Stereotactic adenovirus-mediated gene transfer into spinal cord and spinal cord tumors

Ahmet ÇOLAK*, M.D., Arif Mehmet ALADAĞ*, M.D., Shu-Hsia CHEN**, Ph. D.,
Savio L.C. WOO**, Ph. D. Robert G. GROSSMAN***, M.D., David H. SHINE***, M.D.

The authors evaluated the feasibility of using replication defective recombinant adenovirus vectors to transfer genes to the spinal cord, and spinal cord tumors in vivo. Spinal cord tumors at thoracic region were induced in Fischer 344 rats by a stereotactic intramedullary injection of $1x10^4$ 9L cells in 1.5 µl Hank's balanced salt solution (HBSS). Seven days after cell injection, a replication defective adenoviral vector carrying the bacterial β-galactosidase (ADL-β gal) or saline was injected into tumors. Efficacy of gene delivery was investigated at 9 and 15 days after tumor cell implantation. Stereotactic injection of the adenoviral vector carrying β-gal (ADL-β gal) gene into the spinal cord of animal revealed intense staining of all cells including neurons, astrocytes, and ependymal cells in the adjacent to injection site, and a relatively low staining in the peripheral part of injection site. In the rats bearing 7 day tumors, stereotactic intratumoral injection of ADL-β gal vector stained 5-10 % of the tumor cells. Transduction and gene delivery were successfully achieved in both groups. Expression of reporter gene was more prominent in rats sacrified at 9 days than those sacrified at 15 days.

Our results demonstrate that recombinant adenoviral vectors can efficiently be used to transfer genes into spinal cord and into spinal cord tumors in vivo. This approach can provide successful transfer of therapeutic genes such as the thymidine kinase gene into the spinal cord for tumor therapy. [Journal of Turgut Özal Medical Center 2(1):1-6,1995]

Key Words: *Adenovirus, β-galactosidase, gene therapy, spinal cord tumors*

Adenoviruslar aracılığıyla spinal kord ve spinal kord tümörlerine stereotaktik olarak gen transferi

Yazarlar spinal kord ve kord tümörlerinde in vivo gen transferi için replikasyonu defektif rekombinant adenovirusların kullanılabilirliğini incelediler. Spinal kord tümörü Fischer 344 ratlarının torasik bölgesine 1.5 µl HBS solusyonu içinde $1x10^4$ 9L hücresinin stereotaktik intramedüller enjeksiyonu ile oluşturuldu. Bunu takip eden 7 günde β gal genini taşıyan rekombinant adenoviral vektör (ADL-β gal) veya salin tümör içine verildi. Gen transferinin etkinliği implantasyondan sonraki 9. ve 15. günlerde incelendi. ADL-β gal'in stereotaktik olarak spinal kord içine enjeksiyonu ile enjeksiyon bölgesindeki tüm hücreler; nöronlar, astrositler ve epandim hücreleri yoğun olarak boyanırken enjeksiyon bölgesinin periferindeki hücreler rölatif olarak az boyanma gösterdiler. 7 günlük tümör taşıyan ratlarda ADL-β gal vektörü tümör hücrelerinin %5-10'unu boyadı. Transdüksiyon ve gen nakli her iki gurupda başarılı olarak gerçekleşti. 9. günlerde incelenen ratlardaki reporter gen ekspresyon 15. günlerde incelenen ratlara göre daha belirgindi.

