ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 May; 20 (5): 440-444
http://www.simm.ac.cn; www.chinainfo.gov.cn/periodical

Gene therapy for human hepatocellular carcinoma with cytosine deaminase gene and prodrug flucytosine

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KEY WORDS gene therapy; gene transfer; experimental liver neoplasm; prodrugs; antineoplastic agents; hepatocellular carcinoma; cultured tumor cells; nude mice; flucytosine

ABSTRACT

AIM: To investigate the antitumor effects of cytosine deaminase (CD) gene in combination with prodrug flucytosine (Flu, 5-fluorocytosine) on human hepatocellular carcinoma. **METHODS**: CD gene was transduced into human hepatocellular carcinoma cell line SMMC7721 with retroviral method and the cytotoxicity of Flu on the tumor cells was assayed *in vitro* with clonogenic techniques. The xenograft tumor model in nude mice was used to study *in vivo* therapeutic effects of CD gene/Flu system against human hepatocellular carcinoma. **RESULTS**: CD gene/Flu system had significant antitumor activities on human hepatocellular carcinoma cells *in vitro* and in nude mice. The antitumor activities of Flu 500 $mg \cdot kg^{-1}$ on hepatocellular carcinoma xenografts in nude mice were more potent than those of 5-fluouracil 10 $mg \cdot kg^{-1}$. CD gene/Flu system possessed bystander killing effects on hepatocellular carcinoma xenografts in nude mice. **CONCLUSION**: The experiment demonstrates the potential value of the CD gene/Flu system in the treatment of human hepatocellular carcinoma.

INTRODUCTION

Gene therapy has opened a new avenue for the treatment of cancers. The suicide gene therapy or drug sensitivity gene therapy, is one of the important ways among the cancer gene therapy[1-3]. It uses a replication-defective recombinant viral vector to introduce a foreign gene encoding an enzyme capable of converting a nontoxic prodrug into a cytotoxic compound, and kills the cancer cells selectively. Cytosine deaminase (CD) gene, as a suicide gene, possesses anticancer activities on some cancers in combination with prodrug flucytosine (Flu)[4-6], but there is no report about this gene on human hepatocellular carcinoma (HCC). In this paper, we investigated the anticancer effects of the CD gene/Flu system on HCC.

MATERIALS AND METHODS

Reagents RPMI-1640 medium, Dulbecco's modified Eagle's medium (DMEM) and G418 were purchased from Gibco Life Technologies, and Flu was from Sigma. Lipofectin was a product of Promega Corp. and 5-fluorouracil (FU) was obtained from Shanghai Haipu Pharmaceutical Factory.

Vector and animals The plasmid pLXSN, one of replication-defective retroviral vector, contained eukaryotic expression elements: (5') Moloney murine sarcoma virus long terminal repeat (LTR) promoter, polycloning site, Simian virus 40 early promoter, neomycin phosphotransferase gene, and Moloney murine leukemia virus promoter (3'). Plasmid pCD2 which contained the CD gene controlled under the LTR promoter was a generous gift from Professor Michael BLAESE working in the NIH, and its cloning vector was plasmid pLXSN.

BALB/cpbi-nu nude mice, ♀, 5-6 wk old, specific pathogen free animal, Certificate № (1994)

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Received 1998-07-17 Accepted 1998-10-29

074, were purchased from Laboratory Center of National Institute for the Control of Pharmaceutical and Biological Products.

Cellular techniques NIH3T3 cells and PA317 cells were maintained in DMEM medium supplemented with 10 % (vol/vol) heat-inactivated fetal bovine serum. The human HCC cell line SMMC7721 was grown in RPMI-1640 medium supplemented with 10 % (vol/vol) heat-inactivated fetal bovine serum.

