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ORIGINAL ARTICLE

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Influence of adenoviral vector on expression of angiogenesis regulating factors in non-small cell lung cancer cell lines

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Abstract Gene therapy to suppress the neovascularization of solid tumors may prove to be an effective strategy in cancer therapy. Recombinant adenovirus (Ad) vectors are currently the most widely used vectors for gene transfer. In this study, we examined the expression of angiogenesis-regulating factors in tumor cells infected with the Ad vector. These cells showed an upregulation of basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) transcription, and a higher protein level of intracellular bFGF and increased VEGF secretion. In addition, we observed an increase of angiostatin-generating activity in the supernatants of infected tumor cells. Supernatants of Ad vector-infected cells did not stimulate endothelial cell proliferation, and inhibited their migration. These results indicate that infection of tumor cells with the Ad vector results in an increased expression of angiogenesis-regulating factors, but does not by itself activate angiogenesis.

Keywords Adenoviral vector · Angiogenesis bFGF
NSCLC · VEGF

Introduction

Neovascularization is an important process required by solid tumors to maintain their growth [7]. Angiogenesis also promotes metastatic spread from primary tumors [14]. These processes are regulated by a fine balance between angiogenesis-stimulating and inhibiting factors [10]. The secretion of growth factors by tumor cells stimulates endothelial cell proliferation and migration, and induces angiogenesis.

Vascular endothelial growth factor (VEGF) and members of the fibroblast growth factor (FGF) family, in particular, basic FGF (bFGF), are potent inducers of angiogenesis [6, 20]. By binding to their receptors on endothelial cells, both factors stimulate the proliferation, migration and differentiation of endothelial cells [11, 16]. In addition, bFGF can synergize with VEGF in the induction of angiogenesis [18], e.g. by stimulating VEGF secretion [19]. Upregulation of VEGF and bFGF has been demonstrated in many tumors. Enhanced expression of VEGF and bFGF has been associated with poor prognosis in several reports [2, 5, 8].

Gene therapy strategies for the inhibition of tumor angiogenesis have the potential to provide sustained and elevated high local concentrations of anti-angiogenic mediators in tumors. Adenoviral (Ad) vectors, due to their ability to infect most of the epithelial cells with high efficacy, are widely used for gene transfer. However, the Ad vector still carries most of the adenoviral genes. Several authors have shown that Ad or Ad vector infection induces several signal pathways, resulting in e.g. the synthesis of cytokines [3, 4]. We hypothesized that the production of angiogenesis-regulating factors becomes altered in tumor cells following Ad vector infection, possibly resulting in a change in proliferation and migration of endothelial cells.

In this report we demonstrate that Ad infection induces enhanced expression of angiogenesis-stimulating factors VEGF and bFGF in NSCLC cell lines. In addition, Ad vector infection also results in the production of factors which can generate angiostatin, an angiogenesis-inhibiting factor. In vitro, supernatants of Ad vector-infected tumor cells exhibited no effect on the proliferation of endothelial cells, but did inhibit their migration. These results suggest that in vitro the infection of tumor cells with the Ad vector alone did not stimulate angiogenesis.

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Materials and methods

Cell lines and cell culture

All cell lines used were purchased from ATCC. The lung cancer (NSCLC) cell lines H1299, H460 and H358 and 293 cells (human embryonic kidney cells) were maintained in Dulbecco's modified Eagle medium (DMEM) or RPMI 1640, supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine at 37°C, 5% CO_2 in a humidified atmosphere. For experiments, cells were seeded, treated and analyzed as indicated.

Recombinant adenovirus vectors

The recombinant E1-deleted adenovirus (Ad vector) AdRSV.β-Gal, carrying the cDNA of β-galactosidase (β-Gal) and Ad.Null without expression cassette were kindly donated by Dr. Wolff (Department of Hematology, Oncology and Tumor Immunology, Robert-Rössle-Klinik, University Medical Center Charité, Humboldt University of Berlin, Berlin, Germany). Vectors were propagated on 293 cells and purified by cesium chloride density centrifugation, titered by plaque assay and stored at –80°C. Multiplicity of infection (MOI) resulting in equal levels of infection in the different cell lines was determined by infecting the cells with AdRSV.β-Gal, carrying the reporter gene β-Gal. Infection of cells was carried out for 1.5 h at 37°C in serum-free medium. After 1 day of infection, cells were fixed and stained for β-Gal activity.

