Journal of NeuroVirology (1998) 4, 237–241

http://www.jneurovirol.com

Short Communication

Effects on brain tumor cell proliferation by an adenovirus vector that bears the Interleukin-4 gene

Ming X Wei[1], Fangqin Li[1], Yasuhiro Ono[1], Jack Gauldie[2] and E Antonio Chiocca[1]

[1]*Molecular Neuro-Oncology Laboratory, Neurosurgery Service, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114, USA;* [2]*Department of Pathology, McMaster University, Hamilton, Ontario, Canada*

A recombinant adenovirus vector bearing the IL-4 gene (AD-IL-4) was used to infect rat glioma C6 cells in culture at multiplicity of infections (MOI) from 50 to 1800. C6 cell proliferation was not altered significantly by adenoviral infection. However, IL-4 production increased in a dose-dependent manner. To ascertain effects on *in vivo* cell proliferation, a subcutaneous tumor model was used. Rat C6 glioma cells alone or C6 mixed with the control virus bearing the LacZ gene (Ad-LacZ) produced tumors that measured an average of approximately 3000 mm^3 35 days after implantation. In contrast, C6 cells mixed with Ad-IL-4 produced significant inhibition of tumor growth (P=0.035 compared to C6 tumor; P=0.023 compared to C6+Ad-LacZ tumor. Student's t test). IL-4 levels in mice serum were measured by ELISA and reached a peak of approximately 700 pg/ml at 14 days. These preliminary results showed that adenovirus-mediated delivery of the IL-4 gene may result in a significant inhibition of rat C6 cell tumor growth. Further studies will be necessary to refine this anti-tumor effect for as a potential therapy for cancer.

Keywords: brain tumors; gene therapy; interleukin-4; recombinant adenovirus vectors

Introduction

Malignant glioma represent about 40% of all primary brain tumors in humans (Schoenberg, 1983). They are considered incurable and even multimodal approaches, including surgery, radiation therapy, and chemotherapy, prolong the survival of patients by only a few months (Mahaley *et al*, 1989). This resistance to therapy is due in part to glioma cell migration, phenotypic heterogeneity and a very poor immune response in the brain, as well as to difficulties in chemotherapeutic agents passing through the Blood-Brain Barrier (BBB). Therefore, new approaches to therapy of these tumors should be evaluated. Some of the first applications of gene therapy for tumors in animal models have consisted of introducing the prodrug-activating gene, herpes simplex virus (HSV)-thymidine kinase (tk) into rat glioma cells, followed by ganciclovir administration (Takamiya *et al*, 1992, 1993; Culver *et al*, 1992; Ram *et al*, 1993; Chiocca *et al*, 1994). Ganciclovir acts as a nucleoside analog which is specifically phosphorylated by HSV-tk and then incorporated into the DNA during its replication, resulting in cell death. However, there is a high percentage of resting cells in malignant gliomas which would not be sensitive to this therapy (Yoshii *et al*, 1986; Hoshino *et al*, 1986). In this context, we have developed a new approach which targets tumor cells independently of their phase in the cell cycle: the enzyme, cytochrome P450 2B1, converts the pro-drug, cyclophosphamide, into an alkylating agent, phosphoramide mustard. This binds covalently to DNA and causes strand breaks during DNA replication, resulting in cell death (Wei *et al*, 1994, 1995a,c).

