Kinase domain insert containing receptor promoter controlled suicide gene system selectively kills human umbilical vein endothelial cells

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INTRODUCTION
Gene therapy is a novel technology leading to improved treatments of some types of cancer[1-3]. On the other hand, anti-angiogenic therapy has also been proven to be a rational approach in the treatment of solid tumors also[4-9]. Selective delivery of the suicide gene into tumor endothelial cells and target expression of it to abrogate tumor vasculature may be a good way for tumor therapy.

A suicide gene is a gene encoding an enzyme that converts nontoxic prodrugs into toxic forms. The herpes simplex virus-thymidine kinase (TK) gene is one of the most widely studied suicide genes[10], and the E. coli cytosine deaminase (CD) gene is another widely studied one[11-13]. However, results of numerous investigations aimed at eradicating tumors employing either CD or TK demonstrate their limitations. Whether the limitations...
stem from unfavorable pharmacokinetics, loss of transgene expression or biochemical resistance is not clear. Nevertheless, fusion gene of CD and TK shows exciting superiority in many studies.[14-17]

The adenovirus vector system has many advantages.[18-20] First, adenovirus vectors can be prepared at much higher titers than retroviral vectors and have a high efficiency of gene transfer regardless of the proliferative states of the tissues whereas retroviral vectors insert their genes only into dividing cells. Second, adenovirus genomes usually do not integrate into the host cell chromosome, and the level of therapeutic gene expression is very high. Human umbilical vein endothelial cells (HUVECs) are primary cells that can be used to investigate the mechanisms underlying the role of endothelial cells.[21] The kinase domain insert containing the receptor (KDR) gene is strictly expressed only in vascular endothelium cells. The activity of the KDR promoter in endothelial cells is similar to that of the potent SV40 promoter/enhancer and that this high level activity is specific to endothelial cells.[22]

We constructed two recombinant adenoviruses to transfer the double suicide gene under the KDR promoter or the CMV promoter into HUVECs respectively, and LoVo cells (a cell system of colon carcinoma) were used as the control. Prodrug sensitive experiments were performed to evaluate the killing effect of the fusion double suicide gene under the regulation of the KDR promoter, mediated by the adenovirus vector on HUVEC cells.

MATERIALS AND METHODS

Materials

Shuttle plasmid pAdtrack, pAdtrack-CMV, adenoviral backbone plasmid pAdEasy-1 and E.coli JM1853 were provided by Dr. Belt Vogelstein at Johns Hopkins Oncology Center, Howard Hughes Institute of Medicine. pMD18-T vector was purchased from TaKaRa Biotechnology (Dalian) Co. Ltd. 293 cells, and HUVECs were obtained from American Type Culture Collection (ATCC). All sorts of exonuclease, T4 DNA ligase, and Taq DNA polymerase were purchased from New England Biolabs Co. DMEM, fetal bovine serum (FBS), transfection reagents, and LipofectAMINE2000 were products of Gibco Co. Primers of KDR promoter gene, CD gene, TK gene were synthesized and sequenced by Sangon Biotechnology (Shanghai) Co., Ltd.

KDR promoter sequence and CDglyTK sequence

KDR promoter genes (including the minimus core of the gene sequence -226~+268) were generated by PCR using human blood genome as the template (the upstream primer sequence: 5'-GGATCCGTCCAGTGTGCTGC-3', the downstream primer sequence: 5'-CGCGTTACGCT-3'). The kinase domain insert containing the receptor (KDR) gene was amplified using GoTaq DNA polymerase. Hind III restriction sites at the 5' end and Bgl II restriction sites at the 3' end respectively were added to the KDR promoter sequence to get KDR-CDglyTK fragment. The resulting fragment containing Bgl II at the 5' end, and Hind III restriction sites at the 3' end respectively were digested with Hind III and Bgl II and cloned into pcDNA3-KDR-CDglyTK, which was digested with Hind III and Bgl II. First, KDR-CDglyTK fragments were subcloned into pAdtrack to generate pAdtrackKDR-CDglyTK. pAdtrackKDR-CDglyTK fragment from pcDNA3-CdglyTK (Hind III, Xba I) was subcloned into pAdtrackCMV to construct pAdtrackCMV-CdglyTK.

