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Abstract

Inhibition of angiogenesis has been shown to be an effective strategy in cancer therapy in mice. However, its widespread application has been hampered by difficulties in the large-scale production of the antiangiogenic proteins. This limitation may be resolved by in vivo delivery and expression of the antiangiogenic genes. We have constructed a recombinant adenovirus that expresses murine endostatin which resulted in a significant delay of tumor progression of JC breast carcinoma and Lewis lung carcinoma, and more importantly, in complete prevention of lung metastases formation in Lewis lung carcinoma. The inability to control pre-established tumors may be due to insufficient circulating endostatin levels or to inadequate local endostatin concentrations, both of which have been shown to be important for the anti-tumor effect of endostatin. We now constructed a recombinant adenovirus expressing a murine Ig-endostatin fusion protein resulting in significantly higher circulating endostatin levels in vivo. This construct is now being tested in breast cancer models. Furthermore, we demonstrate that locally deposited endostatin is biological active with respect to inhibition of endothelial cell proliferation and induction of endothelial cell apoptosis. We are now constructing a new tumor-targeted version of endostatin to increase directly local endostatin concentrations in the tumor. In conclusion, the present study clearly demonstrates the potential of vector-mediated antiangiogenic gene therapy in cancer treatment. Changes in vector design, however, resulting in higher transgene expression levels, or tumor targeted delivery of endostatin may even lead to stronger anti-tumor activity.
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Introduction

In recent years it has become clear that angiogenesis plays a pivotal role in tumor progression and metastases formation (1). The target of antiangiogenic cancer treatment is the genetically normal endothelial cell. Therefore, the development of resistance to angiostatic therapy is very unlikely and has not been reported so far (2). If a cancer exceeds the size of approximately 1-2 mm³, recruitment of new blood vessels is needed (angiogenesis) to prevent tumor cell apoptosis. Therefore, continuous overexpression of antiangiogenic factors by gene therapy, for instance, should counteract the tumor-induced angiogenesis. We are thus developing an antiangiogenic gene transfer approach to treat metastatic breast cancer using the potent angiostatic molecule endostatin in conjunction with immunomodulatory therapy with IL-12.
DOD Award DAMD17-99-1-9307: Annual Report for the Second Year (24 Months)

Body

Task 1: 1-18 months
Construction of a gutless ADV expressing endostatin (gADV-end) and gADV-control, its in vitro characterization and in vivo efficacy testing.

Task 2: 19-30 months
Incorporation of Gene regulation with the tetracycline regulatable system (tet-off) in the gutless ADV

As reported in the Annual Report of June 2000, we put the construction of the gutless ADV on hold due to problems with helper virus contamination secondary to insufficient expression of Cre recombinase of the packaging cell lines. Instead of concentrating on vector design, our efforts were rather focused to prove the efficacy of endostatin gene transfer for breast cancer. To this end, we constructed an E1 minus recombinant adenovirus expressing endostatin under the control of the human EF-1α promoter, which resulted in a significant delay in tumor growth and total prevention of metastases formation (1). Regression of pre-established tumors, however, could not be documented with this approach. It has been shown that different doses of endostatin are needed for the successful treatment of primary tumors or metastases (2). In addition, we have shown that the effect of endostatin on the maturation of tumor vasculature is also dose dependent. There is an inverse relationship between endostatin serum levels and tumor vessel maturation (3). Furthermore, local endostatin concentration was also shown to be important for the anti-tumor effect (4). Therefore, the limited efficacy with the gene transfer approach observed in our study could be due to insufficient circulating endostatin levels or secondary to inefficient concentration of endostatin at the tumor site. We thus took a two-fold approach to improve the efficacy of endostatin gene transfer:

A. Changes in vector design in order to increase transgene expression levels and prolongation of circulating half-life of endostatin. Construction of an E1 minus adenovirus expressing a fusion protein between the Fc portion (hinge-CH2-CH3) of a murine immunoglobulin molecule (IgG2a) and endostatin under the control of the human EF-1α promoter.

