RESEARCH ARTICLE

Transcriptional targeting of conditionally replicating adenovirus to dividing endothelial cells

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Conditionally replicating adenoviruses (CRADs) are a novel strategy in cancer treatment and clinical trials using CRADs targeted to tumor cells have been reported recently. We hypothesized that it would be possible to construct CRADs targeted to dividing endothelial cells, which are present in the tumor endothelium. We utilized the regulatory elements of *Flk-1* and *endoglin* genes, which have been shown to be highly overexpressed in angiogenic endothelial cells, to construct two CRADs: *Ad.Flk-1*, which has adenoviral E1A gene under the control of the *Flk-1* enhancer/promoter, and *Ad.Flk-Endo*, which harbors the same *Flk-1* enhancer/promoter as *Ad.Flk-1*, plus it has the adenoviral E1B gene under control of the *endoglin* promoter. Viral titer measurements by plaque assay showed that in human umbilical vein endothelial cells (HUVECs), both CRADs replicated at levels comparable to that of wild-type adenovirus. In *Flk-1* and *endoglin* negative Hep3B and A549 cells, however, the repli-

cation of *Ad.Flk-1* and *Ad.Flk-Endo* was reduced by 30-fold and 600-fold, respectively. Cytotoxicity assays demonstrated that both CRADs killed HUVECs as effectively as wild-type adenovirus and their cytotoxicity in Hep3B and A549 cells was comparable to nonreplicating control adenovirus. Furthermore, there was a striking inhibition (83–91%) of capillary network formation in an *in vitro* angiogenesis assay when HUVECs were infected with *Ad.Flk-1* or *Ad.Flk-Endo* as compared with the nonreplicating control virus. These results demonstrate that CRADs can be transcriptionally targeted to dividing endothelial cells with high specificity, and that the combined use of *Flk-1* and *endoglin* regulatory elements has a synergistic effect on targeting specificity. This principle may be incorporated into novel therapeutic agents to develop anti-angiogenic treatment for cancer.

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Introduction

Conditionally replicating viruses are a novel approach to cancer treatment and the focus of intense study.^{1,2} Most initial cancer gene therapy experiments have been carried out using replication-defective viruses, which are by definition limited in their ability to infect and spread to neighboring cancer cells. Replication-selective oncolytic viruses provide an attractive alternative to cancer treatment, as they are able to replicate in tumor cells and spread to neighboring cells upon cell lysis. In addition to conditionally replicating adenoviruses (CRADs), herpes simplex virus-1, vaccinia virus, reovirus and Newcastle disease virus have been tested for oncolytic purposes in clinical trials.³ Two main strategies have been utilized to construct tumor-targeted CRADs. The first one involves the use of tumor- or tissue-specific promoters, such as MUC1, AFP, PSA, kallikrein-2 and pS2 to drive adenoviral genes that are essential for replication.^{4–8} This approach has been successful in animal experiments and the prostate-specific viruses CN-706 and CV-787 are being tested in clinical trials. The second strategy is based on deleting adenoviral genes that become dispensable in

cancer cells, such as the E1A and E1B genes responsible for inactivating tumor suppressor p53 and Rb genes, thereby enabling the virus to selectively replicate in tumor cells.^{9,10} This strategy has also been effective *in vivo* and some level of efficacy has also been reported in a clinical trial combining the E1B 55 kDa-deleted Onyx-015 virus with chemotherapy.¹¹ However, a major limitation of tumor-targeted CRADs is their relatively poor efficiency to spread in the tumor mass, thereby requiring repeated viral injections administered at multiple sites and over several days.^{11,12}

While most endothelial cells are normally quiescent, angiogenesis, the formation of new blood vessels is essential for certain physiological processes such as female reproductive cycle and wound healing. In addition, many studies have shown that tumor growth is dependent on angiogenesis, thus providing a rationale for anti-angiogenic therapy for cancer.^{13,14} Angiogenesis is a complex multicomponent process, involving many growth factors and their receptors, cytokines, proteases and adhesion molecules, providing multiple targets for therapeutic intervention.^{15,16} In addition, many endogenous proteins or protein fragments have been shown to have anti-angiogenic properties.^{13,17,18} Targeting a CRAD to the tumor endothelium offers a novel approach to anti-angiogenic therapy for cancer.

