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Inhibitory effect of adeno-associated virus-mediated gene transfer of human endostatin on hepatocellular carcinoma

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Abstract

AIM: To investigate the effect of adeno-associated virus-mediated gene transfer of human endostatin on the growth of hepatocellular carcinoma (HCC).

METHODS: HCC cell line Hep3B was infected with recombinant adeno-associated virus containing human endostatin gene (rAAV2-hEndo). The results of transfection were detected by RT-PCR and SDS-PAGE assay. MTT assay was used to observe the effects of supernatant of transfected cells on ECV304 cell proliferation. An animal model of HCC was established by injecting Hep3B cells subcutaneously into the back of nude mice. Intratumoral injection of rAAV2-hEndo, empty virus and phosphate-buffered saline were given sequentially. Serum endostatin was determined by ELISA, the inhibitory effect of endostatin on the growth of xenograft was assessed in 3 wk.

RESULTS: The results of RT-PCR and SDS-PAGE assay confirmed that rAAV2-hEndo successfully transfected Hep3B cells, and endostatin was secreted from Hep3B cells to medium. The supernatant of transfected cells markedly inhibited the proliferation of ECV304 cells ($P<0.01$). Intratumoral injection of rAAV2-hEndo (2×10^{10} v.g.) led to a sustained serum endostatin level of approximately (86.71±5.19) ng/mL. The tumor volume and microvessel density were less in rAAV2-hEndo group than in control groups ($P<0.01$).

CONCLUSION: Human endostatin can be stably expressed by adeno-associated virus-mediated gene transfer and effectively inhibit the growth of HCC.

Key words: Endostatin; Gene; Adeno-associated virus; Hepatocellular carcinoma

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INTRODUCTION

Endostatin, a M_r 22 000 protein, was originally isolated from the supernatant of a cultured murine hemangioendothelioma cell line and represents a COOH-terminal fragment of collagen XVIII α1-chain[1]. It is a potent angiogenesis inhibitor that specifically inhibits the proliferation and migration of endothelial cells but has no effect on tumor cells or nonendothelial cell types[1-5]. Animal studies demonstrated that recombinant endostatin strongly inhibits the growth of a variety of murine and xenotransplanted human tumors[6-9]. The expression of endostatin is also associated with progression and prognosis of human hepatocellular carcinoma (HCC)[10-12]. In addition, elevated serum endostatin can inhibit tumor liver metastases by preferential targeting of hepatic sinusoidal endothelium[13,14]. In the present study, the recombinant adeno-associated virus vector containing human endostatin gene (rAAV2-hEndo) was constructed and used to transfect liver cancer cells. The data revealed that transfected cells could stably express active endostatin. Furthermore, our *in vivo* experiments showed that intratumoral injection of rAAV-hEndo could inhibit angiogenesis and growth of HCC.

MATERIALS AND METHODS

Reagents and cell lines

Recombinant adeno-associated virus (rAAV) was constructed in the Institute of Virology, Chinese Academy of Preventive Medicine. Human HCC cell line Hep3B was purchased from Shanghai Cancer Institute. Human umbilical vein endothelial cell line ECV304 was purchased from Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. The cells were incubated in DMEM (Gibco BRL) at 37 ℃ in a humidified 50 mL/L CO_2 atmosphere. All media were supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine.

Transfection in vitro

Hep3B cells were plated on 24-well plates at a density of

5×10^4 cells/well and incubated for 24 h. One of the wells was chosen to count cells. The cells were washed twice with PBS and incubated in 200 μL of serum-free medium with rAAV2-hEndo (1×10^5 v.g./cell) or AAV2 for 1 h. The medium was replaced by DMEM supplemented with 10% fetal calf serum, and cells were incubated for 72 h.