The plasmid pLXSN and pCD2 were transfected into PA317 cells using Lipofectin. After 72 h, cells were maintained in the DMEM medium containing G418 1 $g \cdot L^{-1}$ for 2 wk, and the individual colonies were selected and expanded. Virus produced by each of these clones was titred on NIH3T3 fibroblasts. A clone producing virus at relatively high titre (3.4×10^8 colony forming units $\cdot L^{-1}$) was clonally expanded and the viral supernatant was collected. The SMMC7721 cells were exposed to viral supernatant in the presence of polybrene (8 $mg \cdot L^{-1}$) for 6 h, and selected with G418 1 $g \cdot L^{-1}$ for 2 wk. The cells transduced with CD gene were designated SMMC/CD cells.

***In vitro* sensitivity to Flu**[6] Cells were diluted to 1×10^7 cells $\cdot L^{-1}$, and 1×10^3 cells were cultured in flat-bottomed 24-well Costar tissue culture dish along with 2 mL of medium containing Flu or G418 at a series of concentrations. On d 7, the wells were stained with methylene blue, and colonies were counted under 40 × magnification and averaged from triplicate wells.

Tumor studies with nude mice Female athymic nude mice were implanted sc 1×10^7 tumor cells in 0.2 mL of normal saline, and Flu 500 $mg \cdot kg^{-1}$ or FU 10 $mg \cdot kg^{-1}$ were injected ip between d 3 and d 16. Tumor volume (mm^3) was calculated by: tumor volume (mm^3) = [Length (mm)] · [Width (mm)]$^2 \div 2$. On d 23, the mice were killed and the tumors were weighed.

Determination of bystander killing effects Nude mice were implanted sc with 1×10^7 cell mixtures of SMMC/CD and SMMC7721 cells at ratios of 100:0, 30:70, and 0:100, and ip injections of Flu 500 mg $\cdot kg^{-1}$ or the same volume of normal saline in control mice between d 3 and d 16. The tumor volume was measured during the course of experiment.

RESULTS

***In vitro* sensitivity of SMMC/CD cells to Flu** The colony counts of SMMC7721 cells were greater than that of SMMC/CD cells in the presence of Flu at 500 or 1 000 $mg \cdot L^{-1}$. In medium containing G418, no colony of SMMC7721 cell was found, but little effects on SMMC/CD cell. These results indicated that retroviral vectors containing the CD gene could effectively transduce human HCC cells and conferred lethal sensitivity to Flu on the transduced tumor cells (Tab 1).

Tab 1. Colony-forming rate of SMMC7721 cells or SMMC/CD cells treated with flucytosine or G418. $n = 3$ wells. $\bar{x} \pm s$. [a] $P > 0.05$ *vs* SMMC7721 + NS. [f] $P < 0.01$ *vs* SMMC7721 + Flu 0.5 $g \cdot L^{-1}$. [i] $P < 0.01$ *vs* SMMC7721 + Flu 1.0 $g \cdot L^{-1}$. [l] $P < 0.01$ *vs* SMMC7721 + G418 0.5 $g \cdot L^{-1}$.

Cell line	Drug /$g \cdot L^{-1}$	Number of colonies	Inhibiting rate/ %
SMMC7721	NS	517 ± 40	
	Flu 0.5	473 ± 53	8
	Flu 1.0	155 ± 25	70
	G418 0.5	0	100
SMMC/CD	NS	471 ± 25[a]	
	Flu 0.5	30 ± 5[f]	94
	Flu 1.0	8 ± 2[i]	98
	G418 0.5	441 ± 2[l]	6

Antitumor effects in nude mice In control group, tumors derived from SMMC7721 or SMMC/CD cells grew at the same rate throughout the experiment (Fig 1A). On d 23, no difference in tumor weight between SMMC7721 tumor xenografts and SMMC/CD tumor xenografts (Tab 2, $P > 0.05$) in untreated mice. In mice treated with Flu, significant difference was found in the appearance of the SMMC/CD tumor xenografts, the tumor growth rate (Fig 1B, $P < 0.01$) and tumor weights (Tab 2, $P < 0.01$), compared to the adjacent SMMC7721 tumor xenografts. These data indicated that prodrug Flu had profound antitumor effects on the SMMC/CD tumor xenografts and less effects on the SMMC7721 tumor xenografts in nude mice.