Targeting the vessels of the tumor instead of the tumor itself potentially offers enhanced viral spread via the

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tumor's blood supply. In addition, as the target is the genetically normal endothelial cell, resistance to treatment due to somatic mutations in the target cell does not occur. In order to transcriptionally target the dividing endothelial cell, promoters of genes with specificity for these cells can be utilized. Flk-1 (also called KDR and VEGFR-2) is a high-affinity tyrosine kinase receptor for the angiogenic growth factor, VEGF. Flk-1 is essential for endothelial cell differentiation and vasculogenesis and is expressed by endothelial cells or their precursors during development.^{19,20} Flk-1 expression is suppressed in adult endothelium, but is highly induced in the newly formed blood vessels in a variety of human tumors.^{19,21,22} In a recent Flk-1 promoter characterization study a 939-bp promoter fragment in combination with a 510-bp enhancer element in the first intron specifically targeted *LacZ* expression to angioblasts and endothelial cells of transgenic mice.²³ When various tumors were grown in these mice, strong tumor endothelium-specific reporter gene expression was observed, while being absent from most blood vessels in normal adult tissue.²⁴ Endoglin (CD105) is a cell surface component of the TGF- β receptor complex, which is preferentially expressed by endothelial cells. A detailed expression study demonstrated that endoglin expression was strongly elevated in the angiogenic endothelium and that endoglin expression correlated with cell proliferation markers.²⁵ A 741-bp fragment of the endoglin promoter displayed tissue-specific activity in dividing human and bovine endothelial cells.²⁶

The goal of the current study was to determine whether replicating adenoviruses could be targeted to dividing endothelial cells. To this end, we constructed two CRADs targeting the dividing endothelial cells via Flk-1 and endoglin regulatory elements.

Results

Construction of recombinant Ad.Flk-1 and Ad.Flk-Endo vectors

In order to construct an adenovirus that replicates selectively in Flk-1-positive cells, plasmid pFlk-1 was created by deleting the proximal adenovirus E1A promoter (bp 357 to 547) from pXC1 containing adenovirus 5 sequences from bp 22 to 5790 and replacing it with the 1433 bp Flk-1 enhancer/promoter construct. To construct a virus that replicates selectively in Flk-1 and endoglin positive cells, plasmid pFlk-Endo was constructed by inserting the 741-bp Endoglin promoter at bp 1691 of pFlk-1, before the transcription start site of the adenovirus E1B gene. Adenoviruses Ad.Flk-1 and Ad.Flk-Endo were produced by co-transfecting the E1-complementing 293 cells with backbone plasmid pBHG10, which harbors the rest of the adenoviral genome except for a deletion of the E3 gene region, with plasmids pFlk-1 and pFlk-Endo, respectively (Figure 1). The genome lengths of Ad.Flk-1 and Ad.Flk-Endo are 97% and 99% of wild-type Ad5, respectively.

Selection and characterization of target cells

Human primary cells and cell lines were used to assess the selectivity of Ad.Flk-1 and Ad.Flk-Endo viral replication and cytotoxicity. HUVECs represent the dividing endothelial cell population and as expected, RT-PCR analysis demonstrated high levels of both Flk-1 and

Figure 1 A schematic representation of Ad.Flk-1 and Ad.Flk-Endo. Numbers above the drawings indicate positions in adenovirus 5 sequence. Numbers inside the boxes indicate sizes of the regulatory elements. Both viruses harbor a deletion of the endogenous E1A promoter and the E3 region, indicated by hatched boxes. Flk-1 E, Flk-1 enhancer; Flk-1 P, Flk-1 promoter; Endoglin P, endoglin promoter.