B. Exploration of potential mechanisms to target endostatin directly to the tumor site.

A. Construction of ADV-Ig-end by overlapping PCR:

1. PCR: Total RNA was isolated from 1x10^7 splenocytes (RN easy kit, Qiagen) and the hinge-CH2-CH3 portion of IgG2a was amplified using template specific primers (One-step RT-PCR, Qiagen): Primer A (sense) with 11 additional nucleotides on the 5' side coding for the 3' end of rat insulin leader sequence (CTGCCAGGGCTGAGCCCAGAGGGCCCACAAT) and primer B (antisense) with additional 11 nucleotides on the 3' end coding for the beginning of the endostatin gene (TGATGAGTATGTTTACCCGGAGTCCGG). A polymerase with proof-reading activity was used in all DNA amplification steps (pfu, Stratagene).

2. PCR: Primer C (sense): 5' end of rat insuling leader including Hind III restriction site and Kozak sequence (GAATTCAAGCTTGCCAGCCCTGCTGGA). Primer D (antisense): 3' end of rat insuling leader sequence plus 11 additional nucleotides of IgG2a Fc (CCTCTGGGCTCAGCTGGGAGGTGGGGCTTC).
3. PCR: Products from PCR 1 and 2 were gel purified, mixed and amplified with primers B and C to generate rat insulin leader—IgG2a.

4. PCR: The previously cloned endostatin cDNA was amplified with primer E (sense) containing on the 5' end 13 additional nucleotides of the 3' end of IgG2a cDNA. (GACTCCGGGTAAACATACTCATCAGGACTTTC) and primer F (antisense) including a Not I restriction site (GAAGAGTAAGCGGCCGCCTATTTGGAGAAA).

5. PCR: The so amplified endostatin cDNA served together with the rat insulin leader — IgG2a PCR product from the 3. PCR as a template for the last PCR using primer C (sense) and primer F (antisense): 3' end of endostatin including a Not I restriction site. The final PCR product was gel purified, cloned into the Hind III and Not I sites of the previously described adenovirus shuttle vector and sequenced.

Summary of IG-End PCR:

<table>
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<tr>
<th>Rat insulin leader</th>
<th>IgG2a-Fc</th>
<th>Endostatin</th>
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<td>HindIII-C</td>
<td>A</td>
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1. PCR 2. PCR 3. PCR 4. PCR 5. PCR

Recombinant E1 minus adenovirus was rescued using calcium phosphate co-transfection of E1 expressing 293 cells with the Ig-end adenovirus shuttle vector and an adenovirus backbone vector (pBHG10, Microbix) followed by agarose overlay. The resulting viral plaques (17) were harvested after 4 weeks. Viral DNA was extracted from the cell lysate of half of each plaque and digested with Hind III to verify the presence of the transgene. From the other half of each plaque, the virus was released from the cells by three freeze/thaw cycles and used for further virus amplification on 293 cells. The plaques were also screened for transgene expression by determining endostatin levels in plaque supernatants using an ELISA for murine endostatin (Cytimmune). Only one of the 17 plaques expressed the transgene (endostatin) at high levels (Fig. 1).
**Fig. 1:** Endostatin expression in supernatant of viral plaques (ELISA). Only plaque number 12 is expressing endostatin at very high levels (~9000 ng/ml).
Plaque number 12 was then used for large scale virus preparation on 293 cells (500x 15 cm dishes). Virus was purified on two sequential CsCl ultracentrifugation steps. The final preparation was negative for both endotoxin and mycoplasm contamination. Viral particles (vp) as measured by OD260 were 2.85x10^12 vp/ml. Infectious titer (plaque forming units; pfu), determined by standard agarose overlay plaque assay on 293 cells, was 4.5x10^10 pfu/ml.

The purified Adv.Ig-end was tested side by side with the previously described Adv.End for transgene expression in vitro. Murine breast cancer cells (JC) were infected at different multiplicity of infection (moi) with the two endostatin viruses or control virus (Adv.LacZ) and endostatin levels were measured in the supernatant after 48 hours (Fig. 2).