Sonuçlarımız in vivo olarak rekombinant adenoviral vektörlerin spinal kord ve kord tümörlerinde gen transferi için etkin bir şekilde kullanılabileceğini göstermektedir. Bu yaklaşım, spinal kord tümörleri veya patolojilerinde terapötik genlerin (örneğin timidin kinaz v.b. gibi) transferini sağlayabilir. [Turgut Özal Tıp Merkezi Dergisi 2(1):1-6,1995]

Anahtar Kelimeler: *Adenovirus, β-galactosidase, gen tedavisi, spinal kord tümörleri*

* : İnönü University School of Medicine, Department of Neurosurgery - Malatya, Turkey.
** : Baylor College of Medicine, Department of Cell Biology and Howard Hughes Institute - Houston, TX
*** : Baylor College of Medicine, Department of Neurosurgery - Houston, TX

Spinal cord tumors comprise 20 % of all central nervous system tumors, and gliomas are the most common type in intramedullary region. In these cases the current best therapy includes surgical resection with/without postoperative radiation[1,2]. Despite advances in microsurgical techniques and intraoperative monitoring, anaplastic gliomas are still significant morbidity and mortality[1,2].

Recently, somatic gene therapy has become an important experimental treatment modality for different types of malignant tumors and diseases[3-11]. Current gene therapy protocols commonly used retroviral vectors to transfer suicide genes such as Herpes Simplex Virus thymidine kinase (HSV-tk) gene into brain tumors[1,8,10,11]. But these vectors show limitations which restrict their uses. Adenoviral vectors have some advantages which make them efficient vector for gene transfer. These are; no capability of integration into the host genome, episomal location and the availibilty of generating high viral titers. Adenovirus mediated gene transfer has been used in a variety of organs and cells, including skeletal muscle, hepatocyte, and central nervous system tissue[3,12]. Badie et al.[12] showed by stereotactic injection of a recombinant adenovirus vector carrying the *E.coli* β-galactosidase gene into tumors could be used as an efficient and safe delivery vehicle for brain tumors. In previous experiment, we successfully produced spinal cord tumor using an intramedullary tumor cell implantation, and treated these tumors with ADV-tk and ganciclovir treatment (Submitted to Brain Research).

The characteristics *E.coli* β-gal gene and replication defective adenoviral vectors have been described previously[3]. In this study, we used the adenoviral vector containing this gene as an intracellular reporter gene to test the feasibility of using this vector to transfer foreign genes into the spinal cord and cord tumors.

MATERIAL AND METHODS

Viral constructs

The replication defective adenovirus carrying the gal gene under control of the RSV-LTR (ADV/RSV-β gal) was kindly provided by M. Perricaudet (Institut Gustave Roussy, Centrel National de la Rechercher Scientifique, Villejuif, France). Construction of this vector has been described previously[3,6,9]. Virus titers were determined by optical absorbance at 260 nm and stored at -80 degrees C in 10 mM Tris-HCl (pH 7.0), 1 mM $MgCl_2$ with 10% glycerol at a titer of $2x10^{11}$ particles/ml.

Cell culture

The 9L glioma cell line was initially generated in Fischer 344 rats and their morphology has been described previously[13]. The 9L tumor cells (kindly provided by P.J. Tofilon of M.D. Anderson Hospital, Houston, TX) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 ug/ml) in 5% CO_2 at 37 degrees C. Tumor cells were mobilized from culture dish by adding 0.05 % trypsin ethylenediamine tetra-acetic acid (EDTA) for 3 minutes and trypsin neutralized with DMEM. The cells were collected and centrifuged and suspended in HBSS at a concentration of $6.6x10^3$ cells/μl for tumor injection. Before and after the implantation cells were counted in hemocytometer (Baxter, McGraw, Park IL) and their viability was determined by trypan blue staining.

Spinal cord tumor generation

Adult female Fischer 344 rats (155-175 grams) were used as host animals. Rats were lightly anaesthetized with Halothane and then anaesthetized with an intramuscular injection of a combination anaesthetic solution (0.6ml/kg) consisting of ketamine (42.8 mg/ml), xylazine (8.6 mg/ml), and acepromazine (1.4 mg/ml). In all animals, laminectomy (Th4-5) was performed with the aid of surgical microscope under magnification of x16 and x25. Animals were placed into a stereotaxic frame and a syringe with 26 gauge needle (Hamilton, Reno, NV) attached to the manipulating arm of the stereotactic apparatus was mechanically lowered under manual control through the laminectomy to a depth of 0.5 mm from dura. $1x10^4$ 9L cells suspended in 1.5 μl Hank's balanced salt solution were injected into the posterior medial sulcus of the spinal cord at thoracic 4-5 level. Injection was completed within 5 minutes and needle was left in place another 5 minutes and then was slowly withdrawn. The paravertebral muscles and fascia were sutured with 6/0 silk suture and skin was closed with autoclips (Clay Adams, Parsippany, NJ).