endoglin expression (Figure 2). Therefore, these cells could be used as target cells in evaluating the efficiency of Ad.Flk-1 and Ad.Flk-Endo. In contrast, no Flk-1 or endoglin expression was detected in Hep3B and A549 cells, making these cell lines suitable negative controls (Figure 2). As very little Flk-1 or endoglin is expressed *in vivo* outside the tumor endothelial cell population, Hep3B and A549 cells can be used to analyze the cytotoxicity of the viruses in non-target cells.^{19,22,27}

In order to analyze the adenoviral transduction efficiency of the different cell types used, HUVECs, Hep3B and A549 cells were transduced with increasing concentrations of E1-deleted β -gal virus and the transduction efficiency was determined by β -gal staining (data not shown). Hep3B cells were transduced most efficiently, with viral doses of 2×10^5 p.f.u./ml and 2×10^6 p.f.u./ml resulting in transduction efficiencies of 10% and 30%, respectively. A 10-fold increase in viral dose was required to achieve comparable transduction efficiencies in A549 cells, with viral doses of 2×10^6 p.f.u./ml and 2×10^7 p.f.u./ml resulting in transduction

Figure 2 RT-PCR analysis of Flk-1 and Endoglin expression on HUVECs, A549s and Hep3Bs. RT-PCRs were performed using 0.6 μ g template RNA from cultured cells and specific primers for Flk-1 and endoglin cDNA. Specific primers for G3PDH housekeeping gene were included in each reaction as a positive control. Arrows indicate the 500 bp, 410 bp and 109 bp amplified products of Flk-1, endoglin and G3PDH, respectively.

efficiencies of 10% and 20%, respectively. HUVECs were the most difficult to transduce, with viral doses of 2×10^7 p.f.u./ml and 2×10^8 p.f.u./ml resulting in transduction efficiencies of 10% and 25%, respectively. The viral concentrations in subsequent experiments were adjusted accordingly, allowing direct comparison for the effect of transcriptional targeting.

Selective replication of Ad.Flk-1 and Ad.Flk-Endo

From the oncolysis point of view, the most important quality of conditionally replicating viruses is their ability to productively and selectively replicate in the target cells. To assess the selective viral replication, HUVECs, A549 and Hep3B cells were infected by Ad.Flk-1, Ad.Flk-Endo and wild-type Ad5, and viral progeny production was measured by a series of plaque assays. In HUVECs, Ad.Flk-1 and Ad.Flk-Endo viruses replicated at levels comparable to that of wild-type Ad5. In Hep3B and A549 cells, however, the viral titer of Ad.Flk-1 was reduced approximately 30-fold. Interestingly, Ad.Flk-Endo had an even higher specificity ratio, with replication levels reduced 120-fold in A549 and 600-fold in Hep3B cells (Figure 3). The high replication specificity of Ad.Flk-Endo demonstrates that the combined use of Flk-1 and endoglin regulatory elements has a synergistic effect on targeting specificity.

Targeted cell lysis by Ad.Flk-1 and Ad.Flk-Endo

To evaluate if selective replication of Ad.Flk-1 and Ad.Flk-Endo would translate into selective cytotoxicity, a series of trypan blue exclusion assays was performed. HUVECs, Hep3B and A549 cells were infected with Ad.Flk-1 and Ad.Flk-Endo, while wild-type and E1-deleted β -gal viruses were used as positive and negative controls, respectively. At 48 h after infection, HUVECs infected with Ad.Flk-1, Ad.Flk-Endo and wild-type virus showed marked reduction of living cells as compared with cells transduced with E1-deleted β -gal virus (Figure 4). At 72 h, the difference was even clearer, with almost complete cytotoxicity in cells infected with Ad.Flk-1, Ad.Flk-Endo and wild-type virus. Ad.Flk-Endo was somewhat more cytotoxic than Ad.Flk-1 at both time-points. In contrast, a clear difference between the cytotoxicity of wild-type and conditionally replicating viruses

Figure 3 Viral replication of Ad.Flk-1, Ad.Flk-Endo and wild-type virus in Flk-1/endoglin positive (HUVEC) and negative (A549 and Hep3B) human cells. Monolayers in six-well plates were infected at a concentration of 2×10^7 p.f.u./ml (HUVEC), 2×10^6 p.f.u./ml (A549) or 2×10^5 p.f.u./ml (Hep3B). Viral titers were assessed by plaque assay on 293 cells.

