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COMBINED IL-2/IL-3 GENE THERAPY FOR G422 MOUSE GLIOBLASTOMA BY INTRATUMORAL INJECTION OF RECOMBINANT ADENOVIRUSES[1]

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Recombinant adenoviruses encoding murine IL-2 gene or IL-3 gene were directly injected into established subcutaneous tumor model of G422 glioblastoma cells. After treatment, the tumor size and survival of the glioblastoma-bearing mice were observed. The splenic NK and CTL cytotoxicities were detected by standard 4-hour ^{51}Cr release assay. We also examined the histopathological changes of tumor by hematoxylin and eosin staining. The results showed that intratumoral injection of adenoviruses encoding murine IL-2 gene or IL-3 gene significantly inhibited the growth of G422 glioblastoma and prolonged the survival period of glioblastoma-bearing mice. The CTL cytotoxicity of the gene therapy groups was significantly higher than that of the control groups, but NK activity remained unchanged, indicating that specific immunity contributes to the in vivo antitumor effect of the direct gene therapy. There were much more tumor necrosis and inflammatory cell infiltration in the tumor of the gene therapy groups. Combined IL-2/IL-3 gene therapy could induce higher level of CTL and enhance the therapeutic potential further. The results suggest that intratumoral injection of recombinant adenoviruses encoding certain kind of cytokines may be a useful approach in the treatment of a malignancy of the central nervous system.

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Patients with malignant glioma have a poor prognosis despite the combined use of surgery, irradiation, chemotherapy, and a variety of immunotherapies.[1-3] The *in vivo* transfer and expression of foreign genes into malignant brain tumor cells represents a new therapeutic approach for these incurable neoplasms. In these gene therapy approaches, retrovirus vector was the most commonly used for gene transfer.[4,5] Retroviruses have the advantage of selectively integration into replicating cells, but their use is limited by low titer of the vector-producer cells, insufficient gene transfer rate *in vivo*, and the possibility of insertional mutagenesis. On the other hand, adenovirus vectors can be proliferated easily and have high titer with low pathogenicity in humans and are not neurotoxic. In addition, the efficiency of adenovirus-mediated gene transfection is high both *in vitro* and *in vivo*.

Direct transfer of cytokine genes into tumor cells can result in sustained local secretion of cytokines, which is similar to the natural course in the development of an immune response. IL-2 and IL-3 are two kinds of cytokines, which play important roles in the immune regulation and have some synergistic effects on induction of antitumor immune responses.[6] In this study, we evaluated the combined IL-2/IL-3 gene therapy for subcutaneous tumor model of G422 mouse glioblastoma by intratumoral injection of replication deficient recombinant adenovirus. Tumor

size, the period of survival of the treated mice, the splenic cytotoxic activity, and the histopathology of the tumors were investigated.

MATERIALS AND METHODS

Recombinant Adenoviruses

The replication deficient recombinant adenoviruses, Adex1-CA-mIL2, Adex1-CA-mIL3, Adex1-CA-LacZ, were kindly provided by Dr. Hamada (Department of Molecular Biotherapy, Japanese Foundation of Cancer Research, Japan). These adenoviral vectors contain an expression cassette encoding the murine IL-2 gene, murine IL-3 gene or E. coli β-galactosidase gene (LacZ) respectively. The recombinant adenoviruses were propagated in cultured renal embryonal cell line 293 and were stored at −75 ℃. The titers of adenoviruses were determined by plaque assay on 293 cells.

Cell Lines, Reagents and Experimental Animals.

Renal embryonal cell line 293 was kindly provided by Dr. Blankenstein T. from Max-Delbruck-Centrum for Molecular Medicine (Berlin, Germany). G422 is a malignant glioblastoma line syngeneic in Qunming mice. The tumor was originally obtained from Beijing Institute of Neurosurgery, it was maintained by serial transfer in histocompatible Qunming mice. Yac-1 cells, which were used as the target cells for splenic NK cytotoxic assay, were maintained in RPMI-1640 medium supplemented with penicillin 100U/ml, streptomycin 100μg/ml, 2-mercaptoethanol 50 mmol/L and 10% fetal calf serum (FCS). $Na_2{}^{51}CrCO_4$ was purchased from Amersham (USA). Female Qunming mice, 6- to 8-week-old, were purchased from the Center of Laboratory Animals, Second Military Medical University, Shanghai.

Treatment of Tumor-Bearing Mice by Direct Injection of Recombinant Adenoviruses

G422 glioblastoma cells were collected, washed twice in serum-free RPMI-1640 medium and resuspended at 2×10^6 cells/ml for subcutaneous inoculation. 2×10^5 G422 glioblastoma cells were injected subcutaneously into the hind limbs of syngeneic Qunming mice. Mice were randomly divided into 6 groups (each group with 12 mice). Except the no-treatment group, mice were treated with 200 μl of serum-free RPMI-1640, Ad-LacZ (2×10^8 pfu), Ad-IL2 (2×10^8 pfu), Ad-IL3 (2×10^8 pfu), or mixture of Ad-IL2 (1×10^8 pfu) and Ad-IL3 (1×10^8 pfu) by injection into the subcutaneously established G422 glioblastomas 4 and 7 days after tumor implantation. The mice were observed for evidence of tumor growth and development and maintained until death. The length and width of the tumor masses were measured with caliber every 3 days and the tumor size was represented as 1/2× (length+width).

