LIVER

Plasminogen fragment K1–5 improves survival in a murine hepatocellular carcinoma model

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Background: The prognosis of patients with hepatocellular carcinoma (HCC) remains poor, and new alternative treatments are needed.

Aims: To comparatively test the angiostatic and antitumour effects of adenoviral gene transfer of angiostatin (PlgK1–4, amino acids 1–440) and full kringle 1–5 (PlgK1–5, amino acids 1–546) in a model of subcutaneously transferred HCC in mice.

Methods: PlgK1–4 and PlgK1–5 were generated from human WtPlg cDNA and used for adenovirus construction. Vector function and angiostatic effects were confirmed in vitro and in vivo. Antitumoral efficacies of intratumoral vector injections were studied in a model of subcutaneously transferred HCC model.

Results: Cell supernatants containing PlgK1–4 and PlgK1–5 reduced endothelial tube formation in vitro by about 30%, whereas WtPlg exerted no inhibitory effect. Endothelial cell infiltration in vivo was decreased by about 60%, but not in AdWtPlg-treated animals. Intratumoral treatment of subcutaneous HCC tumours inhibited growth by 40% for AdPlgK1–4 and 63% for AdPlgK1–5 in surviving mice 12 days after initiation of treatment, whereas treatment with AdWtPlg even led to accelerated growth. Although PlgK1–4 and PlgK1–5 have similar inhibitory effects on intratumoral microvessels, PlgK1–5 markedly improved the survival time compared with PlgK1–4.

Conclusion: PlgK1–5 and PlgK1–4 effectively inhibited HCC growth. As PlgK1–5 could also prolong the survival time, inducing complete tumour elimination in half of the AdPlgK1–5-treated mice, PlgK1–5 might be the most potential plasminogen fragment for treatment of experimental HCC.

Most patients with hepatocellular carcinoma (HCC) are not eligible for curative treatments, and therefore there is an urgent need for innovative treatment strategies to curb HCC disease. As in other tumour entities, it has been acknowledged that HCC progression is associated with tumour angiogenesis.^{1–2} Vascular endothelial growth factor (VEGF) has been identified as a key mediator of tumour angiogenesis and its specific role has also been shown in patients with HCC.^{3–9} Consecutively, a spectrum of angiostatic treatments has been tested to be effective in HCC, embracing—among others—anti-VEGF treatments, endostatin and angiostatin.^{5–15}

Plasminogen derivatives such as angiostatin are well-known antiangiogenic factors. Angiostatin was first described in 1994: O'Reilly *et al*¹⁶ showed that application of angiostatin resulted in a marked inhibition of tumour growth in a murine Lewis Lung Cancer model. So far, several studies have been published, which used angiostatin (PlgK1–4) protein successfully and also adenoviral-mediated gene transfer of angiostatin cDNA to successfully treat distinct tumour entities.^{16–21} Mostly, differing inhibitory effects on tumour growth was achieved, but tumour elimination was rarely observed. The different kringle regions of plasminogen have been characterised and Cao *et al*²² firstly showed that the human kringle region 5 (K5, amino acids 449–547 and amino acids 449–563, respectively), generated via proteolytic digestion of human plasminogen, also exerted antiangiogenic effects compared with K1–4 (amino acids 95–454).²² It was later shown that kringle regions K1–4.5, bearing kringle 1–4 and most of kringle 5 (amino acids 95–529), had even stronger antiangiogenic and antitumoral effects.^{23–25}

To our knowledge, there is no antitumour study that has comparatively analysed antitumour effects of adenoviral-mediated gene delivery of K1–4 and full K1–5. Previous studies showed angiostatic effects by using K1–4.5 obtained by

proteolytic digestion. During the preparation of this manuscript, one study was published, which showed strong angiostatic and antitumoral effects using adenoviral gene transfer of a different sequence of full kringle 1–5 (amino acids 1–566), including the plasmin generation site.²⁴ Further, it is unclear whether antitumour effects that have been achieved in other tumour entities can also be transferred to HCC and, if so, whether human plasminogen fragment containing the five kringle regions (PlgK1–5) is able to induce complete tumour regression in experimental HCC in mice.