Figure 4 Cytotoxicity of Ad.Flk-1 and Ad.Flk-Endo. Monolayers in 24-well plates were infected at a concentration of 2×10^7 p.f.u./ml (HUVEC and A549) or 2×10^5 p.f.u./ml (Hep3B). Cells infected with wild-type and E1-deleted β -gal viruses and uninfected cells were used as controls. Forty-eight or 72 h later, cells were stained with trypan blue and live cells were counted. * $P < 0.05$; ** $P < 0.01$.

was observed in Flk-1 and endoglin negative Hep3B and A549 cells. The wild-type virus efficiently killed most Hep3B and A549 cells by 48 h after infection, whereas Ad.Flk-1 and Ad.Flk-Endo caused only minimal cytotoxicity. At 72 h, when viral replication had progressed, the difference was more profound, in Hep3B cells infected with Ad.Flk-1 and Ad.Flk-Endo the number of living cells was 17.3 and 18.8 times higher, respectively, when compared with cells infected with wild-type virus. Similar ratios were observed for A549 cells, with the number of living cells infected with Ad.Flk-1 and Ad.Flk-Endo being 22.6 and 22.0 times higher, respectively, when compared with cells infected with wild-type virus at 72 h (Figure 4). Although some cytotoxicity was observed

when comparing Ad.Flk-1 and Ad.Flk-Endo to E1-deleted β -gal virus in Hep3B and A549 cells, the difference was not statistically significant. The cytotoxicity of CRADs on a particular cell type depends not only on the rate of replication, but also on the infectivity of the cell.²⁸ This was demonstrated when Hep3B cells, which are very efficiently infected by Ad5, were infected with 2×10^7 p.f.u./ml of Ad.Flk-1 and Ad.Flk-Endo, which resulted in a 60% transduction efficiency, cytotoxicity was observed.

Cytopathic effect was also visualized by examining infected Hep3B and HUVEC monolayers (Figure 5). Infection of HUVECs with Ad.Flk-1, Ad.Flk-Endo and wild-type virus caused profound cytotoxicity at 48 h after infection with rounding and detachment of almost all cells (Figure 5). In contrast, infection of Hep3B control cells with Ad.Flk-1 and Ad.Flk-Endo caused no discernible cytotoxicity at 48 h after infection, whereas infection of Hep3B cells with the same concentration of wild-type virus caused complete cytotoxicity. The results demonstrate that the selective replication of Ad.Flk-1 and Ad.Flk-Endo also translated into specific cell killing of the target cells.

Effect of Ad.Flk-1 and Ad.Flk-Endo on capillary tube formation

Angiogenesis is a complex process characterized by endothelial cell proliferation, differentiation and migration. In order to evaluate the effect of Ad.Flk-1 and Ad.Flk-Endo infection on these processes, HUVECs infected with Ad.Flk-1, Ad.Flk-Endo and control viruses were plated on wells precoated with Matrigel basement membrane matrix. Eight hours after plating, HUVECs infected with the E1-deleted β -gal virus as well as uninfected cells were able to form a network of capillary-like structures and by 24 h after plating these structures covered most of the well. In contrast, HUVECs infected with Ad.Flk-1, Ad.Flk-Endo or wild-type virus failed to differentiate (Figure 6). When compared with cells infected with E1-deleted β -gal virus the number of capillary-like structures was 91% and 83% less than with cells infected with Ad.Flk-1 and Ad.Flk-Endo, respectively. These results were statistically significant ($P < 0.01$) and there was no statistical significance in the inhibition efficiency between Ad.Flk-1, Ad.Flk-Endo and wild-type virus. Thus, infection with Ad.Flk-1 or Ad.Flk-Endo efficiently inhibits angiogenesis *in vitro*.

