COMBINATION THERAPY OF MURINE LIVER CANCER WITH IL-12 GENE AND HSV-TK GENE

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ABSTRACT

Objective: To investigate the synergistic anti-tumor effects of murine IL-12 gene and HSV-TK gene therapy in mice bearing liver cancer. Methods: Mouse liver cancer MM45T. Li (H-2^d) cells were transfected with retroviral vector containing IL-12 gene or HSV-TK gene insert. Gene-modified liver cancer cells, MM45T. Li/IL-12 and MM45T. Li/TK, with stable expression of IL-12 and TK were obtained. Balb/c mice were inoculated subcutaneously with 2×10^5 MM45T. Li cells. When the tumor reached a size of 0.5–1.0 cm, a mixture of MM45T.Li/TK cells and ^{60}Co-irradiated MM45T. Li/IL-12 cell were injected intratumoraly. Ganciclovir (GCV) was injected ip (40 mg.kg^{-1}.d^{-1}) for 10 days. Intratumoral injection of ^{60}Co-irradiated MM45T. Li/IL-12 cells was repeated twice in one week apart. Mice with distant tumors were treated according to the same protocol. CTL activity of spleen cells was measured by ^{51}Cr-release assay and phenotype of tumor infiltrating lymphocytes by immunohistochemical staining. Results: In mice treated with MM45T. Li/IL-12 or MM45T. Li/TK+GCV individually led to moderate reduction in tumor growth, but neither could eradicate the tumor completely, while in 60% of mice treated with a mixture of MM45T. Li/IL-12 and MM45T. Li/TK cells plus GCV, complete tumor regression was observed, with no tumor recurrence for two months. The growth of distant tumor was also inhibited significantly in mice similarly treated. Most of the mice received combined gene therapy plus GCV had abundant CD4$^+$, CD8$^+$T lymphocyte infiltration. Their CTL activity was significantly higher than in mice received single gene therapy. Conclusion Combination therapy with IL-12 gene and HSV-TK gene plus GCV is effective for mouse liver cancer.

Key words: Liver cancer, Interleukin-12 HSV-TK, Gene therapy

Interleukin-12 (IL-12), produced by antigen-presenting cell, is a heterodimeric cytokine that has multiple immune regulatory functions, various studies have shown that IL-12 has multiple anti-tumor effects and anti-metastatic properties for many tumors.[1] Suicide gene approaches are also widely investigated recently, the gene products are capable of converting the non-toxic pro-drug to the active cytotoxic agent. The most commonly used suicide gene and pro-drug is the herpes simplex virus thymidine kinase (HSV-TK) gene and ganciclovir (GCV). However, neither therapy could eradicate the tumor completely. Our investigation was focused on understanding the combination therapy with IL-12 gene and HSV-TK gene plus GCV in mice bearing liver cancer. This is supposed to be a new approach for liver cancer therapy.

MATERIALS AND METHODS

Materials

Lipofect AMINE, G418 and DMEM medium were purchased from Life Technologies, (USA). rIL-12 and polybrene were purchased from Sigma. GCV from Roche. Rat anti-mouse CD4$^+$, CD8$^+$ monoclonal antibodies were purchased from Pharmingen. Goat anti-rat IgG-biotin was purchased from Calbiochem. ^{51}Cr was ordered from Amershan Co. mIL-12 standard biological activity assay kit was a gift from Professor Presky (USA), Murine liver tumor cell line MM45T. Li (ATCC CRL-6421) was a gift from Dr. Cao Guanweng at Shanghai Second Military Medical University, PA317 packaging cell line, murine fibroblast NIH3T3 were purchased for American Type

Received April 22, 2000, accepted July 6, 2000

This work was supported by the National Natural Science Foundation of China (No. 39730440).

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Culture Collection. Female Balb/c mice were purchased from animal center of Chinese Academy of Science and maintained in the animal facilities of our department. Mice were 6–8 w of age at the onset of use.

Construction of Polycistronic Retroviral Vector Containing mIL-12 Gene and Package of Retrovirus

Polycistronic retroviral vector containing p40, p35 cDNA of IL-12 and neomycin phosphotransferase gene were linked by internal ribosome entry site (IRES) fragments from poliovirus and encephalomyocarditis virus (EMCV). The retroviral vector under the control of 5'LTR promoter was named pGCEN/mIL-12.[2] pGCEN/mIL-12 was transfected to PA317 packaging cell line by Lipofect AMINE. Fresh DMEM medium supplemented with 10% FBS was changed at 72 h and selected with G418 (500 mg/l) until positive clones were obtained. The methods used in collection and storage of retrovirus supernatant and measurement of retrovirus titers were described in reference.[3]

Infection of Murine Liver Tumor Cell Line with Retrovirus and mIL-12 Bioactivity Assay

Log phase murine liver tumor cell MM45T. Li were seeded in 100 mm^2 dish at 75% confluence 16–24 h before infection. Cells were infected by retrovirus supernatant at 1 multiplicity of infection (MOI=1) containing 8 mg/l of polybrene. After 8 h, selected with fresh DMEM medium containing 500 mg/l of G418 until positive clones were obtained. mIL-12 bioactivity was detected according to the protocol from Hoffman-La Roche Co.