Assay for Cytotoxicity

Splenocytes of Qunming mice treated with various adenoviruses were used as sources of effector cells for the cytotoxicity studies. Four mice per group were killed 2 weeks after the first time of intratumoral injection of adenovirus. The viability of the cells was >98%, as determined by trypan blue dye exclusion. The splenocytes were directly used as effector cells for NK activity assay. Aliquots of the splenocytes were co-incubated in complete RPMI-1640 medium at 37 ℃ for 6 days with irradiated G422 cells in the ratio of 20 to 1. After incubation, the nonadherent cells were collected and used as effector cells in CTL activity assay.

Cytotoxicity was measured by a standard 4-hour ^{51}Cr release assay. Briefly, 2×10^6 target cells (Yac-1, G422 cells as target cells for assay of NK, CTL activity respectively) were incubated for 2 hours in RPMI-1640 containing 100 μCi of sodium ^{51}Cr (Amersham, USA). After three washes with serum-free medium, the labeled target cells were mixed with the effector cells for 4 hours at 37 ℃ with effector target ratio of 50:1. For the maximal and spontaneous ^{51}Cr release controls, 100 μl of 1% NP40 and 100μl of medium were added to the target cells respectively. The amount of released ^{51}Cr was measured by γ counting on a 1275 Minigamma Counter (LKB-WALLAC, Finland), and cytotoxic activity was calculated as the following equation:

$$\text{Cytotoxic activity}(\%) = \frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Maximal cpm} - \text{Spontaneous cpm}} \times 100$$

Histopathological Examination of Tumors

The subcutaneous tumors were removed for histological examination when the mice were sacrificed for spleen cell-mediated cytotoxicity assay. The tissue was fixed in 10% formalin, prepared for sectioning after paraffin embedding, and 4-μm sections were stained with hematoxylin and eosin according to standard procedures.

Statistics

Group data were compared using ANOVA analysis. Values in the figures are means±SD.

RESULTS

Inhibition of Tumor Growth and Prolongation of Survival of Glioblastoma-Bearing Mice by Direct IL-2 and IL-3 Gene Therapy

Qunming mice were inoculated with 2×10^6 G422 glioblastoma cells on day 0 and treated by intratumoral injection of different kinds of recombinant adenoviruses at day 4 and day 7. The results in Table 1 showed that there were no significant difference of tumor size between every groups at day 12 after tumor inoculation. At day18, day 24 and day30, tumor sizes of the three gene therapy groups were significantly smaller than that of the three control groups ($P<0.01$). In addition, tumor size of the combined IL-2/IL-3 gene therapy group was significantly smaller than that of either IL-2 gene therapy or IL-3 gene therapy group ($P<0.01$).

Table 1. Tumor size of glioblastoma-bearing mice after gene therapy by direct intratumoral injection of recombinant adenoviruses.

	Tumor Size ($\bar{x}\pm s$ cm)			
	Day 12	Day 18	Day 24	Day 30
No-treatment	0.9± 0.1	1.8± 0.2	3.0± 0.4	*
1640	0.9± 0.1	1.9± 0.2	2.9± 0.4	*
LacZ	1.0.± 0.1	1.8± 0.3	2.7± 0.5	*
IL-2	1.0± 0.1	1.3± 0.2a	1.6± 0.3a	2.7± 0.4
IL-3	1.1± 0.2	1.4± 0.2a	1.8± 0.3a	2.8± 0.5
IL-2+IL-3	0.9± 0.2	0.9± 0.2a,b	1.1± 0.2a,b	1.4± 0.3a,b

Notes: * all mice were dead

a P<0.01as compared with the control groups

b P<0.01as compared with the IL-2 or IL-3 gene therapy alone.

As shown in Figure 1, the average survival time of the three control groups (No-treatment, injection of equivalent volume of RPMI-1640 , injection of control adenovirus Ad-LacZ) were 22.6±3.5, 23.4±4.4, 23.5±4.0 days respectively. The average survival time of tumor-bearing mice treated with intratumoral injection of adenoviruses containing mouse IL-2 gene or IL-3 gene were 29.8±6.8 or 28.6±5.4 days, which were significantly longer than that of the control groups (P<0.05). The average survival time of mice treated with combined IL-2/IL-3 gene therapy was 39.6±8.1 days, which was significantly longer than that of the control groups or IL-2, IL-3 gene therapy groups (P<0.01).