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 May; 20 (5)
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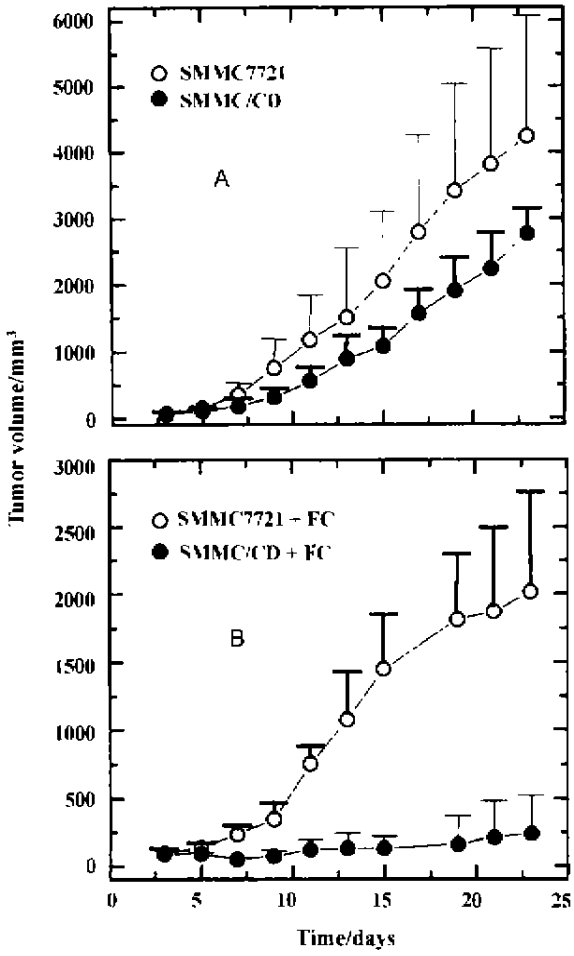

Fig 1. Growth rate of the tumor xenografts derived from SMMC/CD or SMMC7721 cells in nude mice ip (A) 0.5 mL of normal saline ($P > 0.05$ *vs* SMMC7721), and (B) flucytosine 500 mg·kg^{-1}·d^{-1} ($P < 0.01$ *vs* SMMC7721). $n = 5$ mice. $\bar{x} \pm s$.

Tab 2. Antitumor effects of CD gene/Flu system on transgenic human hepatocellular carcinoma in nude mice. $n = 5$ mice. $\bar{x} \pm s$, $^aP > 0.05$, $^bP < 0.05$, *vs* SMMC7721; $^fP < 0.01$ *vs* SMMC7721 + Flu; $^jP < 0.01$ *vs* SMMC/CD + FU; $^jP > 0.05$ *vs* SMMC/CD.

Group	Flu /mg·kg^{-1}	Tumor weight/g	Inhibiting rate/%
SMMC7721	0	2.98 ± 1.06	
SMMC/CD	0	2.15 ± 0.61^a	
SMMC7721 + Flu	500	1.47 ± 0.68^a	51
SMMC/CD + Flu	500	0.097 ± 0.051^{b,f}	95
SMMC7721 + FU	10	1.11 ± 0.92^b	63
SMMC/CD + FU	10	1.51 ± 0.53^j	30

Antitumor effects of Flu and FU The evident antitumor activity was observed in Flu on SMMC/CD tumor xenografts, and FU on SMMC7721 tumor xenografts. However, there was statistically difference in tumor growth rate and tumor weights between Flu 500 mg·kg^{-1} and FU 10 mg·kg^{-1} on the mice bearing SMMC/CD tumors. The results indicated that Flu 500 mg·kg^{-1} possessed higher antitumor activity than that of FU 10 mg·kg^{-1} in nude mice (Fig 2).