ELISA

The concentrations of VEGF and bFGF in the culture supernatant and in the cell lysates (see western blot) were determined by specific enzyme-linked immunosorbent assays (ELISA; R&D Systems). Results were normalized to amount (pg) per 1×10^6 cells/10 ml supernatant or 100 µl cell lysate (see below).

RT-PCR

Total RNA was isolated from harvested cells using the RNeasy mini-kit (Qiagen). One microgram RNA was digested with DNase (Gibco) at room temperature (RT) for 15 min. Digested RNA was reverse-transcribed at 42°C for 30 min (Reverse Transcription System, Promega). Amplification was performed for VEGF, bFGF and β-actin as standard, using the following primer sequences: VEGF (s): 5'-CGA AGT GGT GAA GTT CAT-3', VEGF (as): TTC TGT ATC AGT CTT TCC TGG TGA G-3', bFGF (s): 5'-ACT GGT GTA TTT CCT TGA CC-3', bFGF (as): 5'-AAA ACG GGG GCT TCT TCC TG-3', ß-actin (s): 5'-CCT TCC TGG GCA TGG AGT CCT G-3', β-actin (as): 5'-GGA GCA ATG ATC TTG ATC TTC-3'. Polymerase chain reaction (PCR) was carried out in a final volume of 20 µl, with 1 µl cDNA, 200 µM dNTP, 1 IU Taq polymerase (Perkin–Elmer) and 10 pmol of each oligonucleotide primer. PCR for GAPDH was performed for 22 cycles with an initial denaturation at 94°C for 2 min and cycling times of 30 s at 94°C, 30 s at 62°C, and 30 s at 72°C. PCR for VEGF and bFGF was performed with annealing temperatures of 58°C and 52°C and with 35 and 30 cycles, respectively. Following amplification, PCR products were analyzed in 2% agarose gel containing ethidium bromide. PCR bands were measured and compared using the Chemi Doc System (Bio-Rad).

Angiostatin generation

Serum-free conditioned medium (SFCM) was derived from tumor cells by washing cells three times with phosphate-buffered saline (PBS) followed by 24 h incubation with serum-free basal medium (medium 200, Cascade Biologics). SFCM was spun at 600 *g* for 10 min, and stored at –20°C until further use.

Three micrograms of human plasminogen (Calbiochem) were added to 100 µl of SFCM of tumor cell culture, and the mixture was incubated at 37°C for 12 h. The samples were analyzed for angiostatin generation by western blot.

Western blot

For western blotting, a 10-µl sample (see the section Angiostatin generation) was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and transferred onto nitrocellulose membranes (Hybond-C, Amersham) by standard procedures. Primary monoclonal antibody (mAb) against angiostatin (C9–1) was purchased from Pharmingen. The biotinylated secondary antibody was obtained from Dako. In all cases detection was performed with streptavidin-coupled peroxidase (Dako) and ECL (Amersham). The intensity of the angiostatin bands was determined using the Chemi Doc System (Bio-Rad).

Endothelial cell proliferation assay

Human umbilical vein endothelial cells (HUVEC) were plated in a 24-well plate at a density of 3×10^4 cells/well. The following day, cells were washed twice with PBS and incubated with 300 µl of SFCM from tumor cell culture as described above, and 200 µl basal medium (Cascade Biologics) containing 5% FCS. At 72 h, 50 µl MTT (5 mg/ml, pH 4.8) was added and the mixture was incubated for 4 h. Supernatants were removed and the cells were lysed with 250 µl digestion buffer (20% sodium dodecyl sulfate (SDS), 50% dimethylformamide). Absorption of the lysed cell suspension was measured at 560 nm.

Endothelial cell migration assay

Migration of HUVEC was assayed in 24-well Multiwell plates (Becton Dickinson) with Falcon cell culture inserts (pore size: 8.0 µm). HUVEC were plated in cell culture inserts at a density of 5×10^4 cells in 100 µl serum-free basal medium. Inserts were incubated in the 24-well plate, previously filled with 800 µl/well SFCM. At 6 h, inserts with cells were washed with PBS, stained with methylene blue and the number of cells that migrated through the filter was visually determined.