RT-PCR analysis

Total RNA was extracted from 10^7 transfected-Hep3B cells using the TRIzol reagent (Gibco BRL), and cDNA synthesis was performed using the reverse transcription system (Gibco BRL) according to the manufacturer's instructions. One microliter of cDNA was used as template and PCR amplification was carried out in a 50 μL reaction system . The primers were as follows: endostatin: 5'-AGT CGA ATT CAT GCA CAG CCA CCG CGA CTT CC-3', 5'-CTG TCG TCG ACC TA CTT GGA GGC AGT CAT G-3' (product size, 552 bp); β-actin: 5'-TCA CCC ACA CTG TGC CCA TCT ACG A-3', 5'-CAG CGG AAC CGC TCA TTG CCA ATG G-3' (product size, 295 bp). The following PCR cycling parameters were employed: at 95 ℃ for 5 min, followed by 35 cycles at 95 ℃ for 45 s, at 56 ℃ for 1 min, and at 72 ℃ for 1 min, then at 72 ℃ for 7 min. The PCR products were resolved on a 1.5% agarose gel.

SDS-PAGE analysis

AAV2 and rAAV2-hEndo-transfected Hep3B cells, and untransfected Hep3B cells were incubated in 24-well plates for 72 h respectively. The supernatant was collected, and 20 μL of 50-fold concentrated supernatant was used as SDS-PAGE samples. SDS-PAGE was performed according to a standard procedure under reducing conditions. Samples were loaded on 15% SDS-PAGE gel and run until the prestain molecular weight standard (Amersham Biosciences) revealed a good resolution, followed by staining with Coomassie Blue and destaining with methanol and acetic acid.

Endothelial cell proliferation assay

ECV304 cells (1×10^4 per well) were incubated in 96-well plates for 24 h. The cells were washed with PBS and the medium was replaced by 20 μL of 10-fold concentrated supernatant of transfected cells. Serum-free medium served as control. After 20 min, 80 μL DMEM supplemented with 10% fetal calf serum was added and cells were incubated for 24, 48, or 72 h, respectively. The viability of cells was determined by colorimetric MTT assay. Absorbance at 570 nm was read using a Model-550 ELISA plate reader (Bio-Rad).

Treatment of transplanted tumor

Hep3B cells were harvested and rinsed with serum-free medium. A total of 2×10^6 cells were inoculated subcutaneously into the back of 6-wk-old BALB/c nude mice. When the diameter of tumor reached about 0.5 cm, the mice were divided into three groups (8 mice/group). Two groups of mice were injected 100 μL rAAV2-hEndo or AAV2 (2×10^{11} v.g./mL). The control groups received PBS injection. Tumor size was monitored by measuring the longest and shortest dimensions thrice a week with a dial caliper, and tumor volume was calculated as width$^2\times$length$\times\pi/6$. The experiments were terminated in 3 wk. The serum samples of mice were collected by centrifugation at 10 000 *g*, and ELISA was performed to determine the serum level of endostatin according to the instructions of human endostatin EIA kit.

Detection of microvessel density

Tumor specimens were taken from the mice, embedded in paraffin and stained with hematoxylin-eosin. Five-micrometer-thick sections were mounted on silanized slides, which were processed according to the standard methods with ABC kit. The primary antibody used was polyclonal antibody of factor XVIII-related antigen (Dako, Glostrup, Denmark) at the dilution of 1:100. Diaminobenzine was used for color development. Negative controls were generated by substituting the primary antibody with distilled water. Neovascular hot spots were searched on duplicate slide set for each sample, which were frequently localized at or near the margin of tumors. Sections were scanned at low power (×40). Five areas with the greatest density of distinct positive microvessels were selected and a ×200 field in each was counted by two independent observers under an Olympus BX60 microscope. The mean value of the five fields was considered as the microvessel density (MVD) of each sample. Data were expressed as the number of microvessels per high power field.

RESULTS

Expression of endostatin in transfected cells

To evaluate the transcriptional expression of endostatin in rAAV2-hEndo transfected cells, RT-PCR was carried out and PCR products were analyzed under ultraviolet light after 1.5% agarose gel electrophoresis. A 552-bp fragment was visualized in PCR products amplified from transfected cells, but not from controls (Figure 1), indicating that endostatin gene was successfully transferred into Hep3B cells. On a reducing 15% SDS-PAGE gel, a distinct band of M_r 22 000, corresponding to endostatin, was seen in the supernatant of rAAV2-hEndo transfected cells only (Figure 2), confirming that endostatin could be stably expressed and secreted in Hep3B cells.