Virus injection

Seven days following 9L cell injection, animals were anaesthetized and placed into the stereotaxic

frame. Reoperation was performed to expose the spinal cord at the same level. The generated tumor could be seen on the dorsal surface of spinal cord as a well demarcated ovoid lesion. ADV/β-gal virus ($4x10^8$ particles) in 2 µl of 10mM Tris-HCl (pH 7.0), 1 mM $MgCl_2$ and 10% glycerol or saline was injected over 5 minutes. The needle was left in place for an additional 5 minutes, and then was slowly withdrawn. Before injection the needle was coated with a small portion of carbon particles (30 um in size to mark the needle tract and verify the co-localization of the virus injection with the tumor. Paravertebral muscles and fascia were closed with 6.0 silk suture and skin was closed with Autoclips.

Perfusion and histological analysis

Animals were anaesthetized and fixed by cardiac perfusion with 100 ml of phosphate buffered saline, pH 7.4 (PBS) containing heparine (10 units/ml) followed by 200 ml of 4% paraformaldehyde in PBS. Spinal cords were removed, placed in 4% paraformaldehyde in PBS for 24 hours, then cryoprotected in 21% sucrose in PBS for 24-48 hours at 4 degrees C, mounted in OCT, frozen and sectioned on a cryostat. 10 µm or 40 µm thick sections were cut for routine histologic analysis and X-gal staining. Standard hematoxylin and eosin staining was used for histological analysis.

X-gal staining

10 µm sections were mounted on gelatin subbed slides. These slides and free floating 40 µm sections were immersed in X-gal staining solution for overnight at 37 degrees C and then free floating sections were mounted on gelatine subbed slides. Slides were stained with hematoxylin and eosin (H&E). X-gal staining solution contained 1 mg/ml X-gal , and 1.3 mM $Mg Cl_2$, 15 mM NaCl, 44 mM Hepes 7.4, 3mM $(K_3Fe(CH)_6)$,and 3 mM $(K_4Fe_2(CH)_6$.

EXPERIMENT PROTOCOLS

Eighteen adult female Fischer 344 rats (155-175 grams) were used in this study. Animals were divided into three groups of 6 each.

Group I: After the laminectomies, $4x10^8$ particles of ADV-/β gal virus were injected into the spinal cord of normal animals at thoracic region. Two days after virus injection 3 of animals were sacrified and their spinal cord removed for study. The remaining 3 animals in this group were sacrified 15 days after virus injection to show temporal expression of the gene.

Group II: After laminectomies, $1x10^1$ 9L cells suspended in 1.5 µl HBSS were stereotacticaly injected into spinal cord at thoracic region. Seven days later, operation site of animal was reopened and 2 µl saline was stereotacticaly injected into tumors. Three animals were sacrified in 2 days after tumor injection and their spinal cords were examined as in group I. The remaining three were sacrified in 15 days after tumor cell implantation.

Group III: Laminectomies and tumor cell injection were performed as in group II. Seven days after tumor cell injection, $4x10^8$ particles of ADV-/β gal virus in 2 µl of 10mM Tris-HCl (pH 7.0), 1 mM $MgCl_2$ and 10% glycerol stereotacticaly injected into tumors. Animals were sacrified and removed their spinal cords as in group II.