Transfer plasmids construction

KDRs were removed from pMD-18KDR by Bgl II, Hind III and subcloned into pcDNA3-CdglyTK to generate pcDNA3-KDR-CdglyTK, which was digested with Bgl II and Pvu II to get KDR-CdglyTK fragment. The resulting KDR-CdglyTK fragments were subcloned into pAdTrack to generate pAdTrackKDR-CDglyTK. pAdtrackKDR-CDglyTK fragment from pcDNA3-CdglyTK (Hind III, Xba I) was subcloned into pAdtrackCMV to construct pAdtrackCMV-CdglyTK.

Construction and identification of recombinant adenovirus vector plasmid

pAdEasy-1 plasmid was transformed into E.coli B(1)5183, followed by growing of transformants on LB agar plates containing ampicillin and streptomycin. The transformed bacteria was named "AdEasy-1 bacteria".

pAdtrackKDR-CDglyTK was linearized and transformed into AdEasy-1 bacteria. Transformants were selected on LB agar plates containing 25 μg/ml kanamycin. Plasmid DNA was prepared from individual colonies, and agarose gel electrophoresis was performed. The correct recombinant pAdKDR-CdglyTK could be clearly identified by size, as only the 11.2-kb pAdtrackKDR-CdglyTK plasmid and the 37-kb pAdKDR-CdglyTK were selectable by kanamycin resistance.

The same protocol was performed to transfer pAdtrackCMV-CdglyTK into AdEasy-1 bacteria to recombine with pAdCMV-CdglyTK.

Propagation, purification, titer determination and identification of the recombinant adenovirus

Both pAdKDR-CdglyTK and pAdtrackCMV-CdglyTK were transferred into 293 cells mediated by LipofectAMINE2000 vector. Their further propagation was visualized under fluorescence microscope by GFP expression of transgene, and ultraconcentration in CsCl gradient was performed to purify the viruses, and the titration of AdKDR-CdglyTK and AdtrackCMV-CdglyTK was measured by plaque formation assay.

The resultant recombinant viruses were boiled to be used as templates. PCR protocols were performed either to ensure the presence of CdglyTK gene in the viruses, in which upstream primer sequence of CD and downstream primer sequence of TK were used, or to ensure the
presence of the KDR promoter gene in AdKDR-CdglyTK. Primers of KDR promoter were used.

**Cell culture and virus infection rate**
HUVECs and LoVo cells, obtained from ATCC, were maintained in calorstart of 37°C, with 5% CO2 and in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells (2 × 10^5/well, 6-well plate) were infected with AdKDR-CdglyTK or AdCMV-CdglyTK at different multiplicity of infection (MOI). Percentages of cells expressing GFP were counted under fluorescence microscope within 3 d.

**Prodrug sensitivity assays**
HUVECs or LoVo cells (1 × 10^4 cells/well, 96-well plate, inoculated one day before) were infected with AdKDR-CdglyTK or AdCMV-CdglyTK at MOI of 100, 16 h later. The medium was removed and replaced with fresh medium with different concentrations of 5-FC and/or GCV. Cells were cultured in the presence of prodrugs for 72 h. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's protocol.

**Statistical analysis**
Statistical analyses were made by ANOVA and LSD test. The values were calculated as means ± SD. P < 0.05 was considered statistically significant.

**RESULTS**

**Amplification or construction of gene fragments**
The products of PCR amplification or constructed KDR promoter gene, CD and TK genes and CDglyTK gene were sequenced and verified by Sangon Biotechnology (Shanghai) Co., Ltd.

**Recombinant viral plasmids**
Newly constructed pAdtrackKDR-CdglyTK and pAdtrackCMV-CdglyTK were identified by restriction analysis and transferred into AdEasier-1 bacteria. Recombinant bacteria were selected by kanamycin. Figure 1 is the map of plasmids from 10 clones of transferred bacteria selected by kanamycin. Nine of them were correctly recombined, with a correction rate of 90% (9/10).

