Specific Induction of Tumor Neovasculature Death by Modified Murine PPE-1 Promoter Armed with HSV-TK

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Key Words

Tumor angiogenesis • Antiangiogenic treatment • Gene therapy • Murine PPE-1 promoter • Herpes simplex virus-thymidine kinase

Abstract

Background: One strategy to increase tissue specificity of gene therapy is to use promoters or enhancers. **Objectives:** (1) To enhance the selectivity of a murine preproendothelin-1 (PPE-1) promoter in tumor angiogenesis by using a positive endothelial transcription-binding element. (2) To test the specificity and efficiency of the modified PPE-1 promoter [PPE-1(3X)] in vitro and in vivo by using reporter genes, and the therapeutic gene herpes simplex virus-thymidine kinase (HSV-TK) in a mouse model of Lewis lung carcinoma (LLC). **Results:** The modified PPE-1 promoter specifically induced expression in the tumor angiogenic vascular bed with a 35-fold higher expression compared to the normal vascular bed of the lung. Thus, when the HSV-TK gene controlled by the modified PPE-1 promoter was used systemically, it induced tumor-specific necrosis, apoptosis and mononuclear infiltrates, leading to massive destruction of the neovasculature of the pulmonary metastasis, which suppressed metastasis development. **Conclusions:** These results show that an

adenoviral vector armed with HSV-TK controlled by the endothelial-selective murine PPE-1(3X) promoter is efficient and safe to target tumor neovasculature.

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Introduction

Specifically targeting a therapeutic gene to relevant cells is a challenging aspect of the gene therapy concept. One strategy to increase the specificity of gene therapy is to use promoters and/or enhancers that are specifically activated in the targeted cells, tissues or under defined physiological conditions [1–10]. Indeed it has been shown that specific targeting of foreign gene expression reduces transgene toxicity and prevents its expression in antigen-presenting cells, thereby decreasing the immune response against the transgene and prolonging its expression [11, 12]. In this prospective, angiogenesis, the formation of new blood vessels by sprouting of existing blood vessels, is an attractive target for gene therapy. Angiogenesis is

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Fig. 1. The murine PPE-1 regulatory elements inserted into the wild-type promoter to produce the PPE-1(3X). **a** Fragment of the regulatory element. **b** Schematic diagram of the endothelial-specific regulator cassette inserted into the wild-type murine PPE-1 promoter to generate the PPE-1(3X) promoter. The 3X fragment of the positive endothelial cell regulator containing 145 bp was inserted into the wild-type murine PPE-1 promoter downstream (–286 bp) to the endogenous fragment (–364 to –320 bp). The modified PPE-1 promoter, termed PPE-1(3X) (1.55 kb), was used to generate the PPE-1(3X) adenobased vectors.

regulated by hypoxia and growth factors and the use of gene therapy to induce antiangiogenic treatment has shown promising potential in mouse models [13–15]. We previously demonstrated [16] that the wild-type murine preproendothelin-1 (PPE-1) promoter, delivered by an adenoviral vector, leads to specific expression of the reporter genes luciferase and green fluorescence protein (GFP) in endothelial cells in vitro and in blood vessels in mouse models in vivo. However, two obstacles interfere with utilizing the wild-type murine PPE-1 promoter as an antiangiogenic-antitumor gene therapy tool: first, its relatively low levels of activity and, second, its activity in both resting and angiogenic endothelium. In the present study, we used a positive endothelial cell transcription-binding element, endogenously present in the murine PPE-1 promoter [17] in order to increase the promoter-specific activity in the tumor neovasculature. The specificity and efficiency of the modified PPE-1 promoter, termed PPE-1(3X) promoter, was tested in vitro and in vivo using reporter genes and the therapeutic gene herpes simplex virus thymidine kinase (HSV-TK) in the mouse model of Lewis lung carcinoma (LLC). The HSV-TK gene was used in this work as has been widely studied in gene therapy of animal tumors, providing a conditional suicide system. The enzyme phosphorylates the nucleoside analogue gancyclovir (GCV), leading to DNA polymerase inhibition, DNA replication inhibition and, consequently, cell death [16]. It was suggested that the migration of phospho-GCV to adjacent cells through gap junction augments the TK cytotoxic bystander effect [18]. To target the tumor neovasculature, Mavria et al. [19] used a retroviral vector armed with HSV-TK under the control of a hybrid pPET1-LTR promoter and showed specific

killing of endothelial cells in vitro, and reduction of tumor growth in established tumors.