RESULTS

In vivo gene transfer to spinal cord : In group I, stereotactic injection of ADV-/β gal vector ($4x10^8$ particles in 2 µl solution) into spinal cord of normal animals resulted in staining of cells. Neurons, astrocytes, and ependymal cells of central canal stained with X-gal showing successful gene delivery to normal cells (Figure 1). Cells in the anterior and posterior horns of spinal gray mater were stained

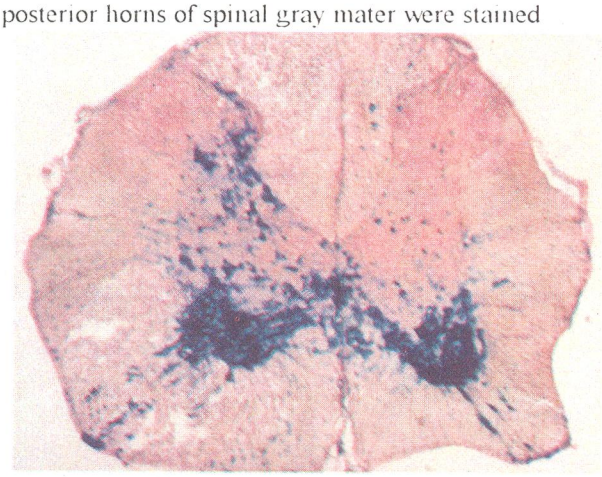

Figure 1: Expression of ß gal gene activity in the spinal cord of the rat following in vivo administration of ADV- ß gal using a stereotactic method. The spinal cords were removed 2 days after virus injection and stained with specific X-gal staining (H&E). Note very prominent activity in gray matter adjacent to injection site.

There was an extension of staining in adjacent ganglia in some animals. Longitudinal extension of staining cells were seen in small restricted area. In close to the injection site where cells were exposed to the high viral particles, staining was observed not only nuclei of cells but also cytoplasm which shows strong staining produced by high viral titers. In the peripheral part of the injection site, only nuclei were labelled. Staining of slides that obtained from animals sacrified 15 day after virus injection was less prominent than those 2 days group, suggesting a spatial and temporal decrease in gene expression with time. All these findings revealed that adenoviral vector successfully transferred this reporter gene into spinal cord cells in high efficacy.

In vivo gene transfer to spinal cord tumors : In group II, control group, stereotactic intratumoral injection saline caused no staining as expected (Figure 2/A). In group III, intratumoral injection of ADV-β gal virus led to extensive staining of tumor cells (Figure 2/B). Cell staining was very prominent in close to needle tract than peripheral part of the injection site. Approximately 5-10 % of tumor cells were transduced with this vector two days following the virus injected. The degree of transduction at 7 days after virus injection was decreased showing a temporal pattern (Figure 3/A-B). Longitudinal distribution of virus within the tumor cells was similar to those observed in group I.

DISCUSSION

These experiments demonstrate that recombinant adenoviral vector can successfully be used to transfer therapeutic gene directly to normal and tumor cells in the spinal cord in vivo. In previous experiments, retroviruses and herpes simplex viruses have been used to transfer gene into malignant tumor cells[1,8,11-16]. Some of the features of these vectors limit their wide-spread use. These are: the difficulty in producing vector in high titers, variable expression of foreign genes, relatively low transduction efficiencies, and the requirement that host cells be dividing for genomic integration. Because of these limitations effective gene therapy has necessitated implantation of xenograft of virus producing cells rather than the purified virus.

Vectors based on adenovirus have several advantages which made them useful candidates for gene therapy[3,17,18]. These are growth to high titers, broad host and cell-type range, episomal location in nuclei and high level of expression of foreign genes.

Adenoviral vectors have recently proven effective vehicles for gene transfer in vitro and in vivo in several cell types[3,5,11,17,18]. Akli et al.[3] analyzed the ability of a replication-deficient adenovirus vector to transfer a foreign gene into neural cells of adult rats, and demonstrated that a large number of neural cells including neurons, astrocytes, and ependymal cells expressed gal gene at least 45 days. Le Gal La Salle et al.[19] injected a recombinant adenovirus carrying gal gene into rat hippocampus and substantia nigra, and showed that expression of β gal was widespread throughout the hippocampus in neuronal and nonneuronal cells including pyramidal neurons, granule cells, and hilar interneuron during the first week after injection. Expression was gradually decreased by one to two months postinjection. Bajocchi et al.[5] injected adenoviral vector carrying β gal gene into the ependymal cells of the lateral ventricles, and showed the intracellular β gal activity was primarily found in the ependymal cells. In our experiments, all types of cells in the spinal cord was appeared to be transduced with this vector. Although expression period of this vector in spinal cord was relatively short, we observed that two week after virus injection expression was still prominent but temporal distribution was evident.