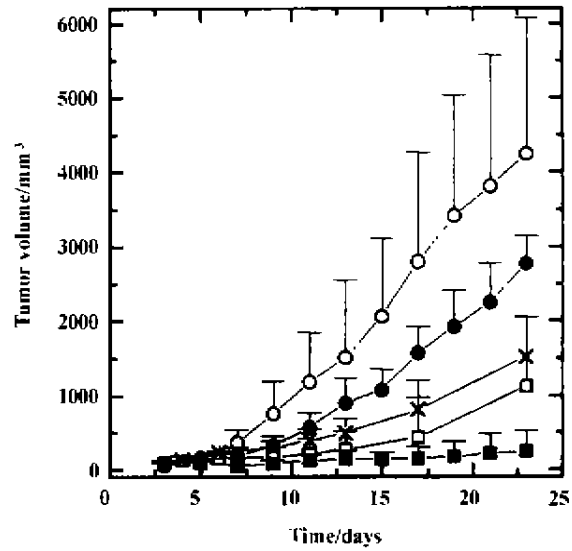

Fig 2. Comparison of flucytosine (Flu) and 5-fluorouracil (FU) antitumor activity targeted to SMMC7721 or SMMC/CD derived tumors. Nude mice were treated wth normal saline (control), or with FU 10 mg·kg^{-1}·d^{-1} ($P < 0.01$ *vs* control), or with Flu 500 mg·kg^{-1}·d^{-1} ($P < 0.01$ *vs* control or FU group). $n = 5$ mice. $\bar{x} \pm s$. (○) SMMC7721 + NS, (●) SMMC/CD + NS, (×) SMMC/CD + FU, (□) SMMC7721 + FU, (■) SMMC/CD + Flu.

Bystander killing effects In control mice not receiving Flu, no statistical difference in tumor appearance, and the average growth rate, were observed for all the sets of tumors. In Flu-treated mice, in the tumor xenografts containing 30 % SMMC/CD cells/70 % SMMC7721 cells, tumor regressions were detected and the growth rate of tumors were less than that of control mice (Fig 3), but no statistical difference compared with 100 % SMMC/CD cells/0 % SMMC7721 derived-tumors treated with

Flu. It demonstrated that only a small percentage of tumor cells in HCC needed to express CD to generate a significant antitumor effects.

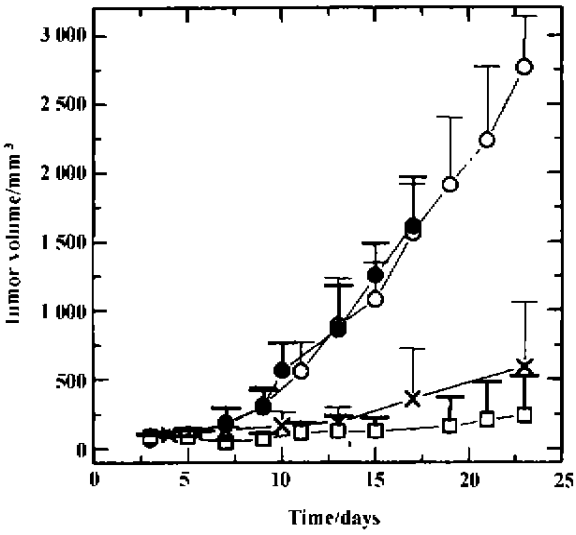

Fig 3. Bystander effects of flucytosine (Flu) on hepatocellular carcinoma in nude mice. The tumors derived from the cells mixture of SMMC7721 with SMMC/CD at raitos: (○) 100:0, (●) 70:30, (×) 70:30 + Flu ($P < 0.01$ *vs* 70:30 group), (□) 0:100 + Flu, which inoculated 1×10^7 cells in nude mice and treated with or without Flu 500 mg·kg^{-1}·d^{-1}. $n = 5$ mice. $\bar{x} \pm s$.