Combination Therapy of Murine Liver Cancer with IL-12 and HSV-TK Gene

2×10^5 MM45T. Li cells were inoculated subcutaneouly into the mice, the mice were divided randomly after the tumor reached the size of 0.5–1.0 cm, 6 mice each group, treated as following: (1) MM45T. Li / IL-12 treatment group: After irradiated by ^{60}Co, MM45T. Li cells modified by IL-12 gene were injected in tumor weekly, 1×10^6 cells each time for 3 times. (2) HSV-TK/GCV treatment group: MM45T. Li cells modified by HSV-TK gene were injected in tumor, 2×10^5 cells each. The next day, GCV was injected intraperitoneally twice daily for 10 days 40 $mg.kg^{-1}.d^{-1}$. (3) Combined genes of MM45T. Li / IL-12 and MM45T. Li /TK group. Mix both 1×10^6 irradiated MM45T/mIL-12 cells and 2×10^5 MM45/TK cells, then injected intratumorally, meanwhile, vector control MM45T. Li cells and MM45T. Li cells were injected as controls after irradiated by ^{60}Co, the radiation dose is 60Gy for each group.

Combination Therapy of Distal Tumor in Mice with IL-12 and HSV-TK Gene

Liver cancer cells MM45T. Li were inoculated subcutaneously in mice, 2×10^5 cells each. On the third day, the mice were injected contralaterally with irradiated MM45T. Li/IL-12 and/or MM45T. Li/TK cells. All the treatment protocols were the same as mentioned in 4.

Cytotoxicity of T Lymphocyte (CTL) Assay

Balb/c mice inoculated with MM45T. Li/IL-12 and/or MM45T. Li/TK +GCV. The mice were treated according to the same protocal as mentioned above. On the 14th day, the mice were sacrificed and the suspension of the spleen cells were cocultured with ^{60}Co irradiated MM45T. Li cells. CTL was detected by ^{51}Cr-release assay after the cells incubated another 5 days with 50U rIL-2.

Histological Analysis and Immunohistochemical Assay

Murine tumor was fixed by 10% formaldehyde, paraffin-embedded sections were stained with H&E for routine analysis. ABC procedure of $CD4^+$ and $CD8^+$ was taken according to the kit protocol.

Management with the Data and Statistic Analysis

The size of the tumor was calculated according to $0.5\times ab^2$,[4] a=longest diameter, b=width, the unit is mm^3, data were deal with analysis of variance.

RESULTS

Recombinant Retrovirus Titer Assay

The titer of retrovirus containing mIL-12 gene or HSV-TK gene is 5×10^8 CFU/L; the titer of retrovirus containing control vector is 1×10^9 CFU/L.

Infection of Murine Liver Cancer Cells and its Identification

The retrovirus containing mIL-12 gene, HSV-TK gene or control vector transduced in MM45T. Li cells respectively, followed selected with G418 until positive clones obtained, and then were amplified. IL-12 bioactivity assay showed that expressing level of IL-12 was 10ng/10^6cells/48 h. The MM45T. Li cells transduced with HSV-TK gene were hypersensitive to GCV. Low concentration treatment of GCV (1 mg/L) for 3 days, 90% of tumor cells could be killed.

Therapeutic Effect of Tumor Vaccine Modified by IL-12 Gene and /or HSV-TK Gene

The results showed that when treated with MM45T. Li/IL-12 or MM45T. Li/TK plus GCV individually led to moderate reduction in tumor growth, but neither could eradicate the tumor completely, while in 60% of mice bearing liver cancer treated with a mixture of MM45T. Li/IL-12 and MM45T. Li/HSV-TK cells plus GCV, complete tumor regression was observed, with no tumor recurrence for two months (Figure 1). In addition, after MM45T.Li cells were inoculated, and injected GCV next day, it was shown that GCV has no significant effect on tumor growth in mice.

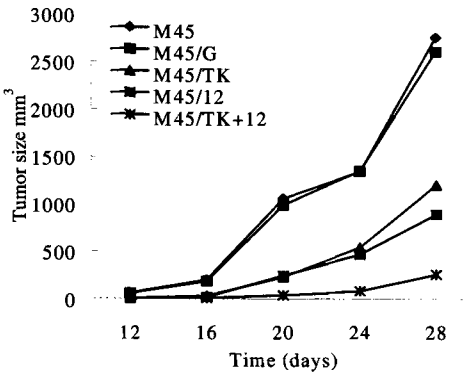

Fig.1 Antitumor effect of irradiated M45/mIL-12 cells combined with M45/TK for murine hepatoma cells *in vivo*

