Construction of Recombinant Adenovirus Containing KDR Promoter-HSV-tk Suicide Gene Using a New pAdeasy System and Its Specific Killing Effect On the Vascular Endothelial Cells In Vitro

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Abstract **Objective** To construct a new recombinant adenovirus containing KDR promoter-HSV-tk suicide gene, using a new pAdeasy system, and evaluate its specific killing effect on the vascular endothelial cells. **Methods** By using pAdeasy system, recombinant adenoviral plasmid containing KDR or CMV promoter-controlled HSV-tk gene (AdKDR-tk and AdCMV-tk) was constructed. After packaging and amplification in 293 cells, the virus was used to infect KDR-expressed human umbilical venous endothelial cells (HUVEC) and KDR-unexpressed human hepatoblastoma cell line (HepG2). After administration of ganciclovir (GCV), the survival rate of gene-transfected HUVEC and HepG2 was evaluated by using MTT method.**Results** The pAdeasy System produced a high titer of the recombinant adenovirus (1×10^{10}pfu/ml). Under infection index of 100, with increasing GCV concentration from 0 up to 50ug/ml, the survival rate of AdKDR-tk-transfected HUVEC and HepG2 decreased from 100% to (28.94±5.67)% and (75.45±2.91)% at proper order, respectively ($P<0.01$), while the survival rate of AdCMV-tk-transfected HUVEC and HepG2 declined from 100% to (17.56±2.48)% and (23.15±5.72)%, respectively ($P>0.05$).**Conclusion** Adenovirus-mediated transfection of KDR promoter-HSV-tk gene could yield specific killing effect on vascular endothelial cells with treatment of GCV in vitro.

Key words Vascular endothelium; KDR promoter; Herpes simplex virus thymidine kinase; Adenovirus vector

It is well known that angiogenesis is required for tumor growth, progression and metastasis, and tumor vascularisation correlated directly with the prognosis of cancer patients[1,2]. One of the most effective methods to prevent tumor metastasis is to block tumor angiogenesis [3,4]. Herpes simplex virus-thymidine kinase (HSV-tk) suicide gene, as it was used before, can effectively damage the neoplasm vascular endothelial cells [5]. But the killing effect of currently used HSV-tk system is non-specific due to employment of cytomegalovirus (CMV) as its promoter. KDR (kinase domain insert containing receptor) is one kind of two receptors of vascular endothelial growth factor (VEGF), which is expressed at high level in the neoplasm vascular endothelial cells, but low level in the normal[6].

In the present study, we construct a new recombinant adenovirus which containing KDR promoter-HSV-tk suicide gene, using a new pAdeasy system, and evaluate its specific killing effect on the vascular endothelial cells.

Xiaoping Liu, Baojin Li, Both authors contributed equally to this manuscript.

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MATERIALS AND METHODS

Main materials

The restriction enzymes,T4 DNA ligase were purchased from TaKaRa Co. Japan and NEC Co,USA. Culture medium DMEM,OptiMEM,Lipofectamine2000 and fetal bovine serum were purchased from Gibco, USA. GCV was purchased from Roche, USA.

Vectors, strains and cell lines

Adenoviral shuttle vector pAdTrack-CMV and pAdtrack were gifts from Dr. Bert Vogelstein (Johns Hopkins University, USA). E.coli strain BJ5183-AD-1, containing adenoviral backbone vector pAdeasy-1, was presented from Dr. Yin Wei(Sun Yat-sen university, China).The plasmid pBluescript Ⅱ KDR-tk was obtained from Dr. Yin Zhengfeng (the second medical university of PLA, China). The human embryonic kidney cell line 293 cells, the human umbilical venous endothelial cells (HUVEC) which expressing KDR, and the heaptoblastoma cell line HepG2 which not expressing KDR were kept in our lab.

Identification of pBluescript Ⅱ KDR-tk

The recombinant of plasmid was transformed into *Ecoli*.DH5αPositive clone was identified by enzyme digestion, and its sequence was measured in Invitrogen, USA.

Construction of shuttle vectors pAdKDR -tk and pAdCMV-tk

The KDR promoter and TK cDNA fragment digested by Xho Ⅰ and Sal Ⅰ from pBluescript Ⅱ KDR-tk was inserted into the multiple cloning sites of pAdtrack to generate the shuttle plasmid pAdKDR-TK. Similarly, the TK cDNA fragment digested by Xho Ⅰ and Hind Ⅲ from pBluescript Ⅱ KDR-tk was inserted into the multiple cloning sites of pAdtrack-CMV to generate the shuttle plasmid pAdCMV-TK.