The PPE-1(3X) promoter showed higher efficiency (3.5-fold) than the wild-type PPE-1 promoter and higher activity (35-fold) in the angiogenic tumor vasculature than in normal lung vasculature.

Moreover, systemic administration of the HSV-TK gene, under the control of the PPE-1(3X) promoter induced necrosis, apoptosis and mononuclear infiltrates and suppressed metastasis development in the LLC model and not in normal tissues. Thus suggesting that the murine PPE-1(3X) promoter is suitable for selectively targeting therapeutic genes into angiogenic tumor beds.

Materials and Methods

Modification of the Wild-Type Murine PPE-1 Promoter

The modified murine PPE-1 promoter, named PPE-1(3X), was developed by inserting extra native copies of the positive transcription element into the *NheI* restriction enzyme site located downstream (–286 bp) to the 43-bp endogenous positive element (–364 to –320 bp). The fragment was synthesized by using two single-stranded DNA oligonucleotides (General Biotechnologies, Israel) of 96 bp each that were annealed at the compatible sites (43 bp) followed by a fill-in reaction with Klenow fragment (MBI Fermentas, Hanover, Md., USA). The double-stranded DNA (145 bp, blunt ended) was introduced intermedially into a Bluescript plasmid (Stratagene, La Jolla, Calif., USA) in *SmaI* restriction sites, followed by introduction of the element into the wild-type PPE-1 promoter in the *NheI* restriction sites (fig. 1).

Preparation of the Recombinant Adenovirus

All recombinant type 5 adenovirus vectors used in this study were generated as previously described. In brief, GFP, luciferase and the (HSV-TK) genes were subcloned under the control of the

PPE-1(3X) promoter by using the *NotI* restriction site. Adenoviruses, termed Ad5PPE-1(3X)Luc, Ad5PPE-1(3X)GFP or Ad5PPE-1(3X)HSV-TK were prepared by cotransfections of HEK-293 cells with pPACPPE-1(3X)Luc/GFP/HSV-TK and the adenoviral DNA plasmid pJM17 [16].

The Ad5CMVLuc virus (kindly provided by R. Gerard from UTsw, Dallas, Tex., USA), Ad5CMV-GFP (Quantum Biotechnologies, Carlsbad, Calif., USA) and AdCMV-TK (Qbiogene Biotechnologies, Calif., USA) containing the CMV immediate early promoter were used as non-tissue-specific controls. The Ad5PPE-1Luc and Ad5PPE-1GFP viruses containing the wild-type murine PPE-1 promoter were previously generated in our laboratory [16] and used as controls for basal PPE-1 promoter activity.

All viruses were prepared in large scale according to the previously described procedure [20]. Viral stocks were tittered and stored at -20°C at a concentration of 10^{12} plaque-forming units/ml (pfu/ml).

Cell Culture

LCC cells (LLC-D122-96, kindly provided by Prof. L. Eisenbach, The Weizmann Institute of Science, Rehovot, Israel), human embryonic kidney (HEK-293), HepG2 (ATCC-HB-8065) and HeLa cells were grown in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), L-glutamine and 100 U/ml penicillin and streptomycin. Bovine aortic endothelial cells (BAEC, kindly provided by Prof. N. Savion, Goldshlager Institute, Sheba Medical Center, Tel Hashomer, Israel) and normal skin fibroblasts (NSF) HeLa (ATCC-CCL-2) were grown in low-glucose DMEM supplemented with 10% FCS, 200 mM L-glutamine and 100 U/ml penicillin and streptomycin. BAEC medium was supplemented with 1 ng/ml basic fibroblast growth factor (bFGF). Human umbilical endothelial cells (HUVEC) were produced as previously described by Jaffe et al. [21] and were grown in RPMI-1640 supplemented with 10% FCS.