Interleukin-4 is a cytokine that has shown evidence of anti-tumor effects (Tepper and Mule, 1994; Yu *et al*, 1993). Our previous study demonstrated that retrovirus-mediated delivery of the IL-4 gene into C6 tumor cells enhanced its anti-tumor effects both in a subcutaneous and in an intracerebral tumor model (Wei *et al*, 1995b). One potential disadvantage of retroviral vectors is that malignant gliomas have a high percentage of resting cells,

Correspondence: EA Chiocca
Received 3 September 1997; revised 2 December 1997; accepted 15 December 1997

which would be resistant to retroviral mediated gene delivery (Yoshii *et al*, 1986; Hoshino *et al*, 1986). Adenovirus vectors do not possess this limitation and thus might provide a useful alternative. Replication-defective adenoviral vectors have been employed to deliver the reporter gene, LacZ, into endogenous neural cells, as well as into tumor cells in the brain (Akli *et al*, 1993; Davidson *et al*, 1993; Bajocchi *et al*, 1993; La Gal La Salle *et al*, 1993; Boviatsis *et al*, 1994; Chen *et al*, 1994). In this study, we expand our previous work by using a recombinant adenovirus vector to delivery an IL-4 transgene into rat brain tumor cells implanted subcutaneously in athymic mice. We show significant inhibition of tumor growth in this model. This effect is associated with an increase in the levels of IL-4 in the serum of treated mice. These results thus provided a basis for further refinements of brain tumor gene therapy using adenoviral vector-mediated delivery of IL-4.

Results

C6 cell growth and IL-4 production after adenoviral infection

To test potential inhibition by adenovirus on tumor cell proliferation as well as subsequent IL-4 production, C6 tumor cells were infected with the recombinant adenovirus (Ad-IL-4) at different MOIs (from 50 to 1800). Tumor cell proliferation was not affected by adenovirus infection at MOIs of 50 to 600 (Figure 1a). At higher MOIs (900 and 1800), there was slight inhibition on tumor cell proliferation. There was a direct correlation between MOI and IL-4 production (Figure 1b). These results indicated that infected tumor cells produced IL-4 and that adenovirus produced minimal effects on cell proliferation.

Anti-tumor effect of the recombinant adenovirus, Ad-IL-4

In order to evaluate the antitumor effect of Ad-IL-4, C6 tumor cells alone or mixed with the recombinant adenovirus Ad-IL-4 or Ad-LacZ, were injected immediately into the flanks of athymic mice. C6 alone or C6 cells mixed with Ad-LacZ formed large subcutaneous tumors, 35 days after implantation (Figure 2). In contrast, the recombinant adenovirus Ad-IL-4 dramatically inhibited C6 tumor growth (P=0.035 as compared to C6 tumor; P=0.023 as compared to C6+Ad-LacZ tumor, Student's t test).

IL-4 production in the serum of athymic mice

IL-4 production was measured in the serum of mice treated with subcutaneous injection of the recombinant adenovirus, Ad-IL-4. Serum IL-4 levels increased rapidly one week after injection, reached the highest level at 2 weeks post-injection, and then decreased gradually (Figure 3). In control mice, inoculated with the C6 cell plus Ad-LacZ mix, serum IL-4 levels could not be measured. These results suggested that infection of tumor cells by the recombinant adenovirus generated systemic levels of IL-4.

Discussion

This work demonstrates that a recombinant adenovirus, expressing the IL-4 transgene in infected tumor cells, can inhibit the growth of tumors *in*