METHODS

Animals and cell lines

Six-week-old male C3H and C57Bl/6 mice were supplied by Charles River (Sulzfeld, Germany) and kept in the local central animal facility of the University Hospital Bonn, Bonn, Germany. The mice were housed under standard conditions and had free access to water and food. Animal procedures were performed in accordance with approved protocols and recommendations for proper care and use of laboratory animals were followed.

The human lung cancer cell line A549 and the embryonic E1-transformed kidney cell line 293 were obtained from American Type Culture Collection (Rockville, Maryland, USA). Cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum.

Abbreviations: BrdU, bromodeoxyuridine; HCC, hepatocellular carcinoma; Hepa129, hepatoma 129; hpf, high-powered field; HUVE, human umbilical vein endothelial; PlgK1–4, angiostatin; PlgK1–5, plasminogen fragment containing the five kringle regions; PCR, polymerase chain reaction; pfu, plaque forming units; BFA, brefeldin A; VEGF, vascular endothelial growth factor; WtPlg, wild-type form of human plasminogen

Hepatoma 129 (Hep129) cells obtained from the NCI-Frederick Cancer Research and Development Center (DCT Tumor Repository; Frederick, Maryland, USA), were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and 200 mM glutamine.

Human umbilical vein endothelial (HUVE) cells were obtained from Cascade Biologics (Portland, Oregon, USA) and were cultured according to the supplier's instructions.

Insertion of the stop codon and restriction site

Internal fragments (PlgK1-4, amino acids 1–440; and PlgK1-5, amino acids 1–546) were synthesised using polymerase chain reaction (PCR). As a template, human wild-type form of plasminogen (WtPlg; cloned in pBluescript s/k-) was used. PlgK1-4 and PlgK1-5 were generated using specific primers (table 1). Each PCR was performed with 35 cycles as follows: a denaturation step for 3 min at 94°C, followed by 20 s at 94°C, 30 s at 55°C and 90 s at 72°C. The predicted sizes of the amplified products were 1419 bp for PlgK1-4 and 1737 bp for PlgK1-5. PCR products were analysed by electrophoresis on a 1% agarose gel. Correct insertion of the stop codon and restriction site were confirmed by sequencing. PlgK1-4 and PlgK1-5 were then subcloned in the construction vector pSTBlue (AccepTor Vector Kit, Novagen, Madison, Wisconsin, USA) according to the manufacturer's protocol.

Generation of adenoviral vectors

First-generation adenoviral vectors encoding PlgK1-4, PlgK1-5 or WtPlg were constructed using the AdEasy System (Howard Hughes Medical Institute, Baltimore, Maryland, USA).²⁶ Recombinant adenoviruses were expanded, and purified by double caesium chloride ultracentrifugation. Purified viruses were dialysed against 10 mM TRIS/1 mM magnesium chloride and stored in glycerol aliquots at –80°C until further use. Virus concentrations were determined by measuring virus particles and by the cytotoxic plaque assay (plaque-forming units (pfu)/ml) in the cell line 293. Virus productions were tested for wild-type adenovirus contamination by the cytotoxic plaque assay in the A549 cell line. To test transduction efficiency in vitro, A549 cells infected with AdLacZ were stained with X-Gal.

Adenoviral DNA-extraction

Correct insertion of the transgene in the adenoviral vectors was confirmed by viral DNA isolation and PCR. DNA was isolated as described previously.²⁷ DNA, 200 ng, was used with a primer for the plasminogen signal sequence (5'-GCGGCCGATGGAACATAAGGAAGTGGTTC-3'). The same reverse primer was used for the generation of PlgK1-4 and PlgK1-5, and the reverse primer 5'-ATATCAAGGTGCCTCCAC-3' for WtPlg. PCR was performed with 35 cycles. PCR was started with a denaturation at 95°C for 90 s, followed by 30 s at 95°C, 30 s at 55°C and 2 min at 72°C. The predicted sizes of the amplified products were 1356 bp for PlgK1-4, 1654 bp for PlgK1-5 and 1806 bp for WtPlg. PCR products were analysed by electrophoresis on a 1% agarose gel.