Luciferase Activity and GFP Expression in Cells and Tissues

Luciferase activity and GFP expression in vitro and in vivo were determined as described previously [16, 22].

To assay the PPE-1 promoter activity quantitatively in vitro and in vivo, we used the luciferase gene expression system kit. 48 h after transfection or transduction, the cells were washed and 200 μl lysis buffer was added for 15 min. Cells lysates were collected and centrifuged for 15 min (14,000 rpm) at 4°C , followed by addition of 10 μl of the supernatants to 50 μl luciferase assay buffer. The activity was measured in a luminometer over a 20-second period.

To assay luciferase activity in mice organs, tissue (20 mg) was excised and homogenized in 1 ml of the homogenization solution and centrifuged for 15 min (14,000 rpm) at 4°C ; 10 ml of the supernatant were assayed for luciferase activity as described above. Results were expressed as luciferase light units per 1 μg protein for cells or 1 mg protein for tissues. Protein was measured using the Bradford assay with bovine serum albumin (BSA) as a standard.

To test the GFP expression in vitro, cells were washed twice with PBS and fixed for 30 min with freshly made 4% paraformaldehyde in PBS, followed by examination in a fluorescence microscope.

To test the cellular distribution of the delivered gene in vivo, tissues were fixed in freshly made 4% paraformaldehyde in 0.1 M phosphate buffer for 6 h at 4°C , soaked overnight in 30% sucrose at 4°C and frozen in OCT compound (Sakura, USA). The tissue blocks were sliced with a cryostat at 10 μm thickness and observed directly in a fluorescence microscope (fluorescein isothiocyanate filter).

The whole protocol was performed under dim light to prevent GFP bleaching.

In vitro Experiments: DNA Transduction

Cells were plated in 16-mm dishes 24 h prior to transduction. DNA transduction of BAEC, HUVEC, HepG2, HeLa and NSF cells was performed by incubation with 1, 5 and 10 m.o.i (multiplicity of infection) of Ad5PPE-1(3X)Luc for 4 h in a total volume of 200 μl growing medium, followed by incubation with the growing medium in a total volume of 2 ml for 48 h. Ad5PPE-1Luc was used as a control for the basal promoter activity and Ad5CMVLuc as a non-tissue-specific control.

Animals

Fourteen-week-old male C57BL/6 mice (Harlan Farms, Jerusalem, Israel) were used. All animal procedures were approved by the Animal Care and Use Committee, Sheba Medical Center, Tel Hashomer, Israel.

Tissue Gene Expression in Normal Mice

To localize the cellular expression, Ad5PPE-1-3X-GFP, Ad5PPE-1GFP or Ad5CMVGFP (10^{10} pfu/ml in 100 μl physiologic saline) was intravenously injected into the mice ($n = 12$ in each group). GFP expression was detected by fluorescence microscopy 5 days after injection. All mice appeared healthy and no toxicity or inflammation was noted in the liver or other tissue.

Tumor Implantation and Metastasis Formation

Implantation of LLC cells was performed as previously described [16]. LLC cells, 5×10^5 cells/ml in 50 μl physiologic saline, were injected into the footpad. When the primary tumor achieved a diameter of 7 mm, the mice were anesthetized followed by amputation of the limb distal segment. Lung metastases developed within 3 weeks after primary tumor removal.