Discussion

In this article, we describe two novel adenoviruses, Ad.Flk-1 and Ad.Flk-Endo, which target the expression of adenoviral E1A and E1B genes to dividing endothelial cells by Flk-1 and endoglin promoters, respectively. We show that Ad.Flk-1 and Ad.Flk-Endo possess significant differential replication ratios in Flk-1 and endoglin positive cells, as compared with cells where these genes are not expressed. Flk-1 is an endothelial-cell specific gene, which in contrast to other endothelial genes that are expressed constitutively, is absent from most vascular beds of the adult organism, but is strongly and specifically induced in the dividing endothelial cells of the tumor endothelium.^{19,22} Endoglin has a similar expression pattern with selective expression in endothelial cells and strong up-regulation especially in the endothelium of the tumor edges, where active cell division occurs.^{25,29} Some endoglin expression has also been reported in cells of the hematopoietic lineage.²⁹ Fortunately, hematopoietic stem cells and erythroid precursors are highly resistant to adenovirus infection.^{30,31} Therefore, a therapeutic agent targeting Flk-1 and endoglin positive cells should be highly specific for tumor vasculature.

We chose E1A as the target for transcriptional regulation because it has been shown to be the major regulator of adenoviral replication. E1B was chosen to further increase specificity as it is also essential for proper viral replication and, importantly, the expression levels of E1B do not depend on E1A, thereby allowing adenoviral replication to be regulated by two independent elements.³² Similar strategies have previously been utilized in constructing viruses which target prostate, breast and liver tumor cells.⁴⁻⁸ However, no replicating viruses targeting the dividing endothelial cells have previously been reported.

In HUVECs, the replication of Ad.Flk-1 and Ad.Flk-Endo was increased 30-fold and 600-fold, respectively, when compared with Flk-1 and endoglin negative control cells (Figure 3). These selective replication ratios are comparable to those obtained with previously reported tumor-targeted CRADs. The high specificity of Ad.Flk-Endo demonstrates that the use of two separate regulatory elements has a synergistic effect on targeting specificity. A similar increase in specificity has been reported for CV764, which has two separate prostate carcinoma-specific promoters driving E1A and E1B.⁷ Ad.Flk-1 and

Figure 5 Cytopathic effects associated with Ad.Flk-1 and Ad.Flk-Endo infection. Hep3B and HUVEC monolayers in 24-well plates were infected with Ad.Flk-1, Ad.Flk-Endo and wild-type virus at a concentration of 2×10^8 p.f.u./ml (HUVEC) or 2×10^5 p.f.u./ml (Hep3B). Forty-eight hours later, the cells were photographed.

Figure 6 The effect of Ad.Flk-1 and Ad.Flk-Endo infection on HUVEC differentiation into capillary-like structures. HUVEC monolayers on six-well plates were infected with Ad.Flk-1, Ad.Flk-Endo, wild-type and E1-deleted β -gal virus at a concentration of 2×10^8 p.f.u./ml. Twenty-four hours later, the infected cells and uninfected controls were seeded on Matrigel and the spontaneous formation of capillary tubes was recorded at 24 h after plating and quantitated as described. * $P < 0.05$; ** $P < 0.01$.