Gene expression in vitro and in vivo

For in vitro gene expression and production of conditioned cell supernatant (conditioned media from cells transduced with the

adenoviral vectors), A549 cells were infected with 250 MOI of AdLacZ, AdWtPlg, AdPlgK1-4 or AdPlgK1-5. After 48 h, the cells were harvested for RNA isolation. For in vivo gene expression, C3H mice were intravenously injected with 5×10^9 pfu/animal AdLacZ, AdWtPlg, AdPlgK1-4 or AdPlgK1-5. Two days later, animals were killed and livers were explanted. Liver tissue samples, 3×3 mm in size, and the harvested cells were used for RNA isolation using the Nucleobond RNAII Kit (Macherey & Nagel, Düren, Germany). RNA concentration was determined and 1 µg RNA was used in the reverse transcriptase reaction with Random Primers (Promega, Mannheim, Germany) and MMLV-Reverse Transcriptase (Promega) according to the protocol. PCR was performed using the same primer pairs as described for the transgene confirmation. Primers for human β-actin were used as controls, the same PCR conditions were applied. For each PCR, 30 cycles were performed as described earlier. As a control for in vivo gene expression, murine β-actin primers (Stratagene, La Jolla, California, USA) were used. PCR was performed according to the manufacturer's protocol. The predicted band size for murine β-actin was 500 bp.

Tube formation in vitro

To evaluate in vitro effects of angiostatin, a tube formation assay was performed. A 24-well plate was coated with 300 µl Matrigel (Gibco/BRL, Karlsruhe, Germany) containing 10 ng/ml VEGF. After 24 h, 2.5×10^4 HUVE cells in 75 µl Medium200 were seeded on the Matrigel and preincubated for 30 min with 75 µl of the conditioned cell supernatant (derived from the A549 cells). A total volume of 150 µl Medium200 (Cascade Biologics, Portland, Oregon, USA) containing LSGS was added and the cells were incubated for 4 h. Intercellular connections and tube-like formations were counted under the microscope (100×, n = 15). Results were expressed as the mean (standard error of mean (SEM), of endothelial-like structures in high-power fields (hpfs).

BrdU proliferation assay in vitro

Although plasminogen derivatives are supposed to have endothelial-specific effects, we analysed inhibition of proliferation in endothelial and tumour cells. A total of 5000 Hep129 and HUVE cells were resuspended in 40 µl of culture medium and dispensed in each well of a 96-culture plate and preincubated with 50 µl of the conditioned cell supernatant. After 30 min of preincubation, 150 µl of RPMI 1640 containing 10% fetal calf

Figure 1 Gene expression of human wildtype plasminogen (WtPlg), angiostatin (PlgK1-4) and full kringle of plasminogen (PlgK1-5) in vivo. Mice were intravenously injected with 5×10^9 pfu/animal. Two days later, animals were killed and the liver was explanted and shock frozen. RNA was isolated and reverse transcribed. Polymerase chain reaction (PCR) was performed using specific primers for human plasminogen. As a positive control, PCR was performed for murine β-actin. C, AdLacZ control; 1, AdWtPlg; 2, AdPlgK1-4; 3, AdPlgK1-5.

