discovery of multiple endogenous inhibitors of angiogenesis (3, 4, 9). Endostatin, an antiangiogenic COOH-terminal fragment of collagen XVIII, has required doses as high as 20 mg/kg/day to demonstrate antitumor responses in mice (10). If similar doses are required in humans, the widespread clinical use of endostatin in cancer patients could present difficult manufacturing and economic considerations.

Endostatin gene therapy may obviate these difficulties by using the...
protein production capacity of the host to generate endostatin in vivo, rather than delivering exogenous recombinant protein. Two previous reports of endostatin gene therapy in mice using nonviral vectors (8, 11) have demonstrated an antitumor effect in the absence of significantly elevated circulating endostatin levels. Chen et al. (11) demonstrated a peak serum endostatin concentration of 33 ng/ml using a liposomal gene delivery system. We believe the antitumor effect noted may have been in part attributable to tumor-specific gene delivery, because we have found serum endostatin levels in untreated nude mice to be ~30 ng/ml (12). Blezinger et al. (8) used i.m. delivery of polymerized plasmid DNA carrying the endostatin gene and also demonstrated an antitumor effect despite peak serum levels of only 8 ng/ml. Differences between the murine endostatin immunoassay used by Blezinger et al. (8) and that used by ourselves and Chen et al. (11) may explain a systemic endostatin effect despite apparently low circulating levels.

Tumor-specific gene therapy designed to produce high local concentrations of an antiangiogenic agent may be a useful strategy, particularly for tumors with minimal metastatic potential. On the basis of the work of Mauceri et al. (13), radiation therapy may augment this antitumor effect. Although tumor-specific expression of an antiangiogenic gene offers clear benefits, a systemic approach avoids the difficult problem of developing tumor-specific gene delivery vectors while taking advantage of the apparent absence of systemic toxicity of most antiangiogenic agents (3). Furthermore, elevation of circulating levels may allow the transgene product access to micrometastatic tumors in a prevascular stage of development, whereas tumor-targeted therapy is likely to require an established tumor blood supply to deliver the vector directly to the tumor.

We selected an adenoviral vector to deliver the murine endostatin gene because of previously reported high circulating levels of transgene product (5), as well as known biodistribution mostly to the liver (14, 15). This model allowed us to investigate the hypothesis that the host, in particular the host liver, can be used as a “factory” for generating angiogenesis inhibitors.

We first cloned the murine endostatin gene preceded by the E3/19K signal sequence (16) into an adenoviral shuttle plasmid (Fig. 1a) and generated recombinant Ad-ss-mEndo. Infection of 293 cells (in which E1-deleted Ad can replicate) yielded dose-dependent elevation of
supernatant endostatin concentrations, up to 920 ± 33 ng/ml at a MOI of 100. Western blotting of the Ad-ss-mEndo-infected cell supernatant revealed a single band with mobility identical to that of recombinant murine endostatin (Fig. 1c). Human melanoma cells (in which E1-deleted Ad cannot replicate) then were infected with Ad-ss-mEndo or Ad-luc. Supernatant endostatin levels (Fig. 2a) and the ability of the supernatant samples to inhibit endothelial cell proliferation (Fig. 2b) were evaluated. A dose-dependent increase in inhibition was observed, up to 61 ± 4% (versus 25 ± 4% in supernatant from Ad-luc-infected cells; P = 0.0006).

The ability of Ad-ss-mEndo to generate endostatin in vivo then was assessed. Mice receiving 10⁹ pfu Ad-ss-mEndo demonstrated 100% survival and appeared healthy. Autopsy revealed only mild hepatomegaly. After receiving 10¹⁰ pfu, 60% of mice died. Surviving mice were markedly lethargic and at autopsy revealed massive hepatomegaly with extensive macronodular changes and bilious ascites. Other organs revealed no signs of toxicity. These findings are consistent with extensive macronodular changes and bilious ascites. Other studies (13) have reported similar findings in a mouse model of hepatic adenoma.

To best evaluate the principle of systemic, rather than tumor-directed antiangiogenic gene therapy, we selected a murine tumor cell line that is relatively resistant to adenoviral infection and gene expression. Although murine hepatocytes were easily infected with Ad-β-gal at an MOI of 100 (Fig. 4a), only occasional MC38 adenocarcinoma cells demonstrated blue staining (Fig. 4b). In a 2-week in vivo treatment model, two weekly treatments were given, based on the time course of endostatin expression presented above. There was no significant difference in tumor size between Ad-luc- and saline-treated animals at all time points (mean of all time points, 55 and 57 ng/ml, respectively). Peak expression after Ad-luc- and saline-treated animals at all time points (mean of all time points, 55 and 57 ng/ml, respectively). Peak expression after Ad-ss-mEndo infection was observed 4 days after injection, at which time plasma endostatin levels were similar between Ad-luc- and saline-treated animals at all time points (mean of all time points, 55 and 57 ng/ml). On day 13, plasma levels remained significantly elevated (187 ± 39 ng/ml versus 56 ± 12 ng/ml in Ad-luc-treated animals; P = 0.041). The peak circulating levels achieved were 50–200-fold higher than those reported previously using nonviral vectors.

In conclusion of the experiment (day 13), at which point 40% tumor group from day 5 onward. This difference was most marked at the conclusion of the experiment (day 13), at which point 40% tumor growth inhibition compared with saline-treated animals was observed (P = 0.012). No toxicity was observed in any of the animals.

To our knowledge, this study represents the first in vivo report of endostatin gene therapy using an adenoviral vector. We demonstrated inhibition of tumor growth associated with high circulating endostatin levels in a tumor model relatively resistant to tumor-directed gene transfer. Because of the toxicity of most modalities available to treat disseminated cancer, tumor specificity has been a major focus of cancer research, including the development of gene therapy vectors. The use of gene therapy to inhibit tumor angiogenesis may represent a change from this traditional paradigm. We consider the adenoviral model presented here a prototype for this non-tumor-directed approach. We are currently investigating new gene delivery systems to prolong systemic expression of antiangiogenic agents, making use of the host as the “factory” for production.

References

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