Akli et al.[3] also demonstrated that low titer virus (up to $3x10^5$ pfu in 10 µl) did not cause any detectible cytopathic effects, but very high titers ($>10^7$ pfu/µl) resulted in cytopathic effect. Badie et al.[12] observed similar results in their experiments. In our study, $4x10^8$ particles of adenoviral vector did not caused any significant cytopathic effects in spinal cord. Therefore, adenoviral vector represents clearly a promising means to transfer therapeutic genes such as HSV-tk into spinal cord tumors and other neurotrophic factor genes into spinal cord for therapeutic goals. We believe that these vectors have great potential in gene therapy of spinal cord tumors and other spinal cord diseases in addition to those of in brain.

ACKNOWLEDGEMENTS

Authors thank Grant Louie for his expert technical assistance. We thank Dr. M. Perricaudet for the use of the ADV-β gal vector and Dr. F. Graham for the cloning vector used to the ADV construct. A portion of this research was funded by a grant to HDS from the Texas Higher Education Coordinating Board Advanced Technology Program.

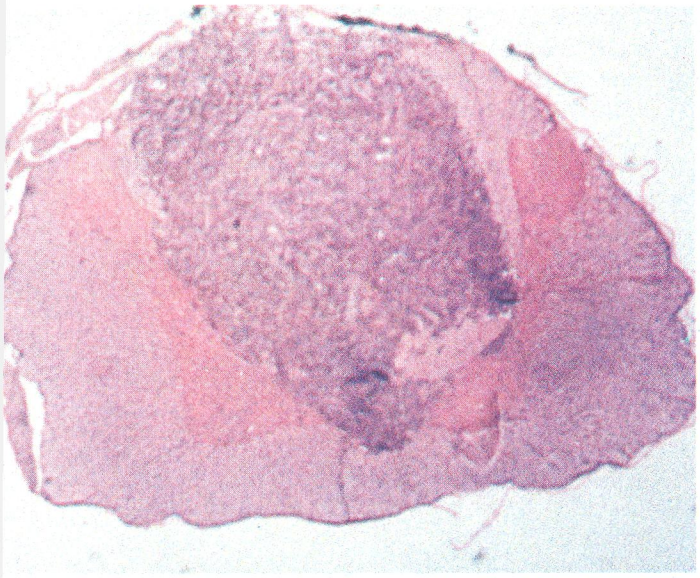

Figure 2 : Photomicrographs showing ß-galactosidase histochemical staining two days after adenovirus injection into spinal cord tumor (A: group II and B: group III). Note prominent transduction of tumor cells with vector and no transduction with saline.

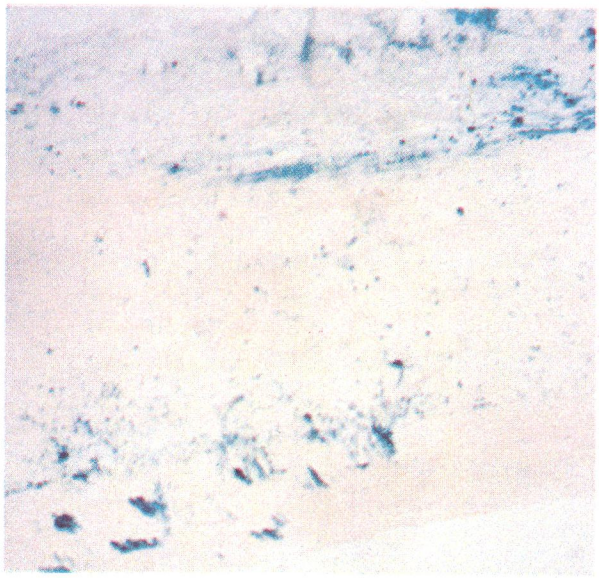

Figure 3: Expression of ß-galactosidase activity in 9L tumor cells at 2 (A) and 7 (B) days after implantation into rat spinal cord. Note a decrease lacZ staining in B.

