The role of angiogenesis in the development of neoplastic diseases has been unravelled in recent years. The underlying attractive rational for angiostatic gene transfer strategies is to let the body itself produce the antitumour drug. While most conventional antitumour strategies attack the malignant tumour cell, the main goal of angiostatic therapies is to prevent tumour progress by inhibiting tumour angiogenesis by high systemic levels of angiogenic proteins. Adenoviral vectors fulfil the basic requirements for angiostatic gene therapy by ensuring a high transduction efficacy allowing high circulating protein levels. Recombinant adenoviruses encoding angiostatin and endostatin have been shown in some studies to exert high antitumour and antimeetastatic effects whereas other data were not as promising. A recent publication by Kuo et al showed for the first time in a comparative study design the responsiveness of different tumour entities towards different angiostatic compounds. This is a striking observation as any antiangiogenic anti-angiostatic antitumour treatment with two recombinant adenoviral vectors encoding angiostatin-like molecule (AdK1-3) and endostatin (Adendo).

RESULTS
AdK1-3 and Adendo exerted inhibitory biological functions on endothelial cell proliferation, migration, and tube formation in vitro. AdK1-3 inhibited significantly endothelial cell infiltration in vascular endothelial growth factor embedded Matrigel plugs in mice whereas Adendo showed only minor effects. Both AdK1-3 and Adendo induced similar antitumour effects in the LLC tumour model in immune competent C57BL/6 mice but AdK1-3 had stronger inhibitory effects in athymic mice. Furthermore, AdK1-3 inhibited tumour growth in a murine CRC and human HCC model but was ineffective in a human CRC model. In contrast, Adendo did not reduce tumour progress in either of these tumour models although AdK1-3 and Adendo effectively reduced intratumoral microvessel density in LLC tumours.

Conclusion: Our data demonstrate that angiostatic gene therapy may form a feasible strategy for the treatment of established hepatocellular carcinomas and that in vivo antitumour efficacy of angiostatic proteins is tumour specific.

METHODS

Animals and cell lines
C57BL/6, Balb/c, ror athymic mice, 5–8 weeks old, were purchased from Harlan (Barcelona, Spain). During the experimental period, mice were housed under pathogen free conditions. All animal procedures were performed according to approved protocols and in accordance with recommendations for proper care and use of laboratory animals.

The human and murine lung cancer cell lines A549 and Lewis lung carcinoma (LLC) cells, human HT-29 CRC cells, and 293 cells (embryonic E1 transformed kidney cell line) were obtained from American Type Culture Collection (ATCC, Rockville, Maryland, USA). Cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS). Human umbilical vein endothelial (HUVE) cells were obtained from Cascade Biologics (Portland, Oregon, USA) and were cultured according to the supplier’s instructions. The human HCC cell line HuH7 and murine colon cancer cells were cultured in DMEM supplemented with 10% FBS.

Abbreviations: CRC, colorectal carcinoma; HCC, hepatocellular carcinoma; LLC, Lewis lung carcinoma; VEGF, vascular endothelial growth factor; DMEM, Dulbecco’s modified Eagle medium; FBS, fetal bovine serum; HUVE, human umbilical vein endothelial; RT-PCR, reverse transcription-polymerase chain reaction; CM, culture medium; BrDU, bromodeoxyuridine; pfu, plaque forming units; MOI, multiplicity of infection.
Construction of recombinant adenoviruses encoding angioatin-like molecule and endostatin

Murine endostatin was generated by a reverse transcription-polymerase chain reaction (RT-PCR) technique using mouse liver RNA as template. Primers used for signal peptide of endostatin were 5’-GGATCCACCTCAGGACACAGGA-3’ and 5’-GATATCCATTTCAATGCGTGAGA-3’. Primers used for endostatin were 5’-GATATCCTAATCCTTAGGA-3’ (underlined sequences indicate restriction enzyme sites for EcoRI and BamHI). PCR fragments for endostatin and endostatin signal peptide were cloned into cloning vector pCR2.1-TOPO (Invitrogen) to form pCR2.1/K1-3. K1-3 fragment was released by BamHI/XhoI from pCR2.1/K1-3 and blunt end ligated into the BamHI site of pSQ1, resulting in pSQ1/K1-3, and put under the control of the CMV immediate early promoter. Recombinant adenoviruses were generated as described previously.