Ad.Flk-Endo were also able to cause selective cytotoxicity as they killed Flk-1 and endoglin positive HUVECs as efficiently as wild-type virus, but in Flk-1 and endoglin negative Hep3B and A549 cells the wild-type virus was approximately 20 times more cytotoxic. It is notable that the therapeutic ratios of many conventional cytotoxic drugs range from 1.5:1 to 6:1.³³ Furthermore, there was no statistical significance when the cytotoxicity of Ad.Flk-1 and Ad.Flk-Endo was compared with the E1-deleted control virus (Figure 4). These results clearly demonstrate that Ad.Flk-1 and Ad.Flk-Endo are able to specifically replicate in, and kill, Flk-1 and endoglin positive cells. It is still possible, however, that some cytotoxicity can be caused by leakiness of the Ad.Flk-1 and Ad.Flk-Endo promoter constructs, especially in cell lines that are very efficiently infected by Ad5.

As angiogenesis is a complex process characterized by endothelial cell proliferation, differentiation, invasion and migration, the effect of Ad.Flk-1 and Ad.Flk-Endo on these processes was also evaluated in Matrigel assays, which mimic angiogenesis *in vitro*. Ad.Flk-1 and Ad.Flk-Endo were very effective in hindering the angiogenic process and it is notable that they already inhibited angio-

genesis at 32 h after infection, well before any cytotoxicity due to viral replication could be observed. This indicates that these viruses efficiently inhibit angiogenesis, at least *in vitro*, at low levels of viral replication.

The development of replication-competent vectors is an attractive alternative strategy to using replication-defective viruses in cancer treatment. By specifically replicating in the target cells, CRADs deliver the therapeutic vector at the intended site of action, keeping the therapeutic dose to a minimum.³⁴ However, the efficacy of CRADs targeting tumor cells has thus far been limited, due at least in part to limited spread of the virus in solid tumor tissues. CRADs targeting the tumor endothelium instead of the tumor itself should be able to spread in the tumor via its blood supply. Furthermore, as the target cell is genetically stable, therapy resistance due to somatic mutations is not anticipated. Other advantages of endothelial targeting include easy reach of target tissue upon intravenous administration and the amplification effect on tumor killing, as one endothelial cell is known to support the nutritional needs of approximately 100 tumor cells.³⁵ The amplification effect was elegantly illustrated in a recent report comparing the antitumor efficacies of

tumor *versus* tumor endothelium infecting retroviruses expressing the herpes simplex virus-thymidine kinase (HSVtk) cytotoxic gene. The therapeutic effect in tumors with HSVtk-infected vasculature comprising less than 5% of the total tumor mass was similar to that of tumors with HSVtk-infected tumor cells comprising over 46% of the total tumor mass.³⁶ This is an encouraging result for targeted anti-angiogenic strategies such as described here, as it indicates that infection of relatively few tumor endothelial cells results in a strong antitumor effect.

The oncolytic effect of CRADs on a particular cell type depends not only on the rate of replication but also on the infectivity of the cell.²⁸ As tumor endothelial cells are not very susceptible to Ad5 infection, the CRADs described here are best suited for local or regional (eg selective canulation of tumor-feeding vessels) administration *in vivo*. In order to improve the selectivity and efficacy of transcriptionally targeted viruses like Ad.Flk-1 and Ad.Flk-Endo, they can be further optimized by combining them with transductional targeting, eg through genetic modification of the viral capsid. Recent advances in adenovirus and tumor biology should make such targeting possible. The binding site of the coxsackie- and adenovirus receptor (CAR) on the adenovirus fiber, which is a major determinant of adenovirus tropism, has recently been described.³⁷ It was also demonstrated that by deleting this binding site and adding a targeting ligand to the adenovirus fiber, the modified vector could be successfully retargeted.³⁷ In order to retarget Ad.Flk-1 and Ad.Flk-Endo, ligands homing to sites of neoangiogenesis must be used. Such ligands have been recently discovered, thereby making it possible to construct CRADs targeting the tumor endothelium by both transcriptional and transductional mechanisms.^{38,39} The construction of such viruses is under way in our laboratory.

In summary, we demonstrate here for the first time the feasibility of targeting replication-competent adenoviruses to dividing endothelial cells with great specificity. This principle has the potential to improve gene therapy strategies directed at disseminated tumors.