Table 1 List of the nucleotide sequences used in the study

Primer	Nucleotide Sequence
T7 (forward)	5'-TAA TAC GAC TCA CTA TAG GG-3'
K1-4 (reverse)	5'-AAG CTT TTA CGC TTC TGT TCC-3'
K1-5 (reverse)	5'-AAG CTT TTA AAA TGA AGG GGC-3'

Figure 2 Tube formation in vitro. Human umbilical vein endothelial (HUVE) cells were seeded on Matrigel (10 ng/ml vascular endothelial growth factor) and incubated with conditioned media from A549 cells transduced with AdLacZ, AdWtPlg, AdPlgK1-4 or AdPlgK1-5 infected A549 cells. (A) Complete tubes were counted under the microscope and compared with controls (mean (SEM); $n = 15$; $*p < 0.003$). (B–E) Exemplary microphotographs (100 $\times$) of HUVE cells incubated with the conditioned cell supernatant from cells infected with (B) AdLacZ, (C) AdWtPlg, (D) AdPlgK1-4 and (E) AdPlgK1-5.

serum or Medium200 containing LSGS was added. Cell culture was continued for 18 h and cells were then labelled with bromodeoxyuridine (BrdU) for another 24 h. The BrdU assay was performed according to the manufacturers protocol (BrdU Proliferation Assay, Roche Diagnostics, Mannheim, Germany).

Apoptosis: determination of the subG₁ fraction

The previous day, 1×10^6 HUVE and Hepa129 cells were seeded on a culture dish. The cells were incubated for 2 h with 15 ml of the conditioned cell supernatant. As a positive control, cells were incubated with 2 μ M Brefeldin A. Culture medium was added to cells and incubation was continued for another 70 h. The cells were harvested, centrifuged and resuspended in 1 ml HBSS. A volume of 10 ml ethanol (70%) was added and the cells were incubated at -20°C for 24 h. Cells were washed two

times with phosphate-buffered saline/ Ca^{2+} / Mg^{2+} , and were resuspended in 40 μ l of extraction buffer and incubated for 30 min at room temperature. To this suspension, 1 ml propidiumiodid solution (50 μ g/ml) and 50 μ l RNase A (10 mg/ml) were added, and cells were incubated at room temperature for 30 min. The subG₁ fraction was analysed using a Beckman Coulter Epics XL cell sorter (Beckman Coulter, Inc., Fullerton, California, USA). Fluorescence was measured by fluorescence channel 2.

In vivo testing of antiangiogenic effects (Matrigel angiogenesis assay)

The Matrigel assay was performed with modifications as described previously.²⁸ VEGF-embedded Matrigel (500 μ l) was injected subcutaneously into the left and right abdominal

Figure 3 In vivo testing of antiangiogenic effects. Matrigel (with 10 ng/ml vascular endothelial growth factor) was injected into the left and right abdominal region of C3H mice. After 24 h, the animals were treated intravenously with 5×10^9 pfu/animal AdLacZ, AdWtPlg, AdPlgK1-4 and AdPlgK1-5. Seven days later, mice were killed and Matrigel plugs were explanted and shock frozen. Cross-sections of the plugs were immunostained for CD31 and microvessels were counted under the microscope. (A) Number of microvessels in the Matrigel plugs compared with the control (mean (SEM); $n = 10$; $*p < 0.05$). Exemplary photomicrographs (100 $\times$) of animals treated with (B) AdLacZ, (C) AdWtPlg, (D) AdPlgK1-4, (E) AdPlgK1-5. E, epidermis; M, Matrigel; P, peritoneum; arrow, microvessels (red-brownish colour).

regions of the mice. After 24 h, the mice were given an intravenous injection of 5×10^9 pfu AdLacZ, AdWtPlg, AdPlgK1-4 or AdPlgK1-5 per animal. Mice were killed after 7 days and Matrigel plugs were removed and shock frozen. Cross-sections were immunostained against CD31. Quantitative analysis was carried out by counting the total number of endothelial vessels/hpf (100 $\times$). Results were expressed as the mean (SEM) number of vessels/hpf.

Tumour induction

In vivo antitumoural effects were studied in a subcutaneous HCC mouse model using syngenic C3H mice. 10^6 Hepa129 HCC cells were resuspended in 150 μ l FCS-free culture medium and injected subcutaneously via a 28 G syringe into the right hind flank.