Results

Ad vector-induced VEGF and bFGF mRNA expression

To determine the effect of the Ad vector on angiogenesis-stimulating factors VEGF and bFGF, the lung cancer cell lines H460 and H1299 were infected with an Ad vector and analyzed for mRNA transcription of VEGF and bFGF by semi-quantitative RT-PCR. Representative examples of RT-PCR separated on agarose gel are shown in Fig. 1. We observed an increase of VEGF and bFGF mRNA levels 24 h following Ad vector infection. Increased VEGF and bFGF mRNA transcription was also observed 96 h after infection (data not shown).

Ad vector-induced protein expression of VEGF and bFGF

To confirm our RNA data, VEGF and bFGF protein were measured in cell lysates of infected cells and in

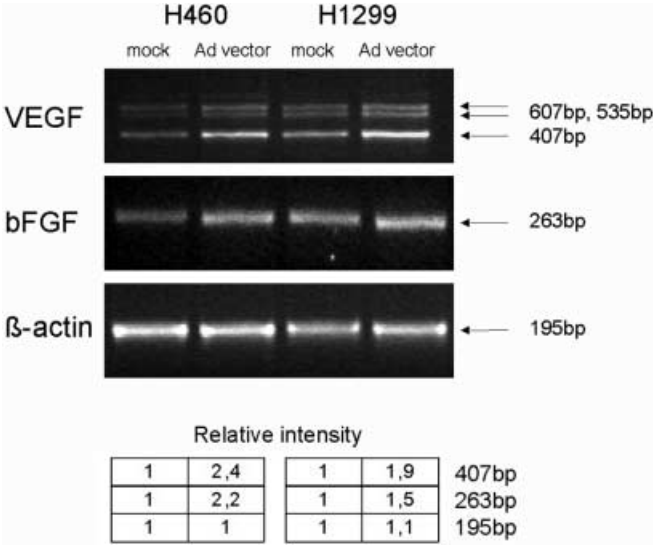

Fig. 1. Ad vector stimulates VEGF and bFGF mRNA expression. Cells were mock-infected or infected with Ad vector carrying the human β-galactosidase gene, AdRSV. β-Gal (200 MOI/H460, 50 MOI/H1299). VEGF and bFGF mRNA was determined by semi-quantitative RT-PCR. The three PCR bands of VEGF represent the splice variants. β-actin was used as control. The data presented are representative of three samples

supernatants by ELISA. In both cell lines, elevated VEGF protein levels were detected in the supernatants of infected cells compared to mock-infected cells (Fig. 2). Significant differences were first detected at 48 h. VEGF protein concentration was also increased in cell lysates of Ad vector-infected cells compared to non-infected (mock) cells (data not shown).

bFGF was first detectable in the supernatants of H460 cells at 96 h following Ad vector infection (H460 mock: 5.2 ± 2.7 pg/ml; Ad vector: 34.3 ± 16.7 pg/ml). However, intracellular bFGF was already present in all samples at 24 h. Similarly to VEGF, the amount of intracellular bFGF was increased in cells infected with the Ad vector compared to mock-infected cells (Fig. 3). The difference in the amount of bFGF between mock- and Ad vector-infected cells was significant at 48 h following infection.

VEGF secretion is stimulated in bFGF-negative cells after Ad vector stimulation

It has been shown that bFGF stimulates VEGF expression [19]. We therefore investigated whether the stimulation of VEGF expression was directly induced by the Ad vector, or indirectly caused by the stimulation of bFGF. The cell line H358, which is negative for bFGF expression, was infected with the Ad vector and VEGF secretion was measured. Interestingly, VEGF concentration was 10-fold higher in the bFGF-negative cells compared to H1299 or H460 cells which express bFGF. Following Ad vector infection, we observed an increase in VEGF secretion, but the difference between