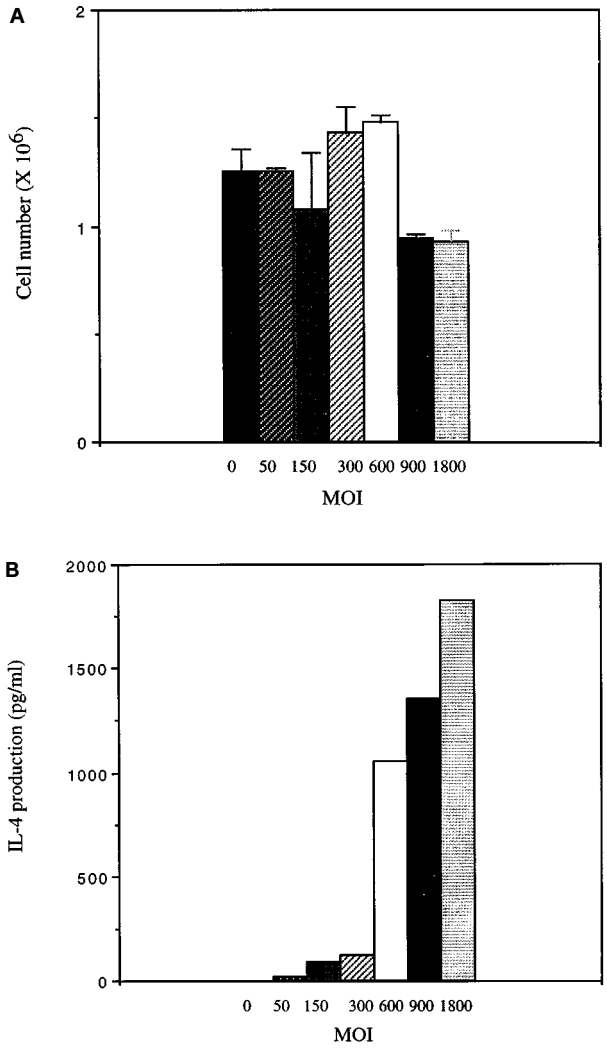

Figure 1 C6 cell growth and IL-4 production after adenoviral infection. C6 glioma cells were infected overnight with the recombinant adenovirus. Ad-IL-4, at different MOIs (Multiplicity of infection). After infection, C6 cells were washed with Hank's Buffered Saline (HBS, Gibco) and replaced in fresh medium. Forty-eight hours later, cells were trypsinized and their numbers were calculated with a Coulter counter (**a**). Conditioned medium was harvested and filtered through 0.45 micron pores. IL-4 activity was then measured by using the Endogen mIL-4 ELISA kit (**b**).

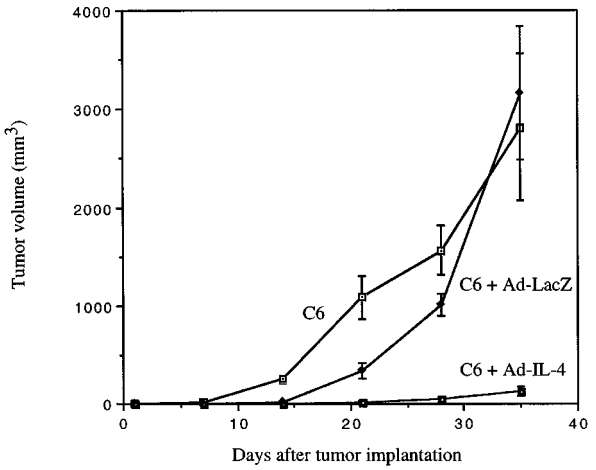

Figure 2 Subcutaneous tumor assay. Athymic mice were inoculated subcutaneously with C6 cells alone or C6 cells mixed together with recombinant adenovirus, Ad-IL-4 or with recombinant adenovirus, Ad-LacZ (five animals per group). Tumor volumes were estimated every week by external caliper measurements.

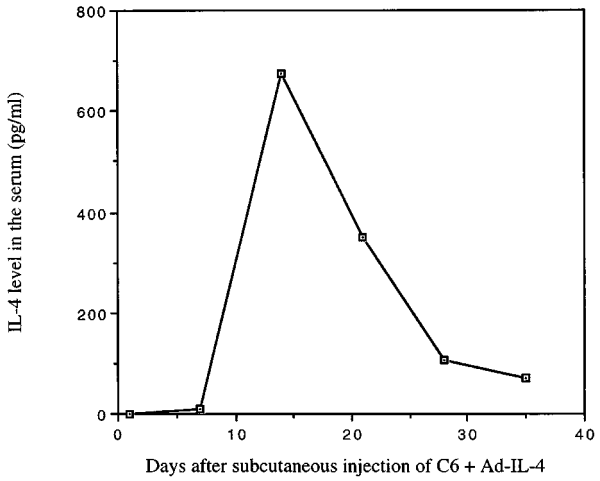

Figure 3 IL-4 production in treated mice serum. Serum was prepared from animals injected with the C6 cells mixed with Ad-IL-4 and IL-4 activity was measured by using the Endogen mIL-4 ELISA kit.