Briefly, human plasmidogen cDNA plasmid was used as template and the K1-3 sequence was cloned into the cloning vector pCR2.1-TOPO (Invitrogen) to form pCR2.1/K1-3. K1-3 fragment was released by BamHI/XhoI from pCR2.1/K1-3 and blunt end ligated into the BamHI site of pSQ1, resulting in pSQ1/K1-3, and put under the control of the CMV immediate early promoter. Recombinant adenoviruses were generated as described previously.

Briefly, pSQ1/K1-3 and pSQ1/endo, respectively, were cotransfected with pJM17 into 293 cells by calcium phosphate precipitation. Recombinant adenoviruses were isolated from a single plaque, expanded, and purified by ultracentrifugation. Purified virus was dialysed against phosphate precipitation. Recombinant adenoviruses were cotransfected with pJM17 into 293 cells by calcium

Migration assay

Inserts (8 μm pores; Costar, Madrid, Spain) for 24 well culture plates were coated with 100 μg/ml of rat tail collagen type I (Becton Dickinson, Bedford, Massachusetts, USA). HUVE cells with a passage number of 4–6 (25 000 cells/50 μl) were seeded into the upper chamber. The lower chamber was filled with Medium 200 containing 1% bovine serum albumin. HUVE cells were preincubated with CM at 37°C for 30 minutes before adding vascular endothelial growth factor (VEGF) at a final concentration of 5 ng/ml to the lower chamber. These chambers were incubated for six hours to allow cells to migrate through the collagen coated pore membranes. Non-migrated cells were thoroughly scraped off the upper surface membrane with cotton swabs. The membrane was stained with Diff-Quick (Dade Behring, Düdingen, Switzerland). Five to eight representative fields in each well were counted at 100× magnification to determine the number of migrated cells. Migration was expressed as percentage of maximal migration (defined as migration with VEGF stimulation without addition of CM).

Tube formation assay

The 24 well plate was coated with 320 μl Matrigel. HUVE cells (25 000 cells/75 μl) were dispensed into each well and incubated with 75 μl of CM for 30 minutes. After adding 150 μl of Medium 200 containing 10% low serum growth supplement (Cascade Biologics, Portland, Oregon, USA), cells were incubated for another six hours. Tube formations were quantified by counting intact tubes in the whole well under microscopy at 40× magnification.

In vivo testing of angiogenic effects (Matrigel angiogenesis assay)

The Matrigel assay was performed with modifications, as described previously.

Athymic mice were treated by intravenous injection of Adeno, AdK1-3, or AdlacZ at a dose of 10^10 plaque forming units (pfu) per animal. Six hours later, 150 μl of VEGF embedded Matrigel was injected subcutaneously into the left and right midabdominal region of mice. After 14 days mice were sacrificed and Matrigel plugs were removed and stained (haematoxylin-eosin). Quantitative analysis was done by counting the total number of endothelial-like cells in the Matrigel plugs with a graticule (ProSciTech, Queensland, Australia; surface patter 21 mm) under a microscope. Results were expressed as mean (SEM) cell number per square scale (mm²).

In vivo testing of antitumoral effects

For establishment of LLC in C57BL/6 and athymic mice, 10^6 LLC cells were injected subcutaneously into the right flank of mice. When the tumours reached 50 mm³ in volume, animals were treated by systemic injection of AdK1-3, Adeno, or the control vector AdlacZ at a dose of 10^10 pfu per animal. In the case of human HCC (Huh7) and human CRC (HT29), 2×10^6 and 10^7 cells were injected subcutaneously into athymic mice, respectively. After 10 days, HCC bearing mice were treated by systemic injection of AdK1-3, Adeno, or control vector AdlacZ at a dose of 10^10 pfu per animal. Murine CRC were established by implantation of 5×10^3 CT-26 cells into the right hind of Balb/c mice. When murine (CT-26) or human (HT-29) CRC tumours reached approximately 5 mm³ in volume, tumour bearing mice were treated with intratumoral injection of AdK1-3, Adeno, or the control vector AdlacZ at a dose of 5×10^6 or 10^10 pfu per animal, respectively. Tumour growth was monitored by caliper measurement. Tumour volume was calculated from the formula: V = length × width² × 0.52.
Assessment of microvessel density

Paraffin sections of tumours that received therapy with adenoviral vectors were immune stained with rabbit anti-von Willebrand factor (factor VIII-related antigen; Dako, Glostrun, Denmark) and followed by sequential incubation of the sections with LSAB2 system (Dako). Enzymatic activity was developed using DAB (Dako) as chromogenic substrate and followed by counterstaining with Mayer haematoxylin.

