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(22) ＢＡＩ１遺伝子の抗腫瘍機能に対する基礎研究

BAI1 遺伝子の抗腫瘍機能に対する基礎研究

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要旨（日本語）

当センターで最近新しくクロニンクされた脳特異的発現する新生血管抑制因子-brain-specific angiogenesis inhibitor 1（BAI1）は in vivo の実験で非常に強い血管新生抑制機能を現し、さらに動物の腫瘍モデルで血管新生抑制による抗腫瘍効果が観察された。現在抗腫瘍遺伝子治療の候補として研究を進めている。

KEY WORDS

BAI1 遺伝子　　抗腫瘍機能

研究報告

目的

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方法

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結果

↓

考察

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参考文献

Antiangiogenic potency of BAI1 in vivo: Implication of gene therapy for human glioblastoma

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ABSTRACT

BAI1(brain-specific angiogenesis inhibitor 1) is an 1584-amino-acid product containing five thrombospondin type 1 repeats and is specifically expressed in the brain. This BAI1 gene is now considered as one of novel p53-inducible genes. As angiogenesis is required for the growth and progression of malignancies. we investigated the in vivo antiangiogenic and antitumor effects of the wild-type BAI1 gene transfer on five humans glioblastoma cells. We constructed BAI1-expressing glioblastoma cells after the transduction with the BAI1 cDNA using a recombinant adenoviral vector (BAI1 cDNA inserted into pAdex1Cawt at Swa1 site). The Northern blot analysis and Western blot analysis demonstrated respectely the mRNA and protein of BAI1 in the transduced tumor cells. In vivo neovascularization assay of adenoBAI1 infected cells was then performed using a transparent skin fold chamber model implanted in SCID mice. In this assays, BAI1 gene transduced glioma cells significantly inhibited angiogenesis in vivo. In tumor inhibition assay, transduced or untransduced human glioma cells were implanted intradermally to the right flank of SCID mice. The presence of detectable tumor was confirmed by palpation and the tumor size was measured periodically. In vivo antitumor assay demonstrated the inhibited tumor growth after the direct injection of BAI1 adenovirus into tumors. Morphologically, tumor showed signs of impaired angiogenesis, such as extensive necrosis and reduced tumor vascular density. Our data demonstrated that a recombinant adenovirus expressing the BAI1 gene had antiangiogenic role for glioma cells and this virus also inhibited the growth of glioma cells in vivo. BAI1 was considered to be one of the potent candidate genes for cancer gene therapy.

Overview summary

Recombinant adenovirus carrying the human BAI1 cDNA (AdBAI1) was constructed using COS-TPC method. In vivo growth inhibition and antiangiogenic effects were observed with BAI1 gene transducced U373MG tumor cells. using Skin fold transparent chamber angiogenesisnmodel, our results also suggested that a AdBAI1 had antitumor effects due to antiangiogenic function of BAI1 protein in human glioblastoma cells carrying codon 237 cgt(Arg)-cat(His)mutation of p53 gene. The coexpression of BAI1 and p53 gene did not enhance the antitumor effects of BAI1 gene. BAI1 was considered to be promising candidate gene for human cancer gene therapy targeting glioblastoma cells with p53 gene mutations.