Viral Vector Injections and GCV Treatment

In the reporter gene experimental groups, viral vectors that contain the reporter genes GFP or luciferase (Ad5PPE-1(3X)Luc, Ad5PPE-1Luc, Ad5PPE-1(3X)GFP, Ad5PPE-1GFP, Ad5CMVLuc or Ad5CMVGFP) ($n = 12$ in each group) were intravenously injected 10 days following leg amputation. In the therapeutic gene experimental groups, Ad5PPE-1(3X)HSV-TK, Ad5CMV-TK (100 μl of 10^{11} pfu/ml) or 100 μl saline were intravenously injected 5 days after leg amputation ($n = 77$). During the following 14 days, the mice received daily intraperitoneal injections of 100 mg/kg GCV according to the different treatment groups: Ad5PPE-1(3X)-TK plus GCV, Ad5CMV-TK plus GCV, Ad5PPE-1(3X)-TK without GCV, and saline plus GCV. The mice were sacrificed 24 days after vector injection; 25% mice of the control group died.

Tissue Analysis

Frozen and paraffin-embedded sections of lungs and livers were stained with hematoxylin and eosin (HE). Immunostaining

of angiogenic endothelial cells was performed on frozen sections using rat antimouse CD31 antibody (PharMingen BD Bioscience, Franklin Lakes, N. J., USA) [23]. For apoptotic cell determination, TUNEL staining was performed on paraffin-embedded slides, cut into 4- μ m slices, using the in situ Cell Apoptotic Detection kit (Boehringer Mannheim, Mannheim, Germany). Anti-caspase-3 staining was performed on paraffin-embedded specimens, cut into 4- μ m slices, using anti-caspase-3 polyclonal antibody (Biocare Medical, Concord, Calif., USA). Nuclei were stained with Hoechst 33258 (Sigma Diagnostic). Blood was collected from the orbital sinus and tested for liver and kidney functions.

Statistical Analysis

ANOVA for parametric variables and the Kruskal-Wallis test for nonparametric variables were used to compare more than two groups in in vitro and in vivo experiments. Student's t test for parametric variables and the Mann-Whitney test for nonparametric variables were used to compare two unpaired groups in in vitro and in vivo experiments. The Tukey multiple comparison test was performed if the ANOVA was found positive.

Results

Generating the PPE-1(3X) Promoter

A 145-bp fragment (termed 3X-fragment) composed of extra copies of the PPE-1 endothelial cell-specific transcription element binding site [17] (fig. 1a) was constructed and inserted into the wild-type murine PPE-1 promoter as described in Materials and Methods (fig. 1b). The PPE-1(3X) was sequenced and aligned with the wild-type murine PPE-1 promoter region (Genebank accession No. U07982; GI:560542).

The PPE-1(3X) Promoter Delivered by Adenoviral Vector Efficiently and Specifically Activates Gene Expression in Endothelial Cells in vitro

PPE-1(3X) promoter activity measured as luciferase activity was up to 450 times higher ($26,320 \pm 2,675$) in endothelial cells (HUVEC) compared to its activity in nonendothelial cells: 152.5 ± 3.5 , 58.0 ± 4.7 , 313.4 ± 59.1 in HeLa, HepG2 and NSF, respectively (fig. 2A). Moreover, its activity in HUVEC was 2.5 times higher than that of the wild-type PPE-1 promoter and 100 times higher than that of the CMV promoter. In nonendothelial cell lines, CMV promoter activity was much higher than that of both PPE-1X and PPE-1(3X).

To study the specific activity of PPE-1(3X) in endothelial cells, GFP expression under the control of the PPE-1(3X) promoter was compared to GFP expression under the control of CMV (fig. 2B). High expression of GFP controlled by PPE-1(3X) was detected in BAEC and no expression was detected in non-endothelial cell lines.

In contrast, no expression was detected in endothelial cell lines under the regulation of the CMV promoter while high expression of GFP was detected in the non-endothelial cells under the regulation of CMV promoter.

The PPE-1(3X) Promoter Is Minimally Active in Normal Tissues of C57BL/6 Mice

Minimal luciferase expression was found in both highly vascularized and nonvascularized tissues of Ad5PPE-1(3X)Luc-injected C57BL/6 mice (data not shown). GFP expression detected in the mouse livers injected with Ad5PPE-1 or Ad5PPE-1(3X) viral vectors was minimal and restricted to the liver blood vessels' endothelial cells (fig. 3a). In contrast, GFP expression detected in mice injected with Ad5CMVGFP was found in the hepatocytes and no expression was detected in the blood vessels (fig. 3b).