### Fig. 2:

**Adv.Endostatin vs. Adv.Ig-Endostatin: in vitro:**

Endostatin expression of purified virus

![Bar chart showing endostatin concentration in supernatant of JC cells 48 hours after virus infection.](image)

- **JC Sup Adv.end 1x10^4 vp moi**
- **JC Sup Adv.end 5x10^4 vp moi**
- **JC Sup Adv.Ig-end 1x10^4 vp moi**
- **JC Sup Adv.Ig-end 5x10^4 vp moi**
- **JC Sup Adv.LacZ 5x10^4 vp moi**
- **JC Sup naive**

**Fig. 2:** Endostatin ELISA in supernatant of JC cells 48 hours after virus infection: Dose response of Adv.End (left two bars), and of Adv.Ig-end (middle two bars). Negative controls (Adv.LacZ and supernatant of non-infected JC cells) right two bars.
With both endostatin viruses, increasing viral dose resulted in significantly more endostatin production as detected in the supernatant of infected JC cells. Importantly, there was no difference in endostatin production with either viral dose when Adv.End was compared with Adv.Ig-end. The fusion protein was as effectively secreted as endostatin alone.

We then went ahead to compare endostatin expression levels in vivo (nude mice) after systemic administration (tail vein injection) of $1 \times 10^{11}$ vp of Adv.End or Adv.Ig-end. Endostatin serum levels were measured at 1 week, 3 weeks and 6 weeks after viral injection. There was no significant difference in expression levels at the three time points. The data of the 6 week time point are shown in Fig. 3.

**Fig. 3:**

**Adv.Endostatin vs. Adv.Ig-Endostatin: in vivo:**

Endostatin serum levels after i.v. administration

![Graph showing endostatin serum levels](image)

**Fig. 3:** Endostatin serum levels measured by ELISA six weeks after i.v. injection of $1 \times 10^{11}$ vp of Adv.End, Adv.Ig-end or control vector (Adv.DL312).
The endostatin levels after the injection of Adv.Ig-end were significantly higher at all time points than those with Adv.End. These two endostatin viruses will now be tested \textit{in vivo} head to head in preestablished murine breast cancers (JC).

\textbf{B. Targeting of endostatin to the tumor site:}

It has become clear that local endostatin concentrations may play an important role in tumor control (4). To explore the natural homing of endostatin to the tumor, we injected $1\times10^{11}$ vp of Adv.end or control vector (Adv.DL312) into tumor bearing mice. The mice were sacrificed after one week and tumors were stained immunohistochmically for endostatin. There was no significant difference in endostatin stainings between treated and control tumors. We therefore wanted to explore if high local concentration of endostatin inhibits endothelial cell proliferation and induces apoptosis in growing endothelium. To this end, we induced an endothelial denudating injury by 3 passages of a 0.25-mm angioplasty guide wire (Advanced Cardiovascular Systems) through a mouse femoral artery one day after i.v. injection of $1\times10^{11}$ vp of Adv.End or Adv.DL312. This model has previously been described in detail (5). Immunostaining for endostatin showed a massive accumulation of endostatin at the site of injury, but not at the contralateral non-injured control leg or after injection of control vector (Fig.4).

\begin{figure}[h]
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\caption{Fig. 4:}
\end{figure}
Fig. 4: Immunohistochemical staining of endostatin protein at the femoral arterial wall 1 week following arterial injury: (A) Injured, endostatin treated: A clearly demarcated brown stained layer (arrows) along the luminal surface of the arterial wall, which is devoid of luminal endothelial coverage, indicates the strong presence of endostatin protein. (B) Non-injured, endostatin treated: Only weak staining of elastic fibers in the media of the non-injured artery can be seen and no endostatin protein is detectable at the luminal surface, which is completely covered by endothelial cells (arrows). (C) Injured, control vector treated: Very little endostatin staining along the denuded luminal surface (arrow).

Removal of endothelium allowed for local concentration of endostatin at the exposed basement membrane at the site of injury. To test for the biological effect of endostatin after the endothelial injury, quantitative computer assisted morphometry was used to measure the degree of re-endothelialization after endostatin gene transfer. Endostatin inhibited re-endothelialization of the denudation injury significantly at two and four weeks after injury when compared with control vector (Adv.DL312). Also, significantly more endothelial cell apoptosis was found in endostatin treated animals vs. controls as measured by activated caspase-3 expression (Fig. 5).

Fig. 5: Comparison of the effects of endostatin overexpression on re-endothelialization and luminal endothelial cell apoptosis in treatment (Adv.End: filled bars) and control (Adv.DL312: open bars) groups at 2 and 4 weeks after arterial injury and adenoviral injection. (A) Difference between percentage of the luminal circumference of the artery covered by endothelium (independent sample t-test $P < .01$) (B) Proportion of caspase-3 expressing endothelial cells in the luminal endothelium (independent sample t-test $P < .01$).