Homologous recombination and purification of shuttle vectors pAdKDR-tk and pAdCMV-tk

Then,the shuttle vectors pAdKDR-tk and pAdCMV-tk were digested with Pme Ⅰ hand they were homologically recombined with pAdeasy in BJ5183-AD-1 bacteria after linealized, named AdKDR-tk and AdCMV-tk. The recombinant clones were obtained after the kanamycin selection.

Package and amplification of recombinant adenovirus vectors AdKDR-tk and AdCMV-tk

After that, 293 cell line was transfected by the recombinant adenoviral plasmid after they were amplified in germ DH5α before being extracted and purified. The infective adenoviruses were packaged and amplified in 293 cells, and then purified by CsCl density gradient purification.

Determination of adenovirus titer[7]

Virus liquid in different concentration by diluting was dropped into each hole. The titer of virus was quantified by a plaque formation assay using 293 cells and expressed as plaque formation units (pfu). Green fluorescent protein (GFP), as an indictor marker, was observed under immunofluoresence microscopy. The titer of virus(pfu/ml)=(GFP positive cells × virus dilution multiple)/0.4ml.

Cell culture

Monolayer cultures of HUVEC and HepG2 cells were grown in DMEM medium supplemented with 10% fetal bovine serum at 37°C in 5% CO_2.

GCV test in vitro

HUVEC or HepG2 Cells were cultured in 96-well plates(2×10^3/well), infected at a MOI (Multiplicity Of Infection)of 0, 1, 10, 100, 1000 with AdCMV-TK or AdKDR-TK separately in the next day. After 16 hours of infection, different concentrations of GCV (0, 1, 10 and 50μg/ml) were respectively added into the medium to incubate for 5 days, but none of GCV was added into the medium to incubate in control group. MTT was added to each well to a final concentration of 200 μg/ml. DMSO 150μl was added 4 hours later. Optical densities (OD) were measured at the wavelength of 490 nm. Each group set three repeated wells and each experiment was performed for three times.

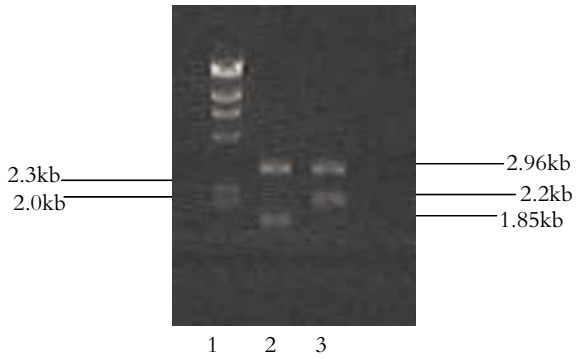

Fig. 1 Enzyme digestive map of pBluescript Ⅱ KS−KDR−tk
Lane1:DNA markers (λDNA/Hind Ⅲ)
Lane2:pBluescript Ⅱ KS−KDR−tk/(Xho Ⅰ /HindIII)
Lane3:pBluescript Ⅱ KS−KDR−tk/ (Xho Ⅰ /Sal Ⅱ)

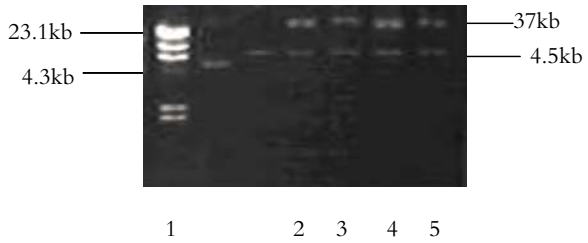

Fig.2 Enzyme digestive map of pAdKDR−tk and pAdCMV−tk
Lane1:DNAmarkers(λDNA/Hind Ⅲ)
Lane2,3:pAdCMV−tk/Pac Ⅰ
Lane4,5:pAdKDR−tk/Pac Ⅰ

Statistical analysis

SPSS V.10.0 software was performed for statistical analysis. Quantitative data were expressed as mean ± standard deviation (SD). Results were evaluated by One−way analysis of variance (ANOVA). The statistilevel of significance was set at $P<0.05$.