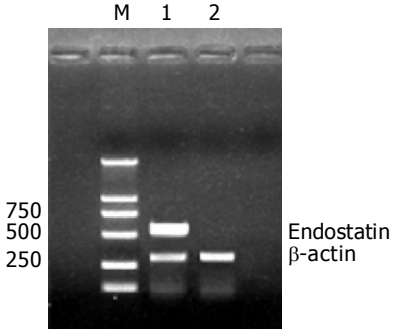

Figure 1 RT-PCR analysis of endostatin in transfected cells. M: DL2000 DNA marker; lane 1: PCR products amplified from rAAV2-hEndo transfected cells; lane 2: PCR products amplified from AAV2 transfected cells.

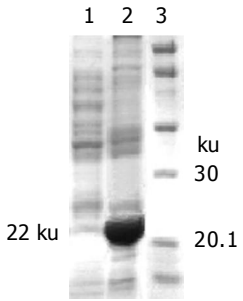

Figure 2 SDS-PAGE of the supernatant of transfected cells. Lane 1: concentrated supernatant of AAV2 transfected Hep3B cells; lane 2: rAAV2-hEndo transfected Hep3B cells; lane 3: molecular weight standard (Amersham Biosciences), myosin (M_r 220 000), phosphorylase (M_r 97 000), albumin (M_r 66 000), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 30 000), trypsin inhibitor (M_r 20 100), lysozyme (M_r 14 300).

Antiangiogenic activities of endostatin

MTT assay was used to investigate the effects of endostatin on endothelial cell proliferation. ECV304 cells were incubated in supernatant of transfected cells for 24, 48 and 72 h. The optical densities in endostatin-transfected group (0.375±0.044, 0.481±0.025, 0.538±0.047) were lower than those in AAV2-transfected group (0.429±0.051, 0.740±0.031, 0.871±0.057) (P<0.05). However, the optical density in AAV2-transfected group had no significant difference from that of DMEM group (Figure 3), suggesting that endostatin expressed by rAAV2/Endo-transfected Hep3B cells could markedly inhibit ECV304 cell proliferation.

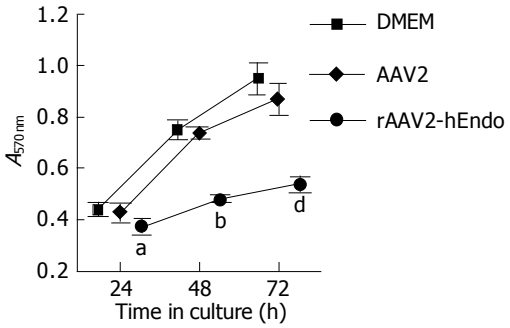

Figure 3 Proliferation inhibition of endothelial cells by supernatant from transfected and untransfected cells. [a]P<0.05, [b]P<0.01 and [d]P<0.01 *vs* AAV2-transfected group.

Determination of endostatin in vivo

The results of ELISA showed that intratumoral injection of rAAV2-hEndo (2×10^{10} v.g.) led to a sustained serum endostatin level of approximately (86.71±5.19) ng/mL. But in empty virus injection group and PBS-injection group, we did not detect the expression of endostatin, indicating that the endostatin could be efficiently expressed *in vivo* by adeno-associated virus-mediated gene transfer.

Effects of endostatin on HCC growth

After 3 wk, the tumor volume and MVD of rAAV2-hEndo injection group were significantly less than those in AAV2-injection group and PBS-injection group (P<0.01, Table 1).

The results of HE staining showed that necrosis of tissue increased, the number of blood vessels decreased and the distribution of tumor cells was asymmetrical in transplanted tumor treated with rAAV2-hEndo, compared with control groups (Figure 4).