PPE-1(3X) Promoter Is Highly Active in LLC Metastasized Blood Vessels

To study the PPE-1(3X) activity in LLC, luciferase expression controlled by PPE-1(3X) was measured following AdPPE-1(3X) injection to mice bearing LLC lung metastases. Luciferase expression controlled by PPE-1(3X) promoter increased 35-fold in the metastasized lungs compared to its expression in normal lungs and 3.5-fold compared to its expression under the control of PPE-1 ($p < 0.001$) (fig. 4). The relative luciferase expression in the metastasis lung compared to the expression in the liver was 30,000-fold higher in Ad5PPE-1(3X)Luc-treated mice. In order to localize the foreign gene expression under the regulation of PPE-1(3X) promoter, adenovirus containing GFP controlled by PPE-1(3X) was used. GFP expression controlled by the PPE-1(3X) promoter was expressed in blood vessels in the lung metastasis, as demonstrated by colocalization with anti-CD31 antibody (fig. 4b, c).

Gene Therapy with AdPPE-1(3X)-HSV-TK Suppressed Metastasis Development in LLC-Bearing Mice

A significant reduction in metastasis mass was found in mice treated with Ad5PPE-1(3X)-HSV-TK plus GCV, while a minimal reduction was found in mice treated with Ad5CMV-HSV-TK plus GCV. No effect was found in mice treated with saline plus GCV or Ad5PPE-1(3X)-HSV-TK without GCV (fig. 5a, b).

The mean weight of lung metastases in mice treated with AdPPE-1(3X)-HSV-TK plus GCV was three times lower than that in AdPPE-1(3X)-HSV-TK without GCV

Fig. 2. The efficiency and specificity of the PPE-1(3X) adeno-based vectors in vitro. **A** Luciferase activity in human cell lines transduced by Ad5PPE-1Luc, Ad5CMVLuc and Ad5PPE-1(3X)Luc. Higher luciferase expression resulted in HeLa, HepG2 and NSF cell transduction by Ad5CMVLuc. While high luciferase expression resulted in HUVEC cell transduction by Ad5PPE-1Luc or Ad5PPE-1(3X)Luc viral vectors, the luciferase expression under the control of PPE-1(3X) promoter was 2.5–25 times stronger in different experiments in endothelial cells compared to the expression under the wild-type promoter. Data represent the triplicate average of one of the three experiments performed. **B** GFP expression in BAEC (**a**), SMC (**b**), HeLa (**c**), HepG2 (**d**) and NSF (**e**) cells transduced by m.o.i. = 1 of Ad5PPE-1(3X)GFP (left column) and Ad5CMVGFP (right column). High GFP expression in BAEC (left column) resulted from Ad5PPE-1(3X)GFP transduction compared to lower GFP expression resulting from Ad5PPE-1GFP transduction (data not shown), and undetectable GFP expression resulting from Ad5CMVGFP transductions (right column).

Fig. 3. GFP expression in the livers of healthy C57BL/6 mice intravenously injected with Ad5PPE-1(3X)GFP (**a**) or Ad5CMVGFP (**b**). GFP under the control of PPE-1(3X) promoters was expressed in endothelial cells of the liver blood vessels. In contrast, GFP expression under the CMV promoter control was localized in the hepatocytes only and not in the blood vessels.

Fig. 4. Reporter gene (luciferase and GFP) expression in lung metastasis of LLC mice, injected with Ad5CMVLuc, Ad5PPE-1Luc, Ad5PPE-1(3X)Luc or Ad5PPE-1(3X)GFP. **a** Luciferase activity expressed as light units/mg protein was measured 5 days after systemic injection of Ad5CMVLuc, Ad5PPE-1Luc, or Ad5PPE-1(3X)Luc. Luciferase expression controlled by PPE-1(3X) promot-

er increased 35-fold in the metastasized lungs compared to its expression in normal lungs and 3.5-fold compared to its expression under the control of PPE-1 ($p < 0.001$). **b** Restricted GFP expression in LLC metastasized new blood vessels. **c** Anti-CD31 antibody staining of new blood vessels.