In summary, accumulated endostatin at the site of endothelial injury effectively induces apoptosis of the re-growing endothelium and inhibits proliferation of activated endothelial cells.
Recently, utilizing phage display techniques, several short peptides (NGR, NG2, etc.) have been identified that specifically home to tumor endothelium/activated endothelial cells (6). We will now incorporate these motifs into the endostatin cDNA and will test if the new molecule with improved homing to tumor endothelium will be more effective in suppressing tumor growth.
Key Research Accomplishments

- Construction of a recombinant adenovirus expressing a fusion protein between the Fc portion of murine IgG2a and murine endostatin (Adv.Ig-end)

- Demonstration of equally effective secretion in vitro from virus infected cells of the Ig-endostatin fusion protein when compared to endostatin alone

- Demonstration of significantly increased endostatin serum levels in vivo after systemic administration of Adv.Ig-end compared to Adv.End

- Demonstration of the potent biological activity of endostatin (inhibition of endothelial cell growth; induction of endothelial cell apoptosis) after local accumulation of endostatin at the site of endothelial repair
Reportable Outcomes


Conclusions

Our data on adenovirus-mediated gene transfer of endostatin has clearly shown the efficacy of an antiangiogenic gene therapy approach for cancer: Significant delay in tumor progression, and more importantly, complete prevention of lung metastases formation. Regression of pre-established tumors, however, has not been achieved, which maybe due to insufficient circulating endostatin levels or secondary to low local concentration of endostatin at the tumor site. Construction of a recombinant adenovirus expressing an Ig-endostatin fusion protein resulted in significantly elevated serum endostatin levels in vivo. This construct will now be evaluated for its anti-angiogenic activity in a murine breast cancer model. Furthermore, local endostatin concentration was also shown to be important for the anti-tumor effect (4). Thus, construction of a specifically tumor-targeted endostatin construct using peptides that home selectively to activated endothelium maybe a promising approach to increase the efficacy of endostatin gene transfer. Finally, the combination with immunomodulatory tumor therapy or even conventional anti-cancer treatments is likely to have additive or even synergistic effects as it was already shown with other angiogenesis inhibitors (7).
References


Inhibition of angiogenesis has been shown to be an effective strategy in cancer therapy in mice. Its widespread application, however, has been hampered by difficulties in the large-scale production of the antiangiogenic proteins. *In vivo* delivery and expression of the antiangiogenic genes may resolve this limitation. We have constructed a recombinant adenovirus that expresses murine endostatin under the control of the human elongation factor 1α promoter which has previously been shown to be exceptionally strong in the liver of mice. The transgene was biologically active both *in vitro* as determined in endothelial cell proliferation assays as well as *in vivo* by suppression of angiogenesis induced by a locally administered adenovirus expressing VEGF<sub>165</sub>. Persistent high serum levels of endostatin (605-1740 ng/ml; mean 936 ng/ml) was achieved after systemic administration of the vector to nude mice which resulted in significant reduction of the growth rates and the volumes of JC breast carcinoma and Lewis lung carcinoma (p<0.001 and p<0.05, respectively). In addition, the endostatin vector treatment completely prevented the formation of pulmonary micro-metastases in Lewis lung carcinoma (p=0.0001). Immunohistochemical staining of the tumors demonstrated a decreased number of blood vessels in the treatment group versus the controls. In conclusion, the present study clearly demonstrates the potential of vector-mediated antiangiogenic gene therapy for cancer. Endostatin gene therapy, however, does not provide a cure in these cancer models, but it may be very useful in conjunction with other cancer treatment modalities.
Endostatin gene transfer inhibits tumor vessel maturation proportionally to transgene expression levels

R. Hutter, E. Reis, S. L.C. Woo, B. Sauter.

Institute for Gene Therapy and Cardiovascular Institute The Mount Sinai School of Medicine, New York, NY, USA 10029