Table 1 Effects of gene transfer of human endostatin on HCC xenograft (mean±SD)

Group	*n*	Volume (cm^3)	MVD (per HP)	Concentration (μg/L)
rAAV2-hEndo group	8	0.493±0.119[b]	18.50±4.38[b]	86.71±5.19
AAV2 group	8	0.992±0.194	44.88±6.22	Not determined
Control group	8	1.073±0.213	46.87±6.60	Not determined

[b]P<0.01 *vs* AAV2 and control group.

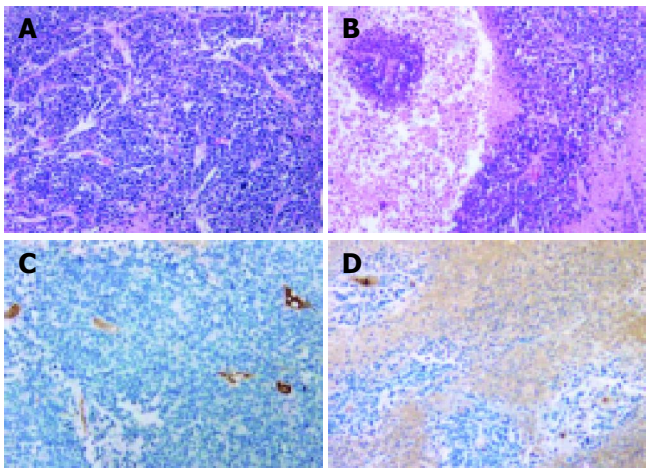

Figure 4 Immunohistochemical and HE staining of HCC xenograft. **A**: AAV2-transfected tissue (HE staining, ×200); **B**: endostatin-transfected tissue (HE staining, ×200); **C**: MVD of AAV2-transfected tissue (stained with factor VIII-related antigen, ×200); **D**: MVD of rAAV2-hEndo transfected tissue (stained with factor VIII-related antigen, ×200).

DISCUSSION

Generally, angiogenesis is essential for tumor growth and metastasis[15-19]. A variety of approaches demonstrate that interference with tumor-induced angiogenesis may be an effective strategy in cancer therapy. However, the most effective strategies require extended suppression of the angiogenic process. Gene therapy offers a possible approach to achieve sustained release of a therapeutically potent transferred gene product[20]. rAAV is currently considered as one of the most promising viral vectors for gene therapy, because of a unique combination of attractive properties[21,22]. Wild-type AAV2 is not associated with any human diseases, and has a broad range of host cells. rAAV vectors have the ability to efficiently transduce both mitotic and postmitotic tissues and the potential of site-specific integration. Previous studies demonstrated that a single administration of rAAV results in persistent expression of transgene and long-lasting phenotypic correction in rat model for more than 6 mo[23]. In addition, Ponnazhagan *et al*[24] reported that rAAV vectors accumulate predominantly in liver cells, suggesting that rAAV may possess hepatotropism.

Here we have constructed a novel recombinant adeno-associated virus vector with endostatin gene (rAAV2-hEndo)[25], which can successfully transfect Hep3B cells. The data indicate that endostatin expressed by transfected cells can markedly inhibit the proliferation of endothelial cells *in vitro*. In our study, intratumoral injection of rAAV2-hEndo (2×10^{10} v.g.) led to a sustained serum endostatin level of approximately (86.71±5.19) ng/mL, the tumor volume and MVD were significantly lower in endostatin-transfected group than in control groups, confirming that transfection of endostatin gene can effectively inhibit tumor cell-induced angiogenesis and suppress the growth of human HCC. Though the antitumor effect of rAAV2-hEndo is evident in HCC, the transplanted tumor did not resolve completely. It is possibly because endostatin has no toxicity to tumor cells and mostly acts on endothelial cells at the hyperplastic stage[26]. Recent studies have shown that combination of antiangiogenesis and conventional therapy improves antitumor effects[27-29], which may be a novel strategy for HCC therapy.

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