**Recombinant viruses**
Three days after transferring pAdKDR-CdglyTK and pAdCMV-CdglyTK into 293 cells, we found that most transferred 293 cells expressed GFP (Figure 2). The titer of the purified virus after being sufficiently propagated was 3.5 × 10^12 pfu/L. No difference between AdKDR-CdglyTK and AdCMV-CdglyTK was observed.

The products of the PCR using the recombinant viruses as templates were proven to be correct by size comparison (Figure 3), with KDR promoter of 580-bp and CdglyTK of 2.5-bp.

**Infection rate of viruses**
HUVECs and LoVo cells infected with AdKDR-CdglyTK or AdCMV-CdglyTK at different MOI were observed 3 d post-infection (Figure 4). It was indicated that cell infection rate increased with the increase of MOI of the virus: when MOI = 1, only a few cells expressed GFP; when MOI = 100, most cells expressed GFP; when MOI = 200, almost all cells expressed GFP. There was no difference between the two recombinant viruses in infecting HUVECs and LoVo cells.
A blood supply is required for a tumor to progress in excess of 1-2 mm in size. Recent data suggest that tumor cells can co-opt surrounding vasculature from an even smaller size in certain tumor types. The expanding growth of a primary tumor is associated with neo-angiogenesis and vessel maturation of the tumor. Abrogation of tumor blood vessel should lead to the eradication or suppression of solid tumor. Moreover, Folkman proposed that the prevention of tumor vascularization essentially stops neoplastic invasion, which may lead to the ablation of metastatic disease. The rapid growth of a tumor results in hypoxia which induces the production of pro-angiogenic factors such as vascular endothelial growth factor (VEGF). VEGF is a potent and specific mitogen for endothelial cells. It plays a major role in angiogenesis and vasculogenesis. KDR and Flt-1 are the two receptor tyrosine kinases that regulate the actions of VEGF and are expressed in endothelial cells. KDR is critically involved in the regulation of angiogenesis, both in the developing and adult animals. The vascular endothelial cell is renewed at a low speed in normal conditions, and its KDR expression level is very low, while tumor vascular endothelial cells proliferate quickly and with a KDR expression level 500 times higher than that of vascular endothelial cells of normal tissues. Therefore, it is possible to realize targeted expression of therapeutic genes in tumor vascular endothelial cells by transcriptional regulation of the KDR promoter, thus markedly reducing the toxicity and side effects of gene therapy targeting vascular endothelial cells of tumors.

In our experiments, we constructed two replication-incompetent recombinant adeno viral vectors with the AdEasy system in a “two-step transformation protocol” to transfer the KDR-promoter-controlled/CMV-promoter-controlled double suicide gene into HUVEC/LoVo cells. The results indicated that “two-step transformation protocol” is a convenient and efficient way to generate adenoviral vectors. The resultant adenoviruses infected HUVECs with a high efficiency and the transgenes were efficiently expressed, which was delineated by the reporter gene GFP as it was expressed in almost all HUVECs when they were infected with the virus at MOI of 200. The subsequent prodrug sensitivity experiments demonstrated that this high level expression was strong enough for cell killing in vitro. The prodrug sensitivity experiment also demonstrated that CMV-promoter-controlled double suicide gene/prodrugs system unselectively killed both HUVECs and LoVo cells; however, the KDR-promoter-controlled double suicide gene/ prodrugs system displayed a targeted killing effect on HUVECs: treated with 40 μg/mL GCV + 400 μg/mL 5-FC, 74.8% KDR-CDglyTK-transferred HUVECs were killed while 77.6% KDR-CDglyTK-transferred LoVo cells were alive; treated with 100 μg/mL GCV + 1000 μg/mL 5-FC, 98.5% KDR-CDglyTK-transferred HUVECs were killed while the killing rate of KDR-CDglyTK-transferred LoVo cells was 42.8%. All these come to an exciting conclusion that the KDR-promoter-controlled double suicide gene/prodrugs system bears a selectively killing effect on HUVEC. This finding may provide an optional way for targeting gene therapy for targeting vascular endothelial cells of tumors.

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