(0.12 ± 0.04 g vs. 0.68 ± 0.16 g, respectively; $p < 0.05$) (fig. 5b). The metastasis weight of mice treated with AdCMV-HSV-TK plus GCV was lower than that in mice treated with saline plus GCV or AdCMV-HSV-TK without GCV. However, this trend was not statistically significant.

The HSV-TK overexpression effect on the liver detected by HE staining showed mononuclear infiltrates and necrotic areas in AdCMV-HSV-TK plus GCV-treated mice but not in PPE-1(3X)-HSV-TK plus GCV-treated mice (fig. 6), indicating liver damage in the CMV- but not in the PPE-treated mice.

Gene Therapy with AdPPE-1(3X)-HSV-TK Induced Mononuclear Infiltration, Apoptosis and Necrosis in the LLC Lung Metastases

AdPPE-1(3X)-HSV-TK plus GCV treatment induced central necrosis of the lung metastasis, while mild peripheral necrosis was detected in metastasis of the AdPPE-1(3X)-HSV-TK without GCV treatment. Mononuclear infiltrates were found in Ad5PPE-1(3X)-HSV-TK plus GCV lung metastasis, while no infiltration was detected in AdPPE-1(3X)-HSV-TK without GCV treatment (fig. 6a). TUNEL staining of lung metastasis demonstrated numerous apoptotic tumor cells in AdPPE-1(3X)-HSV-TK plus GCV treatment compared to few apoptotic cells following treatment with AdPPE-1(3X)-HSV-TK without GCV (fig. 6b). Anti-caspase-3 staining in lung metasta-

sized blood vessels demonstrated massive endothelial cell apoptosis in AdPPE-1(3X)-HSV-TK plus GCV treatment compared to a small number of apoptotic cells in AdPPE-1(3X)-HSV-TK without GCV (fig. 6c).

AdPPE-1(3X)-HSV-TK and GCV Altered Angiogenic Vessel Morphology and Decreased Their Density in LLC Metastasis

Anti-CD31 antibody immunohistochemistry staining revealed that the neovasculature in lung metastasis of AdPPE-1(3X)-HSV-TK plus GCV-treated mice is short and without continuity and branching. In contrast, angiogenic blood vessels from lung metastasis of AdPPE-1(3X)-HSV-TK without GCV or from saline plus GCV-treated mice were long, branched and with distinctive borders (fig. 6d). Vascular density in the AdPPE-1(3X)-HSV-TK plus GCV-treated group was about 1.5 times lower than in the AdPPE-1(3X)-HSV-TK without GCV-treated group ($40,107 \mu\text{m}^2$ versus $61,622 \mu\text{m}^2$, respectively).

Discussion

Transcriptional targeting is one of the strategies used to regulate therapeutic gene expression specifically in a restricted cell population or tissue and increase both the efficiency and safety of gene therapy. In the present work, we modified the murine PPE-1 promoter by inserting ex-

Fig. 5. Effect of HSV-TK recombinant adenoviral vectors on lung metastasis development. **a** Representative pictures of lung metastasis in the different treatment groups indicate significant reduction of lung metastasis in AdPPE-1(3X)-TK with GCV treatment ($n = 11$) compared to AdPPE-1(3X)-TK without GCV ($n = 11$), AdCMV-TK with GCV ($n = 14$) and saline with GCV treatment ($n = 10$). **b** Mean lung weight in the different treatment groups is demonstrated to be significantly lower in the AdPPE-1(3X)-TK with GCV treatment compared to the other treatment groups.

tra copies of the endothelial-specific transcription elements [17] and constructed adenovirus vectors containing GFP, luciferase or HSV-TK under the control of the modified promoter, termed PPE-1(3X). The specificity and efficiency of the promoter in tumor angiogenesis improved as a result of this modification. Moreover, overexpression of the therapeutic gene HSV-TK induced massive necrosis, apoptosis and mononuclear infiltrates, destroying the neovasculature architecture and suppressing metastasis development in the LLC mouse model.