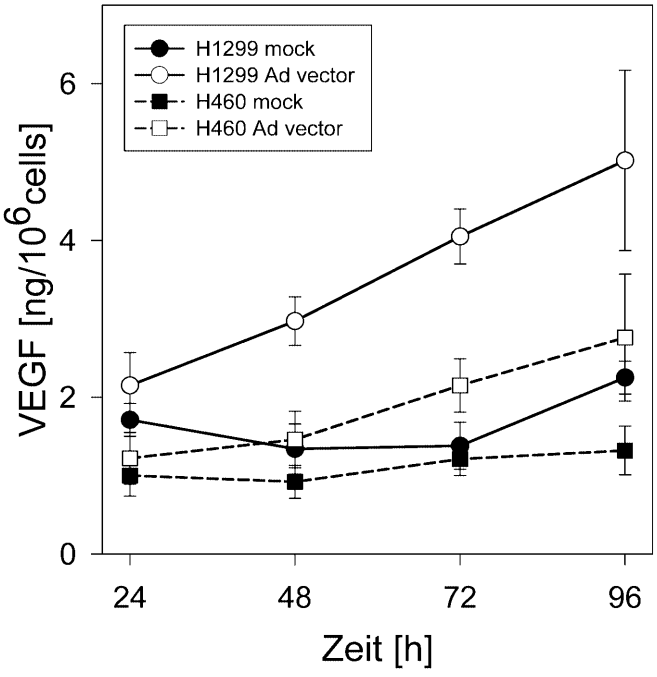

Fig. 2. Ad vector stimulates VEGF secretion. Cells were mock-infected or infected with AdRSV. β-Gal, as described in Fig. 1. Cells were counted at different time periods and the supernatants were subjected to ELISA for VEGF. Each value represents the mean of four independent experiments $\pm$ SD

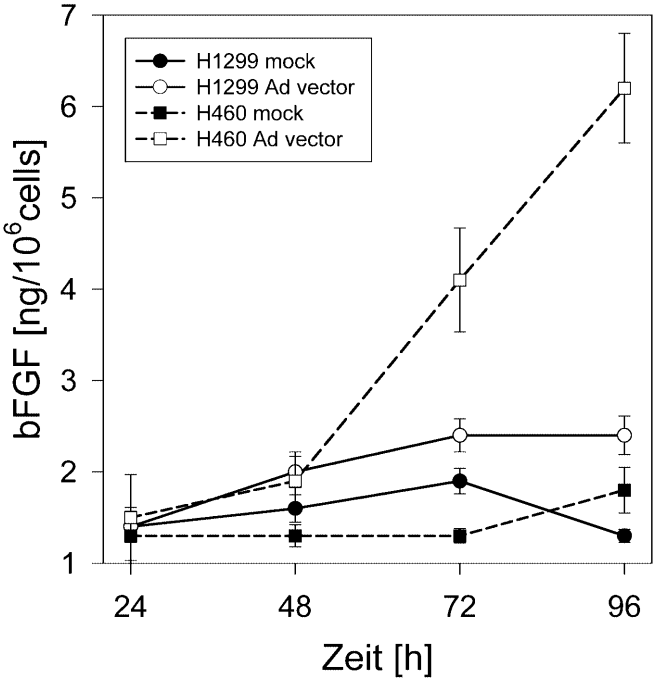

Fig. 3. Ad vector stimulates intracellular bFGF production. Cells were mock-infected or infected with AdRSV. β-Gal, as described in Fig. 1. bFGF production was measured by ELISA in cell lysates. Each value represents the mean of four independent experiments $\pm$ SD

mock- and Ad vector-infected cells was only significant at 96 h (Fig. 4).

Ad vector does not inhibit angiogenesis

Because increased VEGF has been observed in the supernatants of Ad vector-infected tumor cells, we decided to analyze its effect on angiogenesis induction in vitro. Endothelial cells were incubated with supernatants of mock- or Ad vector-infected tumor cells, and were analyzed for cell proliferation and migration. Although there was a higher VEGF concentration in the supernatants of Ad vector-infected cells (mock: 103 ± 18 pg/ml; Ad vector: 278 ± 40 pg/ml), we found no difference in the proliferation of endothelial cells regardless of whether we used the supernatants of infected or uninfected cells (data not shown).

The migration assay showed a reduced migration rate of endothelial cells in culture incubated with supernatants of Ad vector-infected tumor cells, as shown in Fig. 5 (mock: $100 \pm 16.8\%$; Ad vector: $69.1 \pm 8.5\%$). Purified VEGF (300 pg/ml), in contrast, almost doubled endothelial cell migration ($192 \pm 4.7\%$).