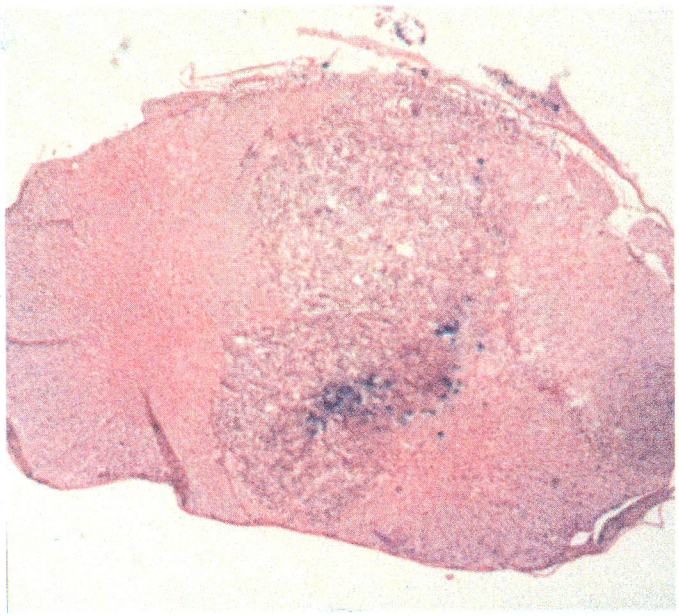

Figure 2B

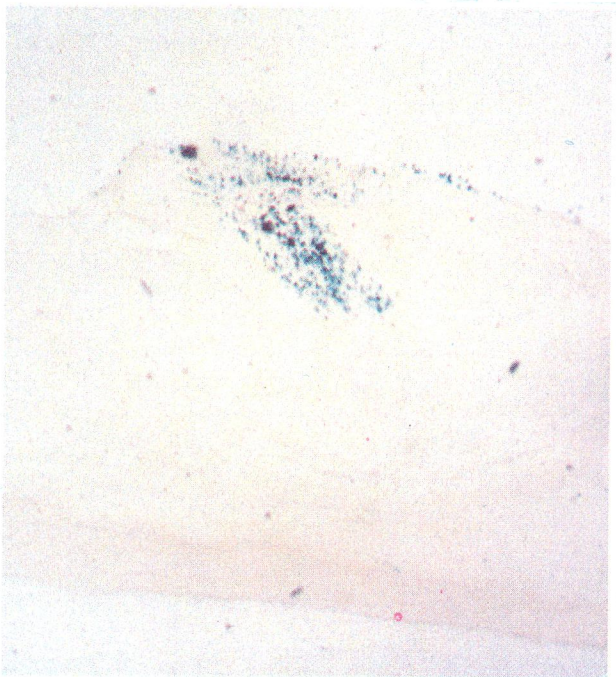

Figure 3B.