vivo. These findings extend previous results that retrovirus-mediated IL-4 gene transfer inhibited the subcutaneous and intracranial growth of rat C6 gliomas into athymic mice and prolonged their survival (Wei *et al*, 1995b). The recombinant adenovirus vector, Ad-IL-4, used in the present study was also shown to produce tumor regression in a breast cancer model (Addison *et al*, 1995).

The *in vitro* experiments demonstrate that the adenovirus expressing the IL-4 gene did not significantly affect C6 tumor cell proliferation at a very high MOI and that IL-4 production increased proportionally with MOI. Interestingly, the MOIs required for a productive infection were relatively elevated compared to those previously used for both human and rodent tumor lines (Addison *et al*, 1995). In fact, Addison *et al* (1995) reported that rodent B16BL6 cells infected with Ad-IL-4 at an MOI=10 generated less than 100 ng of IL-4 for 10^6 cells at 2 days, whereas we had to employ an MOI=50 to generate approximately 10 pg of IL-4 per ml of medium (total of 10 ml of medium) per 2×10^5 cells at 2 days, translating into approximately 0.5 ng of IL-4 for 10^6 cells at 2 days. Evidently, rat C6 glioma cells appear to be relatively resistant to adenoviral-mediated delivery of a transgene, when compared to other cells.

In the subcutaneous tumor experiment, a significant inhibition of tumor growth was observed primarily with the adenovirus vector expressing the IL-4 gene. This inhibition appears to correlate with IL-4 levels observed in the serum of treated mice, since when these levels diminished to less than 200 pg/ml on day 24, a gradual increase in the growth of the subcutaneous C6 tumors was observed. These results imply that sustained levels of the cytokine may be required to maintain tumor growth inhibition, at least in the experimental paradigm used for this report and that involved immunocompromised animals. The transient nature of transgene expression from adenovirus vectors is due to the life cycle of the viral DNA which exists in mammalian cells primarily as an extrachromosomal element. Therefore, upon cell division, loss of transgene expression may occur due to progressive dilution of the adenoviral genome in progeny cells. Recent studies show that adenoviral integration into the mammalian genome can occur at relatively high frequency after radiation (Zeng *et al*, 1997). This may provide a means to obtain long-term gene expression, since integrated genes would be passed onto progeny tumor cells. Alternatively, another type of vector that may be useful to infect resting tumor cells consists of those based on lentivirus (Naldini *et al*, 1996). Further development of the latter vectors may also provide a useful therapeutic means to treat tumors.

The animal model selected for this report was designed to study effects on tumor growth inhibition rather than tumor regression. Current gene therapy protocols for brain tumors involve a gross total surgical resection of the tumor followed by intratumoral injection of the vector into the tumor margin, which is infiltrated by residual tumor cells. Therefore, effects of gene therapy on tumor growth inhibition rather than regression of a tumor mass are being evaluated. In this context, the animal model selected for this study appears to be appropriate. Naturally, additional studies will be required to ascertain the effects of the reported therapy on normal cells in the brain.

Materials and methods

Recombinant adenovirus
Ad-IL-4 and Ad-LacZ recombinant adenovirus were produced as previously described (Addision *et al*, 1995).