Angiostatin generation by Ad vector-infected cells

Angiostatin is a tumor-derived inhibitor consisting of an internal fragment of plasminogen [17]. Supernatants of infected and uninfected tumor cells were incubated with plasminogen. Western blot analysis at 24 h showed the cleavage of plasminogen into multiple bands, as indicated in Fig. 6. Angiostatin was identified as 38-kDa band by staining with an mAb specific for kringles 1–3

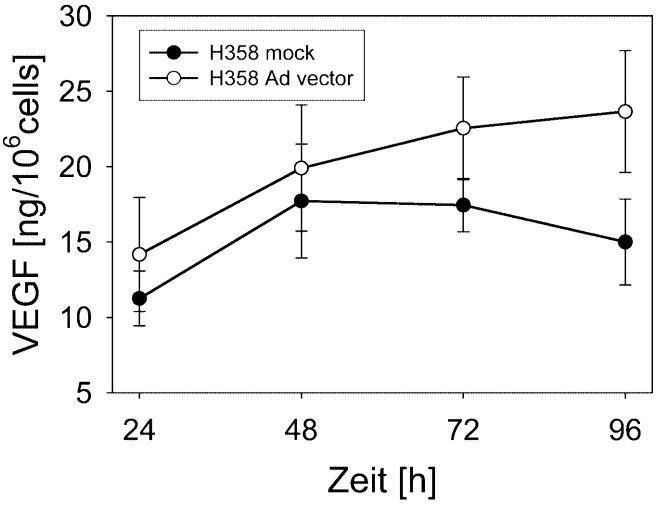

Fig. 4. Ad vector stimulates VEGF secretion in the bFGF-negative cell line H358. Cells were mock-infected or infected with 100 MOI of AdRSV. β-Gal, as described in Fig. 1. Cells were counted at different time periods as indicated, and the supernatants were subjected to ELISA for VEGF. Each value represents the mean of four independent experiments ± SD

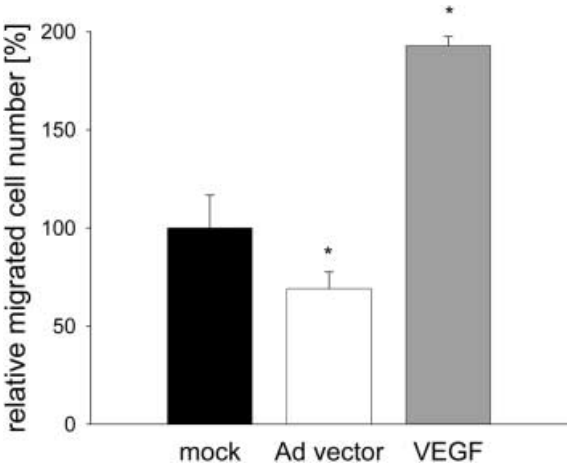

Fig. 5. SFCM of Ad vector-infected cells inhibit endothelial cell migration. HUVEC were plated in cell culture inserts. Inserts were incubated with 800 µl SFCM. VEGF (300 pg/ml) was used as positive control. At 6 h, inserts with cells were washed with PBS, stained with methylene blue and the number of cells that migrated through the filter was visually determined. Data represent mean ($n = 5$) ± SD

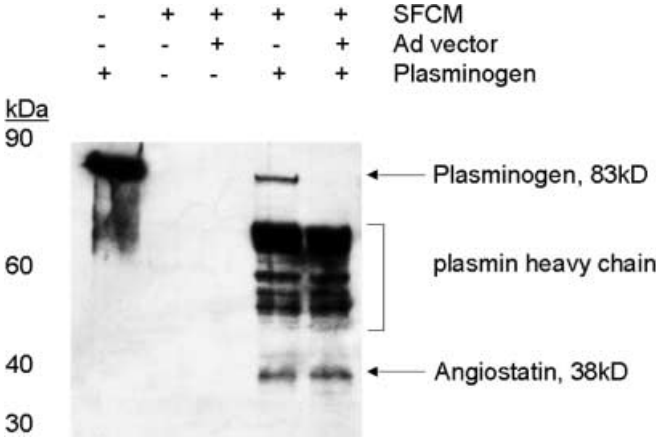

Fig. 6. Conversion of plasminogen to angiostatin by SFCM of Ad vector-infected cells. Three micrograms of human plasminogen was incubated for 12 h at 37°C in 100 µl SFCM of mock- and Ad vector-infected tumor cell cultures. Probes were subjected to SDS–PAGE and western blot analysis for angiostatin determinations. The data presented are representative of three experiments

of plasminogen. The other bands represented cleavage products compatible with kringles 1–4, as previously described [25]. Plasminogen was completely digested in only in the supernatants of Ad vector-infected cells. The semi-quantitative analysis of angiostatin bands showed that angiostatin-generating activity was 1.4 ± 0.17 ($n = 3$) higher in Ad vector-infected cells compared with non-infected cells.