Statistical analysis

Tumour data are given as mean (SEM) tumour volume. Differences between tumour volumes in different experimental groups were analysed for statistical significance by a non-parametric two tailed test (Mann-Whitney test) for unpaired samples; in the case of histology sections, differences between groups were calculated by the Student’s t test for unpaired samples. A p value of <0.05 was considered significant.

RESULTS

Construction of adenoviruses containing angiostatin-like molecule and endostatin (AdK1-3 and Adendo)

Recombinant adenoviral vectors coding for angiostatin-like molecule and endostatin were constructed. The cDNA sequence was confirmed by sequencing, and transgene expression was shown in A549 cells infected with different multiplicities of infection (MOIs) of AdK1-3 and Adendo by RT-PCR. The predicted PCR products were detected in the AdK1-3 and Adendo groups whereas no band was amplified in cells infected with the control vector AdlacZ. Expression of the housekeeping gene, β-actin, was similar in all samples (results not shown). In order to demonstrate whether transgene products can be secreted into the supernatant of A549 cells infected with AdK1-3 and Adendo, supernatant from infected cells was collected for detection of angiostatin-like molecule by western blot and detection of endostatin by ELISA. Figure 1A shows that angiostatin was detected at a molecular weight of 38 kDa only in supernatant from AdK1-3 infected cells and not in supernatant from control vector AdlacZ infected cells. We also found that secretion of endostatin into the cell supernatant of Adendo infected A549 cells was dose dependent, with the highest level of approximately 900 ng/ml at an MOI of 500 (fig 1B). These data demonstrate that infection with AdK1-3 and Adendo induces corresponding protein expression and secretion in infected tumour cells.

To demonstrate in vivo production of endostatin by adenoviral vectors, Balb/c mice that received Adendo or control vector AdlacZ at a dose of $10^{10}$ pfu per animal or saline intravenously were sacrificed seven days later and blood samples collected for measurement of endostatin. Figure 1C shows that high levels of endostatin were found in serum of two Balb/c mice. Compared with the AdlacZ control (fig 2B), while addition of CM to AdlacZ infected A549 cells did not affect the ability of HUVE cells to migrate through a perforated membrane, addition of CM containing K1-3 and endostatin inhibited cell migration by 64% and 86%, respectively (fig 1C).

Biological activity of angiostatin-like molecule and endostatin produced by AdK1-3 and Adendo in vitro

Angiostatic proteins can be characterised by inhibitory effects on different endothelial cell functions: proliferation, migration, and tube formation. To evaluate the antiproliferative effect of CM from A549 cells infected with AdK1-3, Adendo, or control vector AdlacZ, HUVE cells were preincubated with CM at a final concentration of 30%. We discovered that preincubation of HUVE cells with CM from AdK1-3 and Adendo infected cells resulted in inhibition of proliferation by 39% and 38%, respectively, in comparison with AdlacZ (fig 2A). Both proteins also showed similar inhibitory effects on tube formation of HUVE cells. Tube formation was reduced by approximately 43% and 39%, respectively, compared with the AdlacZ control (fig 2B). While addition of CM to AdlacZ infected A549 cells did not affect the ability of HUVE cells to migrate through a perforated membrane, addition of CM containing K1-3 and endostatin inhibited cell migration by 64% and 86%, respectively (fig 1C).

Inhibition of angiogenesis in vivo by AdK1-3 and Adendo

To evaluate whether angiostatin-like molecule and endostatin produced by AdK1-3 and Adendo inhibited angiogenesis in vivo, animals were inoculated subcutaneously with VEGF containing Matrigel, six hours after systemic administration of AdK1-3, Adendo, or the control vector AdlacZ. Matrigel plugs were collected for further histological examination two weeks later. As shown in fig 3, intense invasion by endothelium-like cells was observed in Matrigel plugs from animals that received saline and control vectors AdlacZ. In contrast, Matrigel plugs from mice that had received AdK1-3 showed much less infiltration of endothelium-like cells. Administration of Adendo induced only minor inhibitory effects compared with control animals who received...
saline or AdlacZ. Quantification of endothelium-like cells on Matrigel sections (fig 3) showed that administration of AdK1-3 and Adendo resulted in a reduction in cell infiltration of 48% and 13%, respectively, in comparison with animals who received the control vector AdlacZ.