Introduction

Angiogenesis is considered to be regulated by a net become of positive and negative regulators of blood vessel growth. Recent discoveries of endogenous negative regulators of angiogenesis, thrombospondin (TSP), endostatin, angiostatin and glioma-derived angiogenesis inhibitory factor, all associated with neovascularized tumors, suggest a new paradigm of tumorigenesis(Folkman,1989,1995). Negative regulators would predict how a primary tumor grows and whether metastases appease. Gliomas, particularly glioblastomas are a prominent target of cancer gene therapy because of their poor prognosis despite all current conventional therapies. Intratumoral transplantation of HSV-TK retrovirus producer cells has been under clinical investigation. Gene therapy strategies developed to interfere with the normal function of vascular endothelial growth factor receptor is another new theraputic approach and have been successfully used in different experimental models to block tumor angiogenesis and the growth (Marcia,1999). Tumor cell transduced with the wild type p53 gene inhibited the in vivo tumor growth of adjacent non transduced cells. This data suggest that a recombinant adenovirus expressing the wild type-p53 gene is antiangiogenic, which may explain, in part, the mechanism of the bystander effect induced by the wlid type-p53 gene transfer on adjacent tumor cells (NISHIZAKI, 1999) . Recently, novel P53 inducible gene that encode a 1584-amino acid product containing five thrombospondin type1 repeats has been cleared by one group and has been demonstrated to be specifically expressed in the brain. A recombinant protein corresponding to the TSP- type 1 repeats of this gene product inhibitied in vivo neovascularization induced by bFGF on the rat cornea. The expression of this gene, designated BAI1 (brain-specific angiogenesis inhibitor 1) was absent or significantly reduced in eight of nine glioblastoma cell line, suggesting BAI1 played a significant role of angiogenesis inhibition, as a mediator of p53. (HIROYUKINISHIMORI.,1997)
cancer BAI1 expression was significantly reduced in colorectal cancers as compared to the extra neoplastic tissues. BAI1 expresson was inversely correlated with vascular invasion and metastasis. Moreover, vaeculaty in the colorectal cancer was inversery correlated with BAI1 gene expression. BAI1 expression was seggested to inhibit angiogenesis and metastasis of colorectal cancer (YOSHITAKA FUKUSHIMA., 1998) But whether it has direct inhibit tumor in vivo effect and mechanism remains unclear.
In this study we studied the in vivo growth inhibitory potency of BAI1 gene in using human glioblastoma implanted to SCID mice.

MATERIALS AND METHODS

Construction of recombinant adenovirus

We used the COS-TPC method to construct recombinant adenovirus carrying the human BAI1 cDNA. Briefly, the human BAI1 cDNA was inserted into the cassette cosmid, pAdexlw, which is an 11kb charomid vector bearing an adenovirus 5 genome with deletions of E1 and E3. The recombinant adenovirus was generated by in vivo homologous recombination in 293 cells between the cosmid cassette and Nsil-digested adenovirus DNA tagged with viral terminal proteins. The recombinant adenovirus carrying a LacZ expression unit also controlled by the CAG promoter (AxCALacZ). For in vivo transfer, The adenovirus was purified and concentrated through CsCl step gradients followed by dialysis against phosphate-buffered saline (PBS)-10% glycerol buffer. Titration of recombinant adenoviruses was performed by 50% tissue culture infectious dose (TCID50). Lack of contamination by replication-competent adenovirus (RCA) was confirmed by an inability to amplify a fragment of the E1B region by polymerase chain reaction (PCR). All viruses were purified and expanded on 293 cells by the standard technique. Each viral inoculum was purified by a CsCl step gradient followed by a CsCl equilibrium gradient. The final viral band was diluted 1:1 with sterile glycerol and stored at –80°C. Concentration of the virus was determined by measuring their optical density at 260 nm. The multiplicity of infection (MOI) was calculated on the basis of the ratio of functional virus as assayed on 293 cell is reported before(reference).

Animals .

The experiments were perfomed using SCID mice (SPF/VAF mause, C•B−17/Icr Crj-scid), purchased from Nippon charles River co. Yokohama, bred and maintained in a specific pathogenic germ-free environment in our laboratory. For the surgical procedure, mice (females, 25~30 g body weight). Formulas of the antimicrobial reagents added to the drinking water of the animals were Oxytetracycline 0.3g, Ampicillin 0.3g, Cloxacillin 0.2g and Potassium sorbate 1.35g in one litre , by HCl to pH 2.5.(Knut ,1984)。 A 2μl aliquot of a suspended U373MG cells ($4X10^5$) was injected into the brain of male and female SCID mice under sodium pentobarbital (Nembutal) anesthesia 50mg/kg, i.p.

Cell lines

Five glioblastoma cell lines were used in this study. YKG1 was purchased from HSRRB (Tokyo); U87MG, U373MG, SW1783 and DBTRG05MG were from ATCC. YKG1, U87MG,U373MG and DVTRG05MG cell lines were maintained in Eagle`s . MEM medium with non-essential amino acids, sodium pyruvate, 1mM and Earle`s BSS, (10% fetal bovine serum, 2mM L-glutamine, streptomycin [100μg/ml], and penicillin [100U/ml]). SW1783 was

maintained in Leibovitz`s L-15 medium, 10% fetal bovine serum. In a free gas exchange with at mospheric air. The kidney embryonic cell line 293 was obtained from Microbix Biosistems (TORONTO, Ontario, Canada) were maintaned in complete Dulbecco's medium (DMEM).