In vivo antitumour treatment

Reaching a mean tumour volume of 150 mm³ (about 14 days after tumour-cell implantation), animals were treated with a single, fanned intratumoral injection of the first generation adenoviral vectors AdLacZ, AdWtPlg, AdPlgK1-4 and AdPlgK1-5. The total virus dosage was 5×10^9 pfu/animal. Tumour growth was monitored by Vernier caliper measurement and

survival rates were assessed by using intention-to-treat modalities. Animals were killed when the mean tumour diameter reached 15 mm. This was defined as the point of death. Tumour volumes were calculated by the formula

$$V = \text{length} \times (\text{width})^2 \times 0.52.$$

Histological sections and assessment of microvessel density

Tumour samples were harvested and frozen in liquid nitrogen. To determine intratumoral microvessel density, cryopreserved tumour sample sections were immunostained with rat anti-mouse CD31 (1:200, Dako, Hamburg, Germany) and incubated with a biotinylated rabbit anti-rat antibody (Dako) and streptavidine peroxidase. Enzymatic activity was developed using AEC (Dako) as substrate and sections were counterstained with Mayer haematoxylin. Intratumoral CD31-positive vessels were considered for quantification when they showed a red-brownish, cytoplasmic staining.

Statistical analysis

Tumour data are given as mean (SEM) tumour volumes. Differences between tumour volumes of different experimental groups were analysed for statistical significance by a

non-parametric, two-tailed test (Mann-Whitney U test) for unpaired samples; in the case of histology sections, differences between groups were calculated by Student's t test for unpaired samples. Significance of complete tumour elimination was estimated by two-tailed Fisher's exact test. Survival rates are shown as Kaplan-Maier curves and significance was calculated by the log-rank test. A value of $p < 0.05$ was considered to be significant.

RESULTS

Generation of PlgK1–4 and PlgK1–5

After sequencing and subcloning, correct insertion of PlgK1–4 and PlgK1–5 into the vector was tested by restriction digestion. Agarose gel electrophoresis of the restriction digestion showed the correct band size for PlgK1–4 and PlgK1–5, making both cDNAs usable for adenovirus construction (data not shown).

Propagation of adenoviral vectors and confirmation of correct transgene insertion

Every cloning step in the AdEasy System was confirmed by restriction digestion and agarose gel electrophoresis (data not shown). As a last step, the transgenes were analysed using adenoviral DNA. PCR for the different adenoviral vectors encoding AdWtPlg, AdPlgK1–4, AdPlgK1–5 showed the expected band sizes 1356 bp for PlgK1–4, 1654 bp for PlgK1–5 and 1806 bp for WtPlg. AdLacZ, used as control, showed no band in the agarose gel electrophoresis (data not shown).

Wild-type contamination of the propagated adenoviral stock solutions was analysed in the A549 cells by exclusion of any cytotoxic plaques. Virus concentrations were determined as optical particle units, and ranged from 2×10^{12} to 8×10^{12} opu/ml for AdLacZ, AdWtPlg, AdPlgK1–4 and AdPlgK1–5. Titration by the 293 cell plaque assay showed corresponding concentrations from 1×10^{11} to 1×10^{12} pfu/ml for the adenoviral vectors.

Assessment of gene expression

RNA of the infected A549 cells was isolated, reverse transcribed and amplified by PCR to show gene expression of WtPlg, PlgK1–4 and PlgK1–5. Gel electrophoresis of the PCR for β -actin showed similar bands (800 bp) in all samples. cDNA of cells infected with AdWtPlg, AdPlgK1–4 or AdPlgK1–5 had the expected sizes of 1806 bp for WtPlg cDNA, 1356 bp for PlgK1–4 cDNA and 1654 bp for PlgK1–5 cDNA (data not shown). Liver samples from animals injected with 5×10^9 pfu/animal AdLacZ, AdWtPlg, AdPlgK1–4 and AdPlgK1–5 showed, corresponding to the *in vitro* gene expression, similar bands in reverse transcriptase-PCR. Likewise, the control for murine β -actin showed bands at the expected size of 500 bp (fig 1).