Effect of systemic administration of AdK1-3 and Adendo on established LLC tumours in athymic mice

To evaluate the antitumoral effect of AdK1-3 and Adendo on CRC, murine CT-26 and human HT-29 CRC cells were inoculated subcutaneously into Balb/c or athymic mice, respectively. When CT-26 tumours reached a size of approximately 50 mm³, tumour treatment was initiated by intratumoral injection of the corresponding vectors at a concentration of 5×10⁹ pfu/mouse. Again, AdK1-3 induced a significant reduction in tumour growth by up to 41% on day 8 compared with the saline control whereas Adendo had no inhibitory effect (fig 6). Subcutaneous HT-29 tumours were treated by intravenous (n = 5 each group) or intratumoral (n = 9 each group) injection of AdK1-3, Adendo, or AdlacZ, respectively, at a concentration of 10¹⁰ pfu/mouse.
Antiangiogenic gene therapy treated by intravenous injection of recombinant adenoviruses (10^{10} plaque forming units/mouse) or the control vector AdlacZ or saline. Size of tumour was measured and presented as mean (SEM), n = 7–13. Tumour sizes were measured and are presented as mean (SEM), n = 6–10. *p < 0.05 for AdK1-3 day 3–day 13 compared with the control groups who received AdlacZ (Mann-Whitney).

Inhibition of tumour growth on established colorectal cancer (LLC) tumours in C57BL/6 and athymic mice by systemic administration of AdK1-3 or Adendo. Ten days after implantation of Huh7 cells, mice were treated by intravenous injection of recombinant adenoviruses (10^{10} plaque forming units/mouse) or saline as control. Size of tumour was measured and presented as mean (SEM), n = 6–10. *p < 0.05 for AdK1-3 day 4–day 12 compared with the AdlacZ control (Mann-Whitney).

Figure 4 Inhibition of tumour growth on established Lewis lung carcinoma (LLC) tumours in C57BL/6 and athymic mice by systemic administration of AdK1-3 or Adendo. Established LLC tumours of approximately 50 mm^3 in C57BL/6 mice (A) and athymic mice (B) were treated by intravenous injection of recombinant adenoviruses or the control vector AdlacZ or saline. Tumour sizes were measured and are presented as mean (SEM), n = 7–13. *p < 0.05 for AdK1-3 and Adendo compared with the AdlacZ control (day 4, Mann-Whitney).

Figure 5 Inhibition of tumour growth in athymic mice with hepatocellular carcinoma tumours by systemic administration of AdK1-3 or Adendo. Ten days after implantation of Huh7 cells, mice were treated by intravenous injection of recombinant adenoviruses or saline as control. Size of tumour was measured and presented as mean (SEM), n = 6–10. *p < 0.05 for AdK1-3 day 3–day 13 compared with the control groups who received AdlacZ (Mann-Whitney).

protein specificity of angiostatic tumour therapies is a striking aspect as tumour angiogenesis is considered to be a common pathological process independent of tumour type. Our findings indicate that different tumour entities have to be specifically evaluated for antitumour responsiveness towards distinct angiostatic proteins.

Recently, antiangiogenic tumour therapy attracted broad attention, and experimental data showed promising results with complete tumour elimination in some cases. The underlying concept appears convincing: to cut off the blood supply to the tumour and hence starve the tumour. The two physiological proteins, angiostatin and endostatin, showed antitumour effects in several studies. However, production of the corresponding recombinant proteins is complicated. It has been shown recently that ex vivo gene transfer for angiostatin (representing the first four kringles of plasminogen) reduced effectively the tumorigenicity of HCC cells. However, in vivo treatment of established tumours remains challenging. Adenoviral mediated gene transfer for cDNA encoding angiostatic proteins is a promising approach allowing the body to produce its own angiostatic drug. Indeed, several experimental studies, including our own, demonstrated that recombinant adenoviruses are an appropriate vehicle for gene transfer of angiostatin-like molecule and endostatin.