REFERENCES

1. Cristante L, Herrmann H-D. Surgical management of intramedullary spinal cord tumors: Functional outcome and sources of morbidity. Neurosurgery 1994; 35: 69-74.
2. Nelson DR, McDonald JV, Lapham LW, Quazi R, Rubin P. Central Nervous Tumors. In: P Rubin (ed.), Clinical Oncology, 7 th ed., Chapter 30, Saunders, Philadelphia, 1993: 617-644.
3. Akli S, Caillaud C, Vigne E, Stratford-Perricaudet LD, Poenaru L, Perricaudet M, Kahn A, Peschanski MR. Transfer of a foreign gene into the brain using adenovirus vectors. Nature Genet 1993; 3: 224-228.
4. Barba D, Hardin J, Gage FD. Thymidine kinase-mediated killing of rat brain tumors. J Neurosurg 1993;79:729-735.
5. Bajocchi G, Feldman SH, Crystal RG, Mastrangeli A. Direct in vivo gene transfer to ependymal cells in the central nervous system using recombinant adenovirus vectors. Nature Genet 1993;3:229-234.
6. Chen S-H, Shine HD, Goodman JC, Grossman RG, Woo SLC. Gene therapy for brain tumors:Regression of experimental gliomas with adenovirus-mediated gene transfer in vivo. Proc Natl Acad Sci USA 1994;91: 3054-3057.
7. Culver KW, Ram Z., Walbridge S, Ishii H, Oldfield EH, Blaes RM. In vivo gene transfer with retroviral vector-producer cells for treatment of experimental brain tumors. Science 1992;256:1550-1552.
8. Oldfield EH, Culver KW, Blaese RM, DeVroom HL. Gene therapy for the treatment of brain tumors using intra-tumoral transduction with the thymidine kinase gene and intravenous ganciclovir. Hum Gene Ther 1993;4:39-69.
9. Perez-Cruet MJ, Trask TW, Chen S-H, Goodman JC, Woo SLC, Grossman RG, Shine HD. Adenovirus-mediated gene therapy for experimental gliomas. J Neurosci Res 1994;39:506-511.
10. Ram Z., Culver KW, Walbridge S, Blaese RM, Oldfield EH. In situ retroviral-mediated gene transfer for the treatment of brain tumors in rats. Cancer Res 1993;53: 83-88.
11. Ram Z., Walbridge S, Shawker T, Culver KW, Blaese RM, Oldfield EH. The effect of thymidine kinase and ganciclovir therapy on tumor vasculature and growth of I gliomas in rats. J Neurosurg 1994;81:256-260.
12. Badie B, Hunt K, Economou JS, Black KL. Stereotactic delivery of a recombinant adenovirus into a C6 glioma cell line in a rat brain tumor model. Neurosurgery 1994; 35:910-916.
13. Weizsaecker M, Deen DR, Rosenblum ML, Hoshino T, Gutin PH, Barker M.The I rat brain tumors: Description and application of an animal model, J Neurol 1981;224:183-192.
14. Boviatsis EJ, Chase M, Wei MX, Tamiya T, Hurford RK, Kowal NW, Tepper RI, Breakefield XO, Chiocca AE. Gene transfer into experimental brain tumors mediated by adenovirus, herpes simplex virus, and retrovirus vectors. Human Gene Therapy 1994; 5:183-191.
15. Miller DG, Adam MA, Miller AD. Gene transfer by retrievers vectors occurs only in cells that are actively replicating at the time of infection. Mol Cell Biol 1990;10: 4239-4242.
16. Takamiya Y, Short MP, Moolten FL, Fleet C, Mineta T, Breakefield XO, Martuza RL. An experimental model of retrievers gene therapy for malignant brain tumors. J Neurosurg 1990;79: 104-110.
17. Neve R. Adenovirus vectors enter the brain. Trends in Neurosci 1993;19:251-253.
18. Graham F, Prevec L. Manipulation of adenovirus vectors. In: EJ Murray (ed.). Methods in Molecular Biology: Gene transfer and Expression Protocols. Vol 7, Humana Press Inc., Clifton, NJ, 1993: 109-128.
19. La Gal La Sella G, Robert JJ, Berrard S, Ridoux V, Stratford-Perricaudet LD, Perricaudet M, Mallet J. An adenovirus vector for gene transfer into neurons and glia in the brain. Science 1993;259:988-990.

Correspondence Address : Ahmet Çolak, M.D.,
Assistant professor, Department of Neurosurgry
Faculty of Medicine, University of İnönü
MALATYA 44300, TURKEY.
Fax : 90-422-3410036