In vitro testing of antiangiogenic effects (tube formation assay)

To evaluate the angiostatic function of the generated vector constructs, the influence of WtPlg, PlgK1–4 and PlgK1–5 on the capability of HUVE cells to form tube-like structures (connections of several endothelial cells to form a circular structure) was examined in a so-called tube formation assay. Compared with the AdLacZ control, PlgK1–4 and PlgK1–5 inhibited tube formation by 27% and 30%, respectively ($p < 0.003$; fig 2), indicating biological activity of the secreted gene products with a slight but not significant benefit for PlgK1–5 compared with PlgK1–4. WtPlg showed no inhibitory effects on tube formation. On the contrary, WtPlg led to a slight increase (2%) in tube formation compared with AdLacZ.

BrdU proliferation assay

To examine any direct effects of WtPlg, PlgK1–4 and PlgK1–5 on the proliferation of the Hepa129 tumour cells, a BrdU

Figure 4 Intratumoral treatment of hepatocellular carcinoma (HCC) tumour-bearing mice. In all, 10^6 hepatoma 129 (Hepa129) cells were implanted subcutaneously into the right hind flank of mice. After reaching a mean tumour volume of 150 mm^3 , treatment was initiated by intratumoral injection of 5×10^9 pfu/animal AdLacZ ($n = 10$), AdWtPlg ($n = 10$), AdPlgK1–4 ($n = 10$) and AdPlgK1–5 ($n = 10$). (A) Tumour growth was measured every 2 days. Data are expressed as mean (SEM) tumour volume. * $p < 0.05$ compared with AdLacZ. (B) Survival rates of tumour-bearing mice after intratumoral treatment with AdLacZ, AdWtPlg, AdPlgK1–4 or AdPlgK1–5. The point of death was defined when the mean tumour diameter reached 15 mm or due to death by another cause. Data are expressed as a Kaplan-Maier curve (* $p < 0.05$).

proliferation assay was used. As expected, no direct inhibitory and antiproliferative effects from the conditioned cell supernatant derived from cells infected with AdLacZ, AdWtPlg, AdPlgK1–4 or AdPlgK1–5 on Hepa129 cells were detected in this setting; however, it showed slight inhibitory effects on HUVE cells. The conditioned cell supernatant from cells infected with AdPlgK1–4 reduced cell proliferation by 20% and that from AdPlgK1–5-infected cells reduced significantly HUVE cell proliferation by 37% compared with the control ($p < 0.05$). Compared with PlgK1–4, PlgK1–5 showed stronger but not significant antiproliferative effects. The conditioned cell supernatant from AdWtPlg-infected cells did not show any antiproliferative effect on the HUVE cells (data not shown). Thus, it can be concluded that any potential antitumour effects had to be mediated by indirect (angiostatic) effects.

Apoptosis assay

As effects of vector constructs might be based on different cell functions, we also examined whether the conditioned cell

Figure 5 Microvessel density of hepatocellular carcinoma (HCC) tumours. Tumours of killed animals were explanted and shock frozen. Cryo sections were stained against vessels using anti-mouse CD31. Counterstaining was performed with Mayer haematoxylin. Endothelial vessels show a red-brownish colour. (A) Mean (SEM) number of endothelial vessels/hpf compared with AdLacZ (n=8, *p<0.05) (B–E) Exemplary photomicrographs (400×) of HCC tumours from animals treated with (B) AdLacZ, (C) AdWtPlg, (D) AdPlgK1-4 and (E) AdPlgK1-5.

supernatants of cells infected with AdLacZ, AdPlgK1-4, AdPlgK1-5, or AdWtPlg had proapoptotic effects on tumour and endothelial cells. This was analysed by determining the subG₁ fraction by flow cytometry. In the Hepa129 cells, no change in the subG₁ fraction was determined for PlgK1-4 (34.2%), PlgK1-5 (30.5%) or WtPlg (29%) compared with the control LacZ (32.5%). The Hepa129 cells incubated with 2 μ M BFA (positive control) showed a subG₁ fraction of 64%. The HUVE cells showed a subG₁ fraction of 20.2% for PlgK1-4 and 20.5% for PlgK1-5. SubG₁ fraction was 10% for WtPlg and 19.3% for the LacZ control. The HUVE cells incubated with BFA showed a subG₁ fraction of 94%.