Specific Immunity Generated by Direct IL-2 and IL-3 Gene Therapy

A standard ^{51}Cr release assay was used to detect splenic NK and CTL cytotoxicity. No increasing of NK activity was observed in mice of IL-2, IL-3 or combined IL-2/IL-3 gene therapy groups, suggesting that the antitumor effect of IL-2 or IL-3 gene therapy was not mediated by NK cells. The results in Table 2

indicated that the splenic CTL cytotoxic activity of the IL-2 or IL-3 gene therapy groups were enhanced more significantly than that of the three control groups (P<0.01). Moreover, the splenic CTL cytotoxicity of the combined IL-2/IL-3 gene therapy group was higher than that of the IL-2 or IL-3 gene therapy groups. (P<0.01).

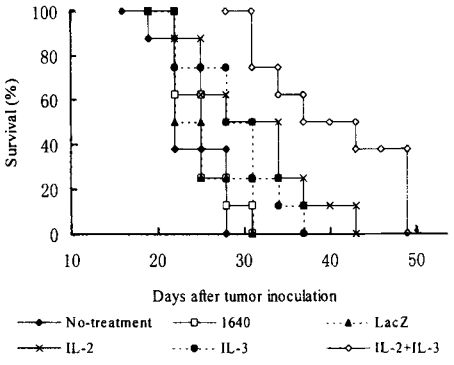

Fig.1. Survival of tumor-bearing mice after intratumoral injection of recombinant adenoviruses.

Table. 2. Cytotoxicity of splenic NK and CTL cytotoxic activity

Groups	Cytotoxity ($\bar{x}\pm s$ %)	
	NK	CTL
No-treatment	15.4± 3.4	19.3± 2.1
1640	17.2± 2.8	17.8± 1.7
LacZ	16.7± 4.1	20.5± 2.8
IL-2	19.1± 4.3	35.1± 2.4a
IL-3	17.6± 2.7	31.9± 1.7a
IL2+IL-3	19.4± 3.9	45.1± 2.6a,b

a. P<0.01as compared with the control groups

b. P<0.01as compared with the IL-2 or IL-3 gene therapy groups.

Massive Tumor Necrosis and Inflammatory Cells Infiltration Induced by Direct IL-2 and IL-3 Gene Therapy

Histopathological analyses of the subcutaneous tumors of the treated and control groups were performed when the mice were sacrificed for splenic cytotoxic assay. Tumors in animals treated with intratumoral injection of recombinant adenoviruses containing IL-2 gene or IL-3 gene revealed much more necrosis and inflammatory cell infiltration than that of the control groups. There were much more tumor necrosis and inflammatory cell infiltration in tumors of the combined IL-2/IL-3 gene therapy group than that of the IL-2 or IL-3 gene therapy groups. Thus, in consistent with an increased survival time and a reduction in tumor size, there was histological evidence of antitumor response in mice treated with IL-2 or IL-3 gene therapy.

DISCUSSION

Adenoviruses, which can be concentrated to a very high titer but are not neurotoxic, have the ability to transduce dividing as well as nondividing cells in a highly efficient manner, and have been shown to successfully transfer foreign genes into tumor and normal cells in the central nervous system.[7,8] These features make adenoviruses attractive vectors for gene delivery into primary brain neoplasm cells. Our experiments have demonstrated the feasibility of adenovirus-mediated gene transfer into glioma cells *in vivo*.

Immunization with cytokines gene-modified tumor vaccines is a new approach to induce antitumor immune responses. This has been shown to be effective in some extracranial tumors.[9,10] For central nervous system tumors, this approach does not insure that the most invasive portion of a glioma, often residing behind an intact BBB, will be accessible to cells participating in the immune response. So, direct injection of recombinant cytokine adenoviruses, which can bypass the BBB, may lead to sustained high level secretion of cytokines and solve the problem mentioned above. On the other hand, cytokine-induced disruption of the BBB should enhance lymphocyte infiltration which had been confirmed in our histopathological findings.

Neither IL-2 or IL-3 has a direct toxic effects on cancer cells. IL-2 is required for growth of cytotoxic T lymphocytes. IL-3 is an important hematopoietic factor and can induce antitumor immunity. In our experiment, the survival time of glioblastoma-bearing mice treated with intratumoral injection of IL-2, IL-3, and combined IL-2/IL-3 recombinant adenoviruses were significantly prolongated. The resistance of the animals to G422 tumor growth may correlate with the histopathological evidence of tumor rejection and enhanced specific CTL response. According to the results of this experiment, the cellular antiglioma

response was mediated predominantly by CTL cells, not by NK cells, indicating that specific immunity plays an important role in the antitumor effect of the direct IL-2 and/or IL-3 gene therapy. The magnitude of the splenic antitumor cytotoxic activity was also clearly higher in mice treated with combined IL-2/IL-3 gene therapy, relative to that of mice treated with either cytokine gene therapy alone. These findings suggest that combined IL-2/IL-3 gene therapy may be useful in treating glioma patients.

In conclusion, direct injection of recombinant adenoviruses encoding murine IL-2 gene or IL-3 gene has therapeutic effects on established subcutaneous tumor model of G422 glioblastoma. Combined IL-2/IL-3 gene therapy has more potent therapeutic potential.

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