In vitro *infection of cell lines*
Rat C6 glioma cells (Benda *et al*, 1971) were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum, 100 000 U/liter penicillin and 100 mg/liter streptomycin (Sigma) in a 5% CO_2 incubator. Before infection with recombinant adenovirus, cells were plated at a density of 2×10^5 cells/10 cm dish (Corning). Cells were then infected overnight with recombinant adenovirus Ad-IL-4 or Ad-LacZ at different MOIs (Multiplicity of infection). After infection, C6 cells were washed with Hank's Buffered Saline (HBS, Gibco) and replaced in fresh medium. Forty-eight hours later, conditioned medium from these cells was harvested, filtered through 0.45 micron pores, frozen at -80°C, and then measured for IL-4 activity, by using the Endogen mIL-4 ELSA kit (Genzyme, Cambridge, MA). Cell numbers were also calculated with a Coulter counter.

Animal studies
Animal studies were conducted according to the institutional guidelines promulgated by the Massachusetts General Hospital Committee on Animal Care. Prior to any procedure, animals were anesthetized with an intraperitoneal injection of Ketamine (100 mg/kg of body weight, Park Davis, NJ, USA) and Xylazine (20 mg/kg of body weight, Mobay Corp, KS, USA). For animal injections, cells in log-phase were trypsinized, counted and washed once with HBS and then resuspended in DMEM without serum for injection.

For subcutaneous injections, 0.2×10^6 cells (in 200 μl) alone or mixed together with recombinant adenovirus Ad-IL-4 or Ad-LacZ (2.5×10^{10}) in 10 μl were injected immediately into the flanks of athymic mice (NCY/Sed, nu/nu; MGH breeding colony; five animals per group). Tumor volumes were estimated every week by external caliper measurements. Mice were routinely euthanized after 1 month. The blood from treated animals was saved, their serum was prepared by centrifugation and then was stored at -80°C. IL-4 activity was measured as described above.

Acknowledgements

We thank M Chase for expert technical assistance. We thank Dr Frank Graham for providing the adenovirus constructs. MX Wei's support derives from the Elsa U Pardee Foundation. This work was supported from NINDS NS24279-08 to EAC.

References

Addison CL, Gauldie J, Muller WJ, Graham FL (1995). An adenoviral vector expressing interleukin-4 modulates tumorigenicity and induces regression in a murine breast cancer model. *Int J Oncol* **7:** 1253–1260.

Akli S, Caillaud C, Vigne E, Stratford-Perricaudet LD, Poenaru L, Perricaudet M, Kahn A, Peschanski M (1993). Transfer of a foreign gene into the brain using adenovirus vectors. *Nature Genetics* **3:** 224–228.

Bajocchi G, Feldman SH, Crystal RG, Mastrangeli A (1993). Direct *in vivo* gene transfer to ependymal cells in the central nervous system using recombinant adenovirus vectors. *Nature Genetics* **3:** 229–234.

Benda P, Someda K, Messer J, Sweet WH (1971). Morphological and immunochemical studies of rat glial tumors and clonal strains propagated in culture. *J Neurosurg* **34:** 310–323.

Boviatsis EJ, Chase M, Wei MX, Tamiya T, Hurford Jr RK, Kowall NW, Tepper RI, Breakefield XO, Chiocca EA (1994). Gene transfer into experimental brain tumors mediated by adenovirus, herpes simplex virus (HSV), and retrovirus vectors. *Hum Gene Ther* **5:** 183–191.

Chen SH, Shine SD, Goodman JC, Grossman RG, Woo SLC (1994). Gene therapy for brain tumors: regression of experimental gliomas by adenovirus-mediated gene transfer *in vivo*. *Proc Natl Acad Sci USA* **91:** 3054–3057.

Chiocca EA, Andersen JK, Takamiya Y, Martuza RL, Breakefield XO (1994). Virus-mediated genetic treatment of rodent gliomas, In: *Gene Therapeutics*, Wolff JA. (ed), Birkhauser Publishers, Boston, MA, pp 245–262.

Culver KW, Ram Z, Wallbridge S, Ishii H, Oldfield EH, Blaese RM (1992). In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. *Science* **256:** 1550–1552.