In vivo testing of antiangiogenic effects (Matrigel angiogenesis assay)

To estimate whether the in vitro activity could be transferred to in vivo conditions, the angiostatic potency of systemic administration of AdWtPlg, AdPlgK1-4 and AdPlgK1-5 was shown in a Matrigel plug assay: endothelial-like structures were significantly reduced by 58% for AdPlgK1-4 and by 66% for AdPlgK1-5 compared with AdLacZ control (p<0.03).

Corresponding to the in vitro data, AdPlgK1-5 showed a slight but not significant reduction (about 18%) in microvessel density compared with AdPlgK1-4. Treatment with AdWtPlg did not lead to a reduction in endothelial vessels (fig 3). In vivo angiostatic activity of the systemic adenovirus application is a pre-requisite for evaluation of antitumour effects in vivo.

Local treatment

Subcutaneous HCC tumours showed 100% tumour take with continuous tumour progression in all control mice. No spontaneous tumour elimination was observed in this model.¹²

In this intention-to-treat study, angiostatic vector activity was associated with an overall reduction in tumour growth for both constructs, AdPlgK1-4 and AdPlgK1-5. Compared with control AdLacZ, tumour growth was significantly reduced by 38% for AdPlgK1-4 and 67% for AdPlgK1-5 in the surviving animals at day 12, with a treatment benefit of another 38% for AdPlgK1-5 compared with AdPlgK1-4 (p>0.05). By contrast, compared with control AdLacZ (fig 4A), treatment with AdWtPlg even showed a marked progression of tumour growth in this model. Inhibitory antitumoural effects were also

translated into an improved survival time and rate (observation time 120 days). The mean survival time was 29 days for AdPlgK1–4, 69 days for AdPlgK1–5 and 12 days for AdWtPlg compared with 19 days for control AdLacZ (fig 4B). Compared with AdPlgK1–4-treated animals survival was improved in AdPlgK1–5-treated animals. Further, AdPlgK1–4 treatment resulted in complete tumour regression in one mouse. But five animals that had received AdPlgK1–5 treatment, had a complete tumour elimination coinciding with long-term survival (>3 months) without any palpable or visible tumour recidive.

Microvessel density

C3H mice that had received local administration of AdPlgK1–4 or AdPlgK1–5 showed a marked reduction in intratumoral microvessel density by about 53.7% and 61.5%, respectively, compared with the control ($n=10$; $p=0.001$; fig 5A), as determined by intratumoral anti-CD31 immunostaining. AdPlgK1–5-treated animals showed the strongest reduction in microvessel density. By contrast, AdWtPlg-treated animals showed no reduction in microvessel density (fig 5B).

DISCUSSION

Therapeutic options for HCC are still rare. Principally, HCC is considered to be well suited for angiostatic, palliative treatment approaches. Angiostatic molecules such as angiostatin were used in a broad variety of experimental antiangiogenic cancer treatments. Most frequently, angiostatin (K1–4) or angiostatin-like molecules (K1–3) were applied to limit tumour burden,^{16 23 25 29–31} but recent studies showed even stronger angiostatic and more potent antitumoural effects for K5 and K1–4.5 compared with angiostatin itself.^{22–24 32–34} Here, we examined for the first time whether the full K1–5 is also suitable to inhibit experimental HCC and to induce complete tumour elimination.