RESULTS

Identification of pBluescript Ⅱ KDR−tk

Plasmids were digested by Xho Ⅰ and Sal Ⅰ, Hind Ⅲ and Xho Ⅰ, the each showing two fragments, 2.2 kb and 2.96 kb; 1.85 kb and 2.96 kb, respectively. Their size and sequence correlated with the provided data. (Fig. 1)

Identification of recombinant adenovirus vectors AdKDR−tk and AdCMV−tk

Recombinant adenoviral vectors pAdKDR−tk and pAdCMV−tk were digested by PacI, the either showing two fragments (4.5kb and 37kb) coincided with anticipated its size(Fig. 2).

Production of recombinant adenovirus and determination of its titer

GFP expression was visible at 24th h after transfection in 20%~30% of the cells, representing the fraction of the population that was transfected. In cells transfected with linearized pAdEasy−GFP, however, this expression never faded and comet−like foci, visualized with GFP fluorescence but invisible by phase contrast microscopy. Either of titer of the virus liquid was 1×10^{10}pfu/ml.(Fig. 3)

Killing effect of GCV in vitro

HepG2 and HUVEC, infected by recombinant adenovirus pAdKDR−tk at various MOI, had different sensitivity to the GCV, while MOI =100 was the most sensitive. At this time, density of GCV increased from 0 to 50μg/ml, and the survival rates of infected HUVEC and the HepG2 were descended to (28.9±45.67)%and (75.45±2.91)% from 100%, respectively. The difference was obvious significance in statistics.($P<0.01$, Fig. 4)

HepG2 and HUVEC, infected by the different MOI of pAdCMV−tk, had similar sensitivity to the GCV. As the MOI and the GCV density increased, the cell survival rate all presented to a obviously descending trend. When MOI was 100, concentration of GCV increased from 0 to 50μg/ ml, the HUVEC and the HepG2 cell survival rates changed into (17.56±2.48) % and (23.15± 5.72) % respectively. (Fig. 4, $P>0.05$)

Under infection index of 100, with increasing GCV concentration from 0 up to 50μg/ml, the survival rate of AdKDR−tk−transfected HUVEC and HepG2 decreased from 100% to(28.94±5.67)% and(75.45±2.91)% at proper order, respectively ($P<0.01$), while the survival rate of AdCMV−tk−transfected HUVEC and HepG2 declined from 100% to (17.56±2.48)% and (23.15±5.72)%, respectively ($P>0.05$, Fig. 4).

DISCUSSION

Angiogenesis plays a key role for tumor growth, progression and metastasis, so antiangiogenesis or de-

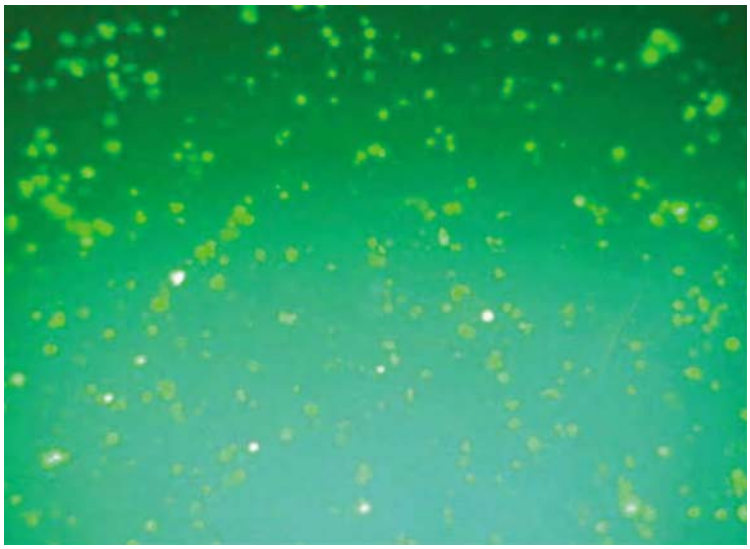

Fig. 3 GFP expression in AdKDR−tk infected 293 cells (×100)