Davidson BL, Allen ED, Kozarsky KF, Wilson JM, Roessler BJ (1993). A model system for *in vivo* gene transfer into the central nervous system using an adenoviral vector. *Nature Genetics* **3:** 219–223.

Hoshino T, Nagashima T, Cho KG, Murovic JA, Hodes JE, Wilson CB, Edwards MS, Pitts LH (1986). S-phase fraction of human brain tumors in situ measured by uptake of bromodeoxyuridine. *International Journal of Cancer* **38:** 369–374.

La Gal La Salle G, Robert JJ, Berrard S, Ridoux V, Stratford-Perricaudet LD, Perricaudet M, Mallet J (1993). An adenovirus vector for gene transfer into neurons and glia in the brain. *Science* **259,** 988–990.

Mahaley Jr MS, Mettlin C, Natarajan N, Laws Jr ER, Peace BB (1989). National survey of patterns of care for brain-tumor patients. *J Neurosurg* **71:** 826–836.

Naldini L, Blomer U, Gallay P, Ory D, Mulligan R, Gage FH, Verma IM, Trono D (1996). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272,** 263–267.

Ram Z, Culver KW, Wallbridge S, Blaese RM, Oldfield EH (1993). *In situ* retroviral-mediated gene transfer for the treatment of brain tumors in rats. *Cancer Res* **53:** 83–88.

Schoenberg BS (1983). The epidemiology of nervous system tumors. In: Walker MD (ed). *Oncology of the Nervous System*. Martinus Nijhoff, Boston.

Takamiya Y, Short MP, Ezzedine ZD, Moolten FL, Breakefield XO, Martuza RL (1992). Gene therapy of malignant brain tumors: a rat glioma line bearing the herpes simplex virus type 1-thymidine kinase gene and wild type retrovirus kills other tumor cells. *Journal of Neuroscience Research* **33:** 493–503.

Takamiya Y, Short MP, Moolten FL, Fleet C, Mineta R, Breakefield XO, Martuza RL (1993). An experimental model of retorvirus gene therapy for malignant brain tumors. *J Neurosurg* **79:** 104–110.

Tepper RI, Mule JJ (1994). Experimental and clinical studies of cytokine gene-modified tumor cells. *Hum Gene Ther* **5:** 153–164.

Wei MX, Tamiya T, Chase M, Boviatsis EJ, Chang TKH, Kowall NW, Hochberg FH, Waxman DJ, Breakefield XO, Chiocca EA (1994). Experimental tumor therapy in mice using the cyclophosphamide-activating cytochrome P450 2B1 gene. *Hum Gene Ther* **5:** 969–978.

Wei MX, Tamiya T, Breakefield XO, Chiocca EA (1995a). Viral vector mediated-transfer of drug-sensitivity genes for experimental brain tumor therapy. In: *Viral vectors: tools for study and genetic manipulation of the nervous system*. Kaplitt MG, Loewy AD. (ed). Academic Press, Orlando. pp 239–253.

Wei MX, Tamiya T, Hurford Jr RK, Boviatsis EJ, Tepper RI, Chiocca EA (1995b). Enhancement of Interleukin 4-mediated tumor regression in athymic mice by *in situ* retroviral gene transfer. *Hum Gene Ther* **6:** 437–443.

Yoshii Y, Maki Y, Tsuboi K, Tomono Y, Nakagawa K, Hoshino T (1986). Estimation of growth fraction with bromodeoxyuridine in human central nervous system tumors. *J Neurosurg* **65,** 659–663.

Yu JS, Wei MX, Chiocca EA, Martuza RL, Tepper RI (1993). Treatment of glioma by engineered interleukin 4-secreting cells. *Cancer Res* **53:** 3125–3128.

Zeng M, Cerniglia GJ, Eck SL, Stevens CW (1997). High-efficiency gene transfer of adenovirus into mammalian cells using ionizing radiation. *Hum Gen Ther* **8:** 1025–1032.