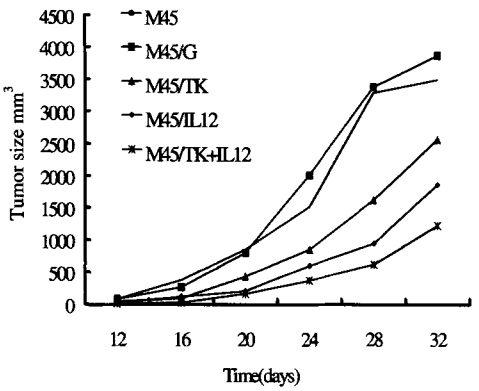

Fig 2. Suppression of the distant tumor in contralateral flank by combination gene therapy of mIL-12 gene and HSV-TK gene for murine hepatoma cells

Therapeutic Effects of Combined mIL-12 Gene Modified Tumor Vaccine and HSV-TK/GCV System for Distal Tumor

The data showed that the outcome of combined therapy group was superior to single gene therapy for distal tumor (Figure 2) ($P<0.05$). The results suggested that combination of mIL-12 and HSV-TK/GCV can induce systemic tumor-specific immune response.

Cytotoxicity of Lymphocyte (CTL) Assay

The results displayed that combination of M45/IL-12 and M45/TK plus GCV could induce cytotoxicity of lymphocyte. And CTL activity in mice treated with MM45T. Li/IL-12 combined with MM45T. Li/HSV-TK plus GCV was significantly higher than that in mice received single gene therapy (Figure 3) ($P<0.05$)

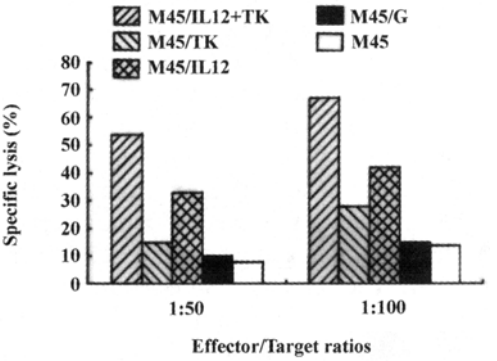

Fig. 3. *In vitro* cytolytic activity of tumor-specific CTL induced by injection of irradiated M45/mIL-12 cells combined with M45/TK-GCV cells *in vivo*

Histological Analysis

It showed that more inflammatory response composed mainly of lymphocytes and macrophages in combined therapy group. Immunohistochemistry assay showed that the combined group had abundant $CD4^+$, $CD8^+$ T lymphocytes infiltration in tumor. In contrast, mice received M45/IL-12 or M45/HSV-TK gene therapy alone had less inflammatory cells infiltration. It indicated that systemic anti-tumor effect is correlated with immune response.

DISCUSSION

IL-12 is a heterodimeric cytokine which can enhance CTL activity, promoting proliferation of T cell and NK cell, inducing T cell and NK cell to secret IFN-γ et al. IL-12 gene modified vaccine can secrete IL-12 which can boost immune response to anti-tumor especially to small and recurring tumors. However, there is no direct cytotoxic effect on tumor, and tumor cell often escape from the surveillance of host due to the rapid growth rate,

which lead to the failure of therapy.[4] HSV-TK/GCV suicide gene system was the most well investigated one. This protein kinase is capable of converting pro-drug GCV to an active cytotoxic factor through phosphorylation, then insert into the extending DNA chain during DNA replication, inhibiting DNA synthesis and causing cell death. The cytotoxic effect of HSV-TK/GCV system not only affect infected cells but also the others, called bystander effect, therefore it is capable of reducing tumor burden effectively. Because of the low transfection efficiency of retrovirus HSV-TK gene and it is only toxic to mitosis phase tumor cells, and other tumor cells which are out of mitosis phase are kept intact, so that recurrence is inevitable after stopping administration with GCV. Suicide gene system is able to reduce tumor burden effectively, but can't eradicate it completely.[5]

According to the reasons mentioned above, combination both of the genes can compensate with each other. The mechanism of synergistic effect of these combined genes is usually surposed to: first, HSV-TK/GCV kills and regresses tumor effectively, meanwhile, deliver some specific tumor peptide which can be taken by antigen-presenting cells, then induce specific CTL. The cytokine, expressed by modified cells, can promote the proliferation of CTL, NK and enhance its cytotoxicity, the amplified systemic anti-tumor reaction can also benefit the bystander effect of HSV-TK/GCV system.[6, 7]

In this report, mice treated with MM45T. Li/IL-12 or MM45T. Li/TK+GCV individually led to moderate reduction in tumor growth, but neither could eradicate the tumor completely, while in 60% of mice treated with a mixture of MM45T. Li/IL-12 and MM45T. Li/TK cells plus GCV, complete tumor regression was observed, with no tumor recurrence for two months ($P<0.01$). The growth of distant tumor was also inhibited significantly in mice similarly treated. Most of the mice received combined gene therapy plus GCV had abundant $CD4^+$, $CD8^+$T lymphocyte infiltration. Their CTL activity was significantly higher than that in mice received single gene therapy, we didn't use another $H\text{-}2^d$ tumor cell as control, the CTL activity may partially non-specific mediated by IL-12. Because it can induce systemic anti-tumor immunoregulatory functions fully, combination therapy with IL-12 gene and HSV-TK gene/GCV is effective for murine liver cancer, which stands for the trend in tumor gene therapy.

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