Background: Inhibition of angiogenesis by endostatin gene transfer has been shown to be an effective strategy in cancer therapy in mice. Endostatin is known to reduce the number of tumor vessels, however, potentially important effects of endostatin therapy on tumor vessel maturation have not been investigated. Methods and Results: To investigate the effect of endostatin on tumor angiogenesis, we first constructed a recombinant adenovirus expressing murine endostatin that was tested for biological activity both in vitro and in vivo. We then systematically evaluated the composition of tumor vasculature in a nude mice breast cancer model comparing mice treated by endostatin gene transfer (group A, n=10) with control vector treated animals (group B, n=10). Persistent high serum levels of endostatin (605-1740 ng/ml; mean 936 ng/ml) were achieved after systemic administration of the vector to nude mice which resulted in significant reduction of the growth rates and the volumes of JC breast carcinoma. Tumor tissue was formalin fixed and processed for HE and immunohistochemical staining detecting alpha-actin and CD31 protein. Quantitative morphometric analysis was performed. Overall mean vessel area per field was significantly lower in the endostatin treated group (p<0.05). In addition, the endostatin vector treatment almost completely prevented the formation of alpha-actin positive tumor vessels compared to controls (p=0.01). Most interestingly, only the density of alpha-actin positive tumor vessels, but not the total number of tumor vessels, correlated negatively with endostatin transgene levels of individual animals (r=-0.58, p=0.018). As a result tumor vessel composition as indicated by the ratio of alpha-actin positive to overall tumor vessel density was significantly changed. Conclusions: Our data indicate that endostatin affects not only the number of tumor vessels but also induces a dose dependent shift from alpha-actin positive to alpha-actin negative tumor vessels. This strongly suggests that endostatin influences the complex process of vessel maturation possibly by interfering with vessel pruning or pericyte recruitment.
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1997/98: "Cancer Gene Therapy" in the laboratory of Dr. Savio L.C. Woo at the Institute for Gene Therapy and Molecular Medicine, Mount Sinai Medical Center, New York.
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1995 - American Gastroenterological Association (AGA).
1995 - American Society for Gastrointestinal Endoscopy (ASGE).
1995 - American College of Gastroenterology (ACG).
2000- American Society of Gene Therapy (ASGT)

GRANT SUPPORT:

1996-1998 "Gene therapy for bilirubin glucuronidation deficiency" by the Swiss National Science Foundation
1997 "Gene therapy for bilirubin glucuronidation deficiency" by the OPO-Pharma Foundation for Research in Basic Science, Switzerland
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1998 "Antiangiogenic Cancer Gene Therapy with Recombinant Adenoviruses Expressing Endostatin" by the Goehner Foundation, Switzerland

1999 Research Fellowship Novartis Research Foundation

1999 Research Fellowship Roche Research Foundation

1999-2002 "Combination Antangiogenic and Immunomodulatory Gene Therapy for Breast Cancer" by the Department of Defense (DOD), USA: Proposal identification #: BC981028

INVITED LECTURES / PRESS CONFERENCES:

Grand Rounds, Institute of Clinical Pharmacology University of Bern 12/1999: "Will Gene Therapy Enter a New Millennium?"

Grand Rounds Division of Gastroenterology, University Hospital of Geneva 12/1999: "Gene Therapy 2000?"

Medical Grand Rounds, University Hospital of Zürich 1/2000: "Gene Therapy in the New Millennium?"


PUBLICATIONS:

Articles:


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In preparation for publication:

Book Chapters/Reviews:


2. Five chapters in UpToDate:
   B. Sauter, N. Roy Chowdhury, and J. Roy Chowdhury.
   In UpToDate, Burton D. Rose (ed.), UpToDate, Inc.; submitted.

   1. Bilirubin Metabolism
   2. Jaundice: Causes, Classifications and Approach to Diagnosis
   3. Neonatal Jaundice
   4. Inherited Disorders (Unconjugated Bilirubin): Gilbert’s Syndrome, Crigler-Najjar Syndrome, Type I and II
   5. Inherited Disorders (Conjugated Bilirubin): Dubin Johnson Syndrome, Rotor Syndrome
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N.B.: UpToDate is a CD ROM book aimed at the “intellectually oriented subspecialist” and is more detailed than general medical texts. It is updated three times a year, and, therefore, it includes the cutting edge of the current medical research of a specific field.


Abstracts:


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