Materials and methods

Construction of Ad.Flk-1 and Ad.Flk-Endo

Plasmid pXC1, which contains human adenovirus 5 sequences from bp 22 to 5790, was purchased from Microbix (Microbix Biosystems, Toronto, Canada). For insertion of the Flk-1 enhancer/promoter a unique *AgeI* site was created in the adenovirus E1A promoter of pXC1 by overlapping PCR as follows. The first primer pair (TCGTCTTCAAGAATTCTCATG (sense) and TTTCAGTCACCGGTGTCGGA (antisense)) produced a PCR fragment from the unique *EcoRI* site in the PBR322 backbone of pXC1 to the new *AgeI* site at position 547. The second primer pair (TCCGACACCGGTGACTGAAA (sense) and GCATTCTCTAGACACAGGTG (antisense)) produced a PCR fragment from the new *AgeI* site to a unique *XbaI* site at position 1339. Combining equal amounts of the two PCR products, a third PCR was performed with the two outside primers, cut with *EcoRI* and *XbaI*, and cloned into similarly cleaved pUC19 to yield pUCE1A. This plasmid was cut with unique enzymes *SacII* at position 357 and *AgeI* at position 547 to delete the endogenous adenovirus E1A promoter.

In order to amplify the Flk-1 enhancer and promoter liver tissue from a BALB/c mouse was homogenized, and DNA was extracted using DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). A 923-bp Flk-1 promoter element was amplified using specific primers based on published sequence: ATTTAGCGGCCGCagttcacaaccgaaatgtcTTC (sense primer with *NotI* linker) and AGTTTACCGGTATCCTGCACCTCGCGCTG (antisense primer with *AgeI* linker).⁴⁰ A 510-bp Flk-1 enhancer element was amplified using specific primers based on published sequence: TCCCCGCGGTAAATGTGCTGT-CTTTAGAAG (sense primer with *SacII* linker) and AATATGCGGCCGCTCCAATAGGAAAGCCCTTC (antisense primer with *NotI* linker).²³ The promoter and enhancer fragments were cut with *NotI*-*AgeI* and *SacII*-*NotI*, respectively, and cloned into similarly cut pUCE1A to yield pUCE1A-Flk1. The modified E1A fragment containing the Flk-1 enhancer/promoter was released from pUCE1A-Flk1 by *EcoRI*-*XbaI* digestion and cloned into *EcoRI*-*XbaI* digested pXC1 to yield pFlk-1 (Figure 1).

In order to construct pFlk-Endo a unique *BlpI* restriction was created in pFlk-1 by overlapping PCR as follows. The first primer pair (TCACCTGTGTCTAGAGAATGC (sense) and GTAACCAAGCTTAGCCCACG (antisense)) produced a PCR fragment from the unique *XbaI* site of pFlk-1 to the new *BlpI* site at position 1690 of adenovirus sequence. The second primer pair (CGTGGGCTAAGCTTGGTTAC (sense) and CCAGAAAATCCAGCAGGTACC (antisense)) produced a PCR fragment from the new *BlpI* site to a unique *KpnI* site of pFlk-1. Combining equal amounts of the two PCR products, a third PCR was performed with the two outside primers, cut with *XbaI* and *KpnI*, and cloned into *XbaI*-*KpnI* digested pFlk-1 to yield pFlk-BlpI, which now has a unique *BlpI* site before the transcription start site of E1B.

In order to amplify the endoglin promoter human DNA was extracted from whole blood using QIAamp DNA Blood Mini Kit (Qiagen). A 741-bp endoglin promoter was amplified using specific primers based on published sequence: GATCATGCTAAGCGATCCCAGCGCTACCATCTTC (sense primer with *BlpI* linker) and TATAATGCTTAGCGTGGGGGCCTGTGCGCTGG (antisense primer with *BlpI* linker).²⁶ The promoter fragment was cut with *BlpI* and cloned into similarly cleaved pFlk-1-BlpI to yield pFlk-Endo (Figure 1). The sequence of all PCR fragments in pFlk-1 and pFlk-Endo were verified by sequencing.