Western blot analysis

Western blot analysis was essentially performed following standard method. Briefly, cells were homogenized and the centrifuged supernatant was electrophoreses in 7% gel and blotted onto a nitrocellulose membrane. The membrane was locked with 3% bovine serum albumin (BSA) in PBS, and incubated for 2 hr with rabbit anti-human BAI1 serum diluted to 1:2000 in 1% BSA-0.05% Tween 20 in PBS. After several washings, the membrane was incubated for 1 hr with horseradish peroxidase-labeled goat anti-rabbit IgG (Jackson Immuno Research Laboratories. INC) diluted to 1:5000 in 1% BSA-0.05% Tween 20 in PBS. For detection, enhanced chemiluminescence (ECL; Amersham , Arlington Heights , IL) was used and the membrane was exposed to Fuji (Tokyo, Japan) RX-U film for 10-30 sec.

Northern blot analysis

Northern blot analysis of BAI1 transduced glioblastoma cells showed BAI1 mRNA expressed properly. RNA was extracted from cultured cells with TRIzol (Life Technologies). RNA from tumor cell lines and tumor tissue was separated on formaldehyde-agarose gels and blotted onto nitrocellulose filter (Micron Separations, Westboro, MA). BAI1 mRNA was detected by hybridization of membranes with ^{32}p labelled BAI1 cDNA. As previously reported (Nishimori,1997). As control for quality and loding of mRNA, blots were also hybridized with a ^{32}p labeled ß actin cDNA. This cDNA probes were labeled with [^{32}p] dCTP (Amersham life Sciences) by the random-primer method to a specific activity of >2x10^{9} dpm/μg. Blots were hybridized in QuickHyb solution (Stratagene) at 68°C for 1 hr followed by washing in 0.1xSSC-0.1% SDS at 65°C and autoradiography (1xSSC is 0.15M NaCl plus 0.015M sodium citrate).

The radioactivity of each band was quantitated with a Fuji Bas 1000 bioimaging analyzer and MacBas software. For experiments designed to determine mRNA , Glioblastoma tumor cells were grown to confluence in a six-well plate and infected with 5x10^{8} PFU/ml AdBAI1 or any infection virus. Twenty-four hours after infection, cells were harvested at indicated times, and the RNA was extracted and subjected to Northern analysis, as described above.

Dorsal skin fold transparent chamber

Dorsal skin fold transparent chamber (DSFTC) was composed of two titanium frames and the total weight was 1.8g. The frame was implanted to mice so as to sandwich the extended double layers of skin perpendicularly to the animal's back. One layer of skin was completely

removed circulaly with the diameter of 15mm, and the remaining layer composed of epidermis, subcutaneous tissue and muscle was covered with a cover slip incorporated in one of the frames. Using stainless steel nuts as spacers, frame-to-frame distance of 400~450um was maintained to prevent the compression of nutritional blood vessels. Fine polyethylene catheters were inserted into the jugular vein and the caroid artery, respectively, passed subscutaneously to the dorsal side of the neck, closed, and sutured to the titanium frames. The animals tolerated to the chambers and showed no signs of discomfort. In particular, no effect on sleeping and feeding habit was observed. The chamber implantation time was about 20 min per mouse. For intravital microscopy, unanesthetized animals were immobilized in polyethylen tubes attached to the stage of a microscope (OPTIPHOTO 66, Nikon, Tokyo, Japan). Observations were made with the following objectives: Nikon X2 (numerical aperture, 0.05), Nikon X4 (numerical aperture, 0.10), Nikon X10 (numerical aperture, 0.25) and Nikon X20 (numerical aperture,0.40) using transillumination and a filter for converting artificial light into daylight. Observations were captured with an intensified CCD camera TEC-470 Optronics Co., Chelmsford, MA, USA), attached to the microscope and serially connected to a TV monitor (BM-1400S Vitor Co., Tokyo, Japan) and recorded on a S-VHS video tape recorder (HR-X1 Video Co., Tokyo, Japan). During off-line analysis of the video tape recordings, stil video frames (680x480 pixels) were captured at a resolution of 72 pixele/inch with Avid Video Shop 3.0 (Avid Technology, Inc., Tewksbury, MA, USA) on a Power Macintosh 7100/80AV computer (Apple Computer, Inc., Cupertino, CA, USA). The video frames taken with X10 objective were subsequently processed in order to obtain vascular parameters. The vessel out line of each of the frame was carefully drawn in a separate transparent layer using Adobe Photoshop 4.0 (Adobe systems, Inc., Mountain View, CA, USA) with the pencil tool of one pixel line width after the defective channels of the RGB color images of the video captured frame were eliminated and the image transformed to the grey scale mode. In this study, observations were perfomed at days 0, 3, 6, 9 and 12 after tumor inoculation.