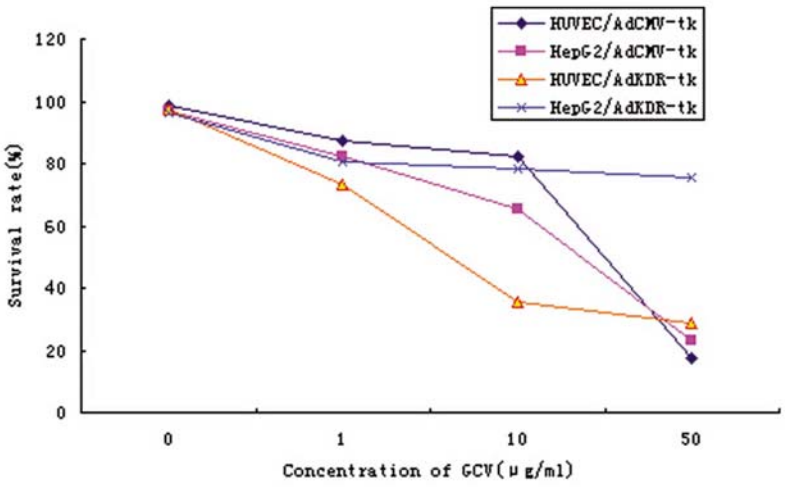

Fig.4 Survival rate of cells transfected by AdKDR−tk or AdCMV−tk after GCV administration

struction of tumor neovessels is an effective strategy to prevent tumor growth [8]. Endothelial cells in tumors have abnormalities in gene expression. Rapid vascular regrowth reflects the ongoing drive for angiogenesis that promote vascular abnormalities and thereby create therapeutic targets[9].

Whereas most antiangiogenic agents prevent new blood vessel formation, vascular targeting agents destroy pre−existing blood vessels of solid tumors. Similarly, the systemic delivery of viral vectors can lead to non specific uptake by various different tissues and results into systemic toxicity because of transgene expression. Hence, transgene expression in the targeted tissue is very important [10]. The tumor vasculature offers an ideal target for anti−cancer gene therapy which has the advantages both of good accessibility to systemically delivered therapy and comparative homogeneity across solid tumor types.

KDR/flk−1 is one of the two receptors (named flk−1 and flt−1 respectively) for vascular endothelial growth factor, a potent angiogenic peptide [11,12], and its expression is restricted to endothelial cells *in vivo*. It has a wider pattern of expression among endothelial cell populations than does flt−1. In addition, only KDR has been shown to autophosphorylate in the presence of

VEGF [13]. Thus, the presence of KDR is absolutely required for the development of endothelial cells from hemangioblastic precursors. In 1995, Patterson C[14] firstly cloned and characterized the promoter of the human KDR gene. They found that it directed high level promoter activity in endothelial cells but not in other type cells. So, the KDR promoter is considered to be endothelial cell-specific and to uncover methods for targeting gene delivery specifically to endothelial cells [15]. Jaggar RT [16]designed promoter sequences from the endothelial-specific KDR/VEGF receptor, and it indicates that KDR has been shown to be upregulated on tumor endothelium.

The herpes simplex virus thymidine kinase gene therapy with ganciclovir forms the basis of a widely used strategy for suicide gene therapy [17]. Mavria G [18] demonstrates that vascular targeting combined with HSV-TK is more effective.

Recombinant adenoviruses provide a versatile system for gene expression studies and therapeutic applications. Adenoviruses transfer genes to a broad spectrum of cell types independent of active cell division. Additionally, high titers of viruses and high levels of transgene expression generally can be obtained[19].

We report herein a new strategy that simplifies the generation and production of such viruses. Recombinant adenoviral plasmids were generated with a minimum of enzymatic manipulations, using homologous recombination in bacteria rather than in eukaryotic cells. After transfections of such plasmids into a mammalian packaging cell line, viral production was conveniently followed with the aid of GFP, encoded by a gene incorporated into the viral backbone. And the GFP tracer made it possible to follow all stages of the viral production process in a convenient fashion. In the case of cells that were inefficiently infected by adenoviruses, the GFP tracer additionally made it possible to isolate expressing cells through fluorescence-activated cell sorting and thereby facilitates several kinds of experiment. The ability to recover reasonable quantities of homogeneous viruses, without plaque purification, represented a major practical advantage.

We infected the vascular endothelial cells HUVEC which expressing KDR or hepatoblastoma cell line HepG2 which not expressing KDR with recombinant adenovirus AdKDR- tk or AdCMV-tk, followed by treatment of GCV. The results showed that adenovirus-mediated transfection of KDR promoter-HSV-tk gene could yield a specific killing effect on vascular endothelial cells, and it would may be a promising approach for cancer anti -angiogenesis therapy.

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