Recombinant adenoviruses Ad.Flk-1 and Ad.Flk-Endo were prepared by co-transfecting 293 cells with plasmids pFlk-1 and pFlk-Endo with backbone plasmid pBHG10 (Microbix), which contains an E1 deletion of bp 188–1339 and an E3 deletion of bp 28133–30818, as described.⁴¹ Recombinant adenovirus was isolated from a single plaque, expanded in 293 cells and purified by double cesium gradient ultracentrifugation. The viral particles were measured by optical absorbance at 260 nm, and the plaque-forming units (p.f.u.) were determined by standard agarose-overlay plaque assay on 293 cells. The genome lengths of Ad.Flk-1 and Ad.Flk-Endo are 97% and 99% of wild-type Ad5, respectively.

Cells and viruses

Hep3B, A549 and 293 cell lines were obtained from American Type Culture Collection. The cells were cul-

tured in DMEM medium supplemented with 10% FBS, 0.2 mM glutamine, and penicillin/streptomycin. In addition, Hep3B medium was supplemented with 1 mM sodium pyruvate. HUVECs were isolated from umbilical cords (Institutional Review Board-approved cord blood program) by collagenase type IV (Sigma-Aldrich, St Louis, MO, USA) perfusion (0.2% in Hanks' balanced salt solution) for 20 min at room temperature. The cells then were cultured in EGM-2 medium (Clontics, Walkersville, MD, USA). The E1-deleted β -gal virus was produced in our vector core laboratory by co-transfecting pCMV-lacZ shuttle plasmid with pBHG10 backbone plasmid as described above.

RT-PCR

RNA was isolated from cultured cells using RNeasy Mini Kit (Qiagen). RT-PCR was performed with OneStep RT-PCR kit (Qiagen) using 0.6 μ g template total RNA and specific primers for Flk-1 and endoglin cDNA. Specific primers for G3PDH housekeeping gene were included in each reaction as a positive control. The samples were run on a 1% agarose gel and photographed.

In vitro replication assay

Twenty-four hours before infection, 2×10^5 cells/well were plated on six-well dishes. Cells were infected with 2×10^7 p.f.u./ml (HUVEC), 2×10^6 p.f.u./ml (A549) or 2×10^5 p.f.u./ml (Hep3B) of virus in 1 ml serum-free medium. After a 2-h incubation at 37°C, cells were washed once with prewarmed PBS and 2 ml complete medium was added to each well. After an additional 48 h, the cells were scraped into the culture medium, and lysed by three freeze-thaw cycles. The lysate was centrifuged at 3000 r.p.m. for 5 min and the supernatant was analyzed for virus production by duplicate plaque assay on 293 cells.

Trypan blue exclusion assay

Twenty-four hours before infection, 5×10^4 cells/well were plated on 24-well plates and infected as described above. Forty-eight or 72 h later, the medium was removed and attached cells were detached by trypsin. The cells were stained with trypan blue and live cells were counted using a hemacytometer.

In vitro angiogenesis assay

Matrigel basement matrix (Becton Dickinson, Bedford, MA, USA) was diluted 1:2 in cold DMEM medium. 200 μ l diluted Matrigel was plated into 24-well plates and allowed to gel for 30 min at 37°C before cell seeding. HUVECs were plated on six-well plates at a density of 2×10^5 cells/well 24 h before infection and infected at a concentration of 2×10^8 p.f.u./ml as described above. Twenty-four hours after infection, the cells were collected with trypsin/EDTA, resuspended in EGM-2 medium and plated at a density of 5×10^4 cells/well on 24-well plates precoated with Matrigel as described above. After 24-h incubation, the plates were photographed and quantitated. Capillary tubes were defined as cellular extensions linking cell masses or branch points.

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