In a previous work we used the endothelial cell-specific, wild-type PPE-1 promoter to express reporter genes in tumor angiogenesis; however, its activity in tumor angiogenesis was relatively low and was not restricted to angiogenesis and, therefore, was detected also in resting endothelium. Similar to the PPE-1 promoter, other endothelial cell-specific-promoters such as von-Willebrand factor, intracellular adhesion molecule 2 (ICAM-2), Tie 2, vascular endothelial growth factor receptor (VEGFR-2), VE-cadherin and E-selectin [24–27] were either weak or

Fig. 6. HE and immunohistochemical staining of lungs excised from LLC-implanted C57BL/6 mice treated with Ad5PPE-1(3X)-TK with GCV (left column) or Ad5PPE-1(3X)-TK without GCV (right column). **a** HE staining shows mononuclear infiltrates in lung metastasis treated with Ad5PPE-1(3X)TK and GCV, but no infiltration detected after treatment with AdPPE-1(3X)-TK without GCV. **b** TUNEL staining of lung metastasis demonstrated numerous apoptotic tumor cells in AdPPE-1(3X)-TK treated with GCV compared to AdPPE-1(3X)-TK without GCV. **c** Anti-caspase-3 staining of lung metastasized blood vessels demonstrated massive endothelial cell apoptosis in AdPPE-1(3X)-TK treated with GCV compared to AdPPE-1(3X)-TK without GCV. **d** Anti-CD31 staining of lung metastasis showed short blood vessels without continuity and borders in the mice treated with AdPPE-1(3X)-TK and GCV, compared to numerous blood vessels with distinctive borders and branching found after AdPPE-1(3X)-TK injection without GCV.

lack the specificity for tumor angiogenesis and, for that reason, systemic administration of gene therapy vectors containing these promoters was not efficient or safe. In a recent study [25], the human VEGFR-2 was used for transcriptional targeting of a viral vector to angiogenesis. Activation of caspase-9 by the human VEGFR-2 disrupted angiogenic blood vessels in vivo. However, the viral vectors were injected into the endothelial cell implant in the mouse model, but systemic administration has not yet been studied.

In order to increase the specific activity of the PPE-1 in tumor angiogenesis, we inserted 145 bp as 3X of endothelial cell-specific transcription element [17] into the wild-type murine PPE-1 promoter. This modification led to increased promoter activity in tumor-angiogenic blood vessels in Lewis lung metastasis in mice but not in the resting endothelium. This system of using the PPE-1(3X) promoter to control therapeutic gene expression selectively in neovasculature beds was proved in other angiogenesis models by using different sets of anti- or proangiogenic genes [28, 29].

In the present work, we combined the angiogenesis-specific promoter with the well-known HSV-TK/GCV system in order to induce toxicity in tumor angiogenesis. Since the toxic effect is dependent on GCV administra-

tion, the cytotoxic effect in this system can be externally controlled. The AdCMV-HSV-TK showed liver toxicity, resulting in necrosis and mononuclear infiltrates, indicative of immune response induction (fig. 6). In contrast, the TK-restricted expression in the lung metastasis of Ad5PPE-1(3X)-HSV-TK-treated mice protected their livers from toxicity. Hence, using the PPE-1(3X), which is restrictively active in endothelial cells that are poor antigen-presenting cells [12], may protect against liver damage and prolong the therapeutic effect. Indeed, we found that PPE-1(3X)HSV-TK plus GCV treatment led to tissue necrosis, apoptosis and significant reduction in metastasis mass without causing any side effects due to the non-specific expression (fig. 5, 6).

In summary, our results show the selectivity of the PPE(3X) system against tumor angiogenesis and demonstrate that HSV-TK enables a relatively safe and efficient treatment in the LLC model.

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