Histology

Specimens were fixed in phospho-lysine-paraformadehyde solution at 4°C over night, embedded in Tissue-Tek O.C.T. Compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and stored at -80°C. Five μm sections were cut for immuno histochemical staining. Expression of von Willebrand factor was detected using rabbit polyclonal antibodies, 8mg/ml (Dako, Tokyo, Japan). After the standard protocol from Dako Sheet. Tissue sections were incubated with 3% hydrogen peroxide in distilled water for 5minutes and rinsed with distilled water and placed in Tris –buffered saline (TBS) for 5 minutes. After the rinse , the specimen were covered with 2-3drops of DAKO EPOS

Anti-Von Willebrand Factor/HRP (one drop equals 50μ l) and Incubated about 60 minutes at room temperature. As negative control, DAKO EPOS Negative Control (code No.U 0951) was used following manufactures protocal,The specimen were than rinse in TBS for 5 minutes, incubated with DAB for 5-15 minutes, rinssed with distilled water, counterstained and mounted with coverslip as reported before.

BAI1 protein immuno histochemical staining as described above.

X-gal staing

Cells were spinned down and fixed with a methanol-formaldehyde mixture for 1 hr and 0.2% gllutaraldehyde in PBS for 30min, stained for β -galactosidase activity by incubating in PBS including 5-bromo-4-chloro-3-indolyl-beta- D-galactopyranoside (X-Gal, 400 mg/ml), 5 mM potassium ferricyanide, 5 mM potassium ferocyanide, and 2mM MgCl2 for 4 hr at 37℃, and lightly counter stained with eosin. Section were immersed briefly in 4% paraformaldehyde, washed extensively with PBS and immersed in a solution containing 1mg/ml X-gal, 2 mM $MgCl_2$, 5mM $K_3Fe(CN)_6$ and 5 mM $K_4Fe(CN)_6$ in PBS overnight at 37C. Sections were rinsed in PBS and counterstained with hematoxylin/eosin and coverslipped in PBS/glycerol for microscopic observation.

Human Glioma Therapy Model in SCID Mice

$1x10^7$ each of human glioma cells were implanted intradermally to the right flank of SCID mice. Tumor with diameters of 5-10mm developed in 14 days and 25mm in 56 days after transplantation. $2X10^{9-10}$ pfu in 50-100μl volume of either AdBAI1, AdLacZ, AdV-RR5 were injected into tumor using a 26-gauge needle. The injection was repeated every other day (Figure5). The presence of detectable tumor was confirmed by palpation and the tumor size was followed periodically.

Data Analysis

The significance of differences between two groups was determined by unpaired t test. Statistical analyses were performed on a mocrocomputer with the aid of the SigmaStat program(Jandel Scientific, San rafael,CA). Data are prosented as mean ±SEM.

Results

BAI1 expression

Northern blot analysis of BAI1-transduced SW1783, YKG1, DBTRG05MG, U87MG and U373MG glioblastoma cells showed BAI1 mRNA expressed properly. But non transduced wild type cells did not expressed BAI1 mRNA (Figure 2).

Western blot analysis showed BAI1 transduced YKG1, DBTRG05MG, U87MG and U373MG glioblastoma cells expressed BAI1 protein. But