Discussion

A major handicap in achieving the goal of effective gene therapy with adenovirus vectors is the inflammatory

response elicited by the vector [13, 21]. An inflammatory response may reduce the efficiency of gene expression, but may also antagonize the effect of the gene directly if the use of an antiinflammatory therapy is intended, or may act synergistically if the opposite is the case. Similarly, if the gene to be delivered is aimed at influencing angiogenesis, it is important that vector-induced changes in angiogenesis should be considered. In this study we analyzed the effect of an E1-deleted adenoviral vector on VEGF and bFGF protein and gene expression, major angiogenesis-stimulating factors. In addition, we studied the effect on angiostatin-generating activity of one of an emerging number of endogenous angiogenesis inhibitors. Finally, the global effect of the supernatants of infected tumor cells on proliferation and migration was tested in cultures of endothelial cells.

We observed the activation of VEGF and to a lesser extent bFGF gene expression and protein production. VEGF secretion was stimulated in Ad vector-infected cells, but bFGF level was only increased intracellularly. The role of intracellular bFGF is not quite clear, but the intracellular presence and increase of bFGF following stimulation without a concomitant increase in the culture medium has been described previously [12, 15, 22]. bFGF may be a mitogenic factor in tumor cells and is downregulated when cells grow to confluence [15], which may explain the observed decrease in bFGF protein concentration in the lysate of H1299 cells at 96 h. Remarkably, this decrease did not occur in infected cells demonstrating the vector-induced continuation of stimulated expression. VEGF expression as well as secretion may be stimulated by bFGF [16, 19]. However, bFGF was not required for VEGF stimulation since it also occurred in the bFGF-negative cell line H358. Interestingly, VEGF concentration was 10-fold higher in the supernatants of this bFGF-deficient cell line, suggesting a role for bFGF in angiogenesis.

The mechanisms of Ad vector-induced upregulation of VEGF and bFGF remain to be elucidated. However, it has previously been demonstrated that the Ad vector stimulated the mitogen-activated protein kinase (MAPK) signaling pathway [3] and stimulated NF-kappaB [4]. VEGF is regulated by the MAPK signaling cascade [1]. It is therefore possible that activation of the MAPK signaling pathway could be the mechanism responsible for VEGF upregulation. bFGF has been shown to be stimulated by protein kinase C [22], and this will also result in the activation of NF-kappaB [23]. However, it is unclear whether the Ad vector results in protein kinase C activation.

Despite our observations of upregulated VEGF and bFGF expression, no stimulation of proliferation and an even reduced rate of migration was observed in the in vitro model for testing the supernatants of infected or control tumor cells in endothelial cell cultures. This suggested that antagonistic factors were also upregulated.

Angiostatin, a proteolytic fragment of human plasminogen including kringles 1–4, is a potent inhibitor of angiogenesis and of the growth of tumor cell metastases [17]. In vitro, angiostatin inhibits endothelial cell migration and proliferation [9]. The ability to generate angiostatin is a common feature of many tumor cell lines [24, 25]. For this reason, we investigated the cleavage of plasminogen–angiostatin-converting activity of the supernatants of Ad vector-infected tumor cells. Angiostatin-generating activity was indeed found to be higher in the supernatants of Ad vector-infected cells compared to that in the supernatants of mock-infected cells. Whether angiostatin induction by Ad vector finally accounts for antagonism of the effects of VEGF and bFGF cannot be concluded.

In summary, infection of tumor cells with the Ad vector did not stimulate angiogenesis in vitro, although both VEGF and bFGF expression were enhanced. Infection with the Ad vector concomitantly resulted in the stimulation of anti-angiogenic activity. It is therefore concluded that the Ad vector constitutes an appropriate vehicle for the transfer of genes to be used for the development of anti-angiogenic strategies, insofar as VEGF and bFGF induction is involved.

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