DISCUSSION

CD (E. C 3.5.4.1) gene as one of important suicide genes, only presents itself in some bacteria and fungi but not in mammalian cells. Molecular cloning and sequencing of the CD gene in *Escherichia coli* was succeeded in 1992 [7]. This gene encoded the enzyme protein that has an ability to convert Flu to FU, a highly toxic metabolite that is lethal to the cells. Flu is an antifungal agent used in clinical practice and non- or low toxic to mammalian cells, since the mammalian cells do not express CD gene and is unable to convert Flu to FU. Taking advantage of different distribution of CD gene between bacteria and mammalian cells, the gene was transferred into HCC cells, then Flu as the prodrug could be converted to FU in the transgenic cancer cells. It locally enhanced the effects of cytotoxicity of the chemotherapeutic drugs within neoplastic cells and had low or no injury to the normal tissues of the host. FU is a potent anticancer agent of treatment of HCC in clinical routine, and it is metabolized to 5-fluorouridine triphosphate and 5-fluoro-2′-deoxyuridine monophosphate, resulting in inhibiting of DNA and RNA synthesis, and cell death. It was found in our experiments that CD gene/Flu system had a significant anticancer activity on CD gene transduced HCC cells *in vitro* and in nude mice. Moreover, it was of interest to discover that Flu 500 mg·kg^{-1} had a more potent anticancer activity on the CD gene transduced HCC in nude mice than that of FU at 10 mg·kg^{-1} (body weight). Though the mechanism was not clear, to increase the cytotoxicity on the tumor cells might be due to the formation of the high concentration of FU converted from high dose of Flu in the cancer cells in Flu-treated animals. The other reason might be the activity of nature killer cells and other immunological functions remained in nude mice, which could be stimulated and enhanced by cancer cells expressing *Escherichia coli* CD gene that encoded the foreign protein acting as antigen [8].

The bystander killing effects were observed in our experiments for the CD gene/Flu system on the HCC xenografts in nude mice, meaning that the CD gene/Flu system not only had cytotoxicity on CD-expressing tumor cells, but also killed neighbor cells which did not express CD gene. It is believed that this phenomenon should enhance its therapeutic efficiency and benefits for the treatment of cancer. However, the mechanism has not been fully elucidated. FU production could be detected in the supernatant of the CD-expressing cell lines with HPLC assay [4]. This would suggest that the bystander killing effect might be due to the direct transfer of FU via diffusion. As a new method to treat cancer, the efficiency of CD gene/Flu system needed to be further improved. Otherwise, the suicide gene system only had an ability to kill the local cancer cells, but less or no effect on the distant cancer (metastases). As to overcome these shortcomings, our next work will be focused on cancer gene therapy using CD gene in combination with some cytokines.

In summary, our experiments confirmed that CD could be expressed and caused cell death with Flu in human HCC cells *in vitro* and in nude mice, and bystander killing effect was found with CD gene/Flu system in animals. It demonstrated the potential value of the CD gene/Flu system in the treatment of HCC in basic and clinical field.

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 1999 May; 20 (5)
E-mail aps@server shcnc.ac.cn Phn/Fax 86-21-6474-2629

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胞嘧啶脱氨酶基因与前体药物 5-氟胞嘧啶对人肝癌的基因治疗作用

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R978.1

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关键词 基因治疗；基因转移；实验性肝肿瘤；前体药物；抗肿瘤药物；肝细胞癌；培养的肿瘤细胞；裸小鼠；氟胞嘧啶

目的：探讨胞嘧啶脱氨酶基因(CD)与前体药物 5-氟胞嘧啶(Flu)对肝癌的抗肿瘤作用. **方法**：应用逆转录病毒法，将 CD 基因转导入肿瘤细胞，体外应用克隆分析法测定 Flu 对肿瘤细胞的细胞毒性；应用裸鼠移植瘤模型研究 CD 基因/Flu 体系对肝癌的抗肿瘤作用. **结果**：前体药物 Flu 对人肝癌在体外和裸鼠体内具有明显的抗癌作用. 与 5-氟尿嘧啶 10 mg·kg^{-1}相比，前体药物 Flu 500 mg·kg^{-1}对裸鼠肝癌移植瘤有更强的抗癌作用. CD 基因/Flu 对人肝癌裸鼠移植瘤具有旁观者杀伤效应. **结论**：CD 基因/Flu 体系在治疗肝癌中有潜在的价值.

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