Inhibition of tumour angiogenesis by systemic administration of AdK1-3 and Adendo in a subcutaneous LLC model

The underlying antitumoral mechanisms could be attributed, at least in part, to angiostatic effects of angiostatin-like molecule and endostatin as intratumoral vessel density was decreased significantly in both treatment groups compared with the AdlacZ control. For this purpose, intratumoral microvessels were immune stained against von Willebrand factor in LLC tumours. LLC tumours were removed seven days after treatment initiation. Tumours from control animals, AdlacZ and NaCl, showed intensive staining against von Willebrand factor, indicating extensive angiogenesis in these tumours. In contrast, tumour samples from AdK1-3 treated animals showed significant reduction of microvessel density of approximately 60%. (Fig 7)

DISCUSSION

In this study, we showed that generation of angiostatin-like molecule (representing the first three kringles (K1-3) of plasminogen) and endostatin by adenoviral mediated gene transfer caused tumour and protein specific antitumoral effects in three different tumour entities in mice: colorectal, hepatocellular, and Lewis lung carcinoma. Tumour and
et al found strong antimitostatic effects of endostatin by gene transfer but only minor effects on primary tumour growth.\(^8\) In a comparative antitumour study, angiostatin and endostatin were less effective than VEGF receptor antagonists when tested in T241 fibrosarcomas.\(^7\)

To our knowledge, the latter publication was the first to focus on a direct comparison of different angiostatic gene therapies. Our study focused on gene delivery for angiostatin-like molecule and endostatin, and confirmed the reported antitumoral effects in subcutaneous LLC tumours in immune competent mice. In addition, we observed that only angiostatin-like molecule maintained significant antitumoral effects in immune deficient mice. Furthermore, we showed for the first time in a direct comparative study that administration of AdK1-3 resulted in significant antitumoral effects in a human HuH7 HCC and a murine CT-26 CRC model whereas no effects of AdK1-3 were detected in a human HT-29 CRC tumour model. Despite similar in vitro effects on endothelial cell functions and in vivo effects in the LLC tumour model, endostatin had no effect in these particular tumour models. Several mechanisms may be involved in these tumour specific antitumour efficacies: (1) protein expression and protein levels may be mouse strain specific and thereby influence antitumour effects; (2) angiostatic antitumour effects may involve primary or secondary immune responses dependent on the applied protein; and (3) tumour angiogenesis is a tumour specific process.

Wen et al showed large discrepancies between time periods and serum levels of protein expression in different mouse strains after gene delivery of endostatin.\(^8\) Although this may contribute to our findings, it is probably not the sole reason as we documented antitumoral effects over a short time period of only two weeks. Interestingly, the authors found strong antimitostatic effects for endostatin in an EOAMA metastasis model in 129/J mice but there was no effect for B16 melanoma in a metastasis model in C57BL6/J mice. However, in our study, treatment of LLC tumours by adenoviral mediated gene transfer for angiostatin and endostatin was effective in the C57BL6/J mouse strain. Our findings correspond well to the antitumour and antimetastatic effects found for LLC tumours described by Sauter and colleagues.\(^8\) Thus it can be concluded that the antimitumoral effects of endostatin do not depend on the mouse strain but on the tumour entity that is treated, indicating that tumour angiogenesis may have to be reconsidered as a complex tumour specific process.

Considering the different antitumour effects for LLC tumours in immune competent and immune deficient mice, secondary or primary immune responses contribute essentially to the antimitumoral effects of endostatin but are less important for the antitumour efficacy of angiostatin-like molecule. This is supported by our observation that reduction of microvessel density in LLC tumours was similar for endostatin and angiostatin-like molecule.

Taking into account the previous studies of Kuo and colleagues\(^7\) and Wen and colleagues,\(^11\) our data further strengthen the hypothesis that tumour vascularisation is a tumour specific process and therefore requires the use of different antitumoral angiostatic proteins. As this aspect is complicating angiostatic antitumour therapy, further studies focusing on basic molecular mechanisms of tumour angiogenesis are warranted. Nevertheless, this study also underlines the fact that angiostatin-like molecule has significant antitumour effects in a human hepatocellular carcinoma model and therefore may contribute towards alternative therapy strategies for this tumour entity.

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**Authors’ affiliations**

V Schmitz*, L Wang, M Barajas, C Gomar, J Prieto, C Qian, Division of Hepatology and Gene Therapy, Department of Medicine, Medical School, University of Navarra, Pamplona, Spain

Present address: Department of Internal Medicine, University Hospital Bonn, Germany

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Treatment of colorectal and hepatocellular carcinomas by adenoviral mediated gene transfer of endostatin and angiostatin-like molecule in mice

V Schmitz, L Wang, M Barajas, C Gomar, J Prieto and C Qian

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