To further elucidate differential effects of different kringles, we decided to comparatively use human PlgK1–4 (amino acids 1–440) and human PlgK1–5 (amino acids 1–546) to test for in vitro and in vivo angiostatic and antitumour activity. PlgK1–4 and PlgK1–5 showed inhibitory effects specifically on endothelial cell proliferation and tube formation in vitro, corresponding well to previous publications.³³ In contrast with other groups, we found no proapoptotic effects in this study.^{25 35} More importantly, angiostatic effects clearly could be seen in vivo. Invasion of endothelial cells in Matrigel plugs was inhibited by application of AdPlgK1–4 and AdPlgK1–5, fitting well to data of other groups that examined angiostatic effects in a mouse corneal micropocket assay.^{31 36} Subsequently, the in vitro and in vivo findings could be translated into marked antitumour effects in this HCC mouse model. Suitable with other non-gene transfer studies, AdPlgK1–5 exerted stronger antitumoural effects than AdPlgK1–4 in our setting.^{23 24 34}

Previous findings by Schmitt *et al*³⁷ and Andreasen *et al*³⁸ implying proangiogenic and pro-tumoral effects of WtPlg were confirmed in our study, showing an accelerated tumour growth with a similar intratumoral microvessel density in this treatment group compared with LacZ-treated animals. This might be explained by the ability of plasmin and plasminogen to degrade extracellular matrix components, secondarily inducing tumour cell invasion and migration.

Corresponding to a study by Galaup *et al* who used a related plasminogen fragment (PlgK1–5-embracing amino acids 1–566), gene delivery of PlgK1–5 (amino acid 1–546) improved the survival rate and also completely eliminated HCC tumours in 50% of treated mice in our study.^{19 24} Davidson *et al*³⁴ showed pro-apoptotic effects of PlgK1–5 on endothelial and tumour cells, but we did not detect direct effects of PlgK1–5 on the hepatoma cells.

Considering published data that have shown a prolonged half life of K1–5^{23 24} and a 1/50 half-maximal concentration of K1–5 compared with K1–4,²² it can be hypothesised that even slightly improved angiostatic effects add up to improved overall antitumoural effects. However, the mechanism of difference of antitumour effects of PlgK1–4 and PlgK1–5 in this experimental model remains unclear.

In conclusion, we showed marked angiostatic and antitumoural effects of adenovirus-mediated gene transfer of PlgK1–4 and PlgK1–5 in vitro and in vivo, whereas treatment with AdWtPlg was not beneficial. As AdPlgK1–5 was capable of completely eliminating experimental HCC in a considerable percentage of treated mice, these data can be used to further focus on PlgK1–5 as an antitumour approach for the treatment of HCC.

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EDITOR'S QUIZ: GI SNAPSHOT

Answer

From question on page 167

The diagnosis is small intestinal diverticulosis. Enteroscopy revealed multiple diverticula in the jejunum. There were shallow ulcerations in the diverticular orifice (fig 1) and mild luminal stenosis around the diverticulum (fig 2). More diverticula were revealed after instillation of the contrast agent (fig 3).

Small intestinal diverticulosis is rare, and 60–70% of cases are asymptomatic. The incidence ranges from 1.1% to 2.3% at enteroclysis, during postmortem examination or during surgical procedures. Most cases are found in patients who are in their seventh decade of life or older. The diverticula are most commonly distributed in the proximal small intestine, with 80% located in the jejunum, 15% located in the ileum and 5% located in both. Symptomatic diverticula have either acute or chronic manifestations. Chronic complications include malabsorption, chronic abdominal pain, intestinal pseudo-obstruction and chronic gastrointestinal bleeding. Acute complications include diverticulitis, abscess, perforation, intestinal obstruction and massive gastrointestinal bleeding.

The diagnosis of small intestinal diverticulosis is usually made after radiographic examination, at surgery or at autopsy. Endoscopic examination, however, has rarely been used to diagnose or treat small intestinal diverticulosis. In this case, double-balloon enteroscopy revealed ulcerations associated with the diverticula, providing stronger evidence to explain the bleeding episodes. Areas of luminal stenosis and adhesions of the bowel loops most likely resulted from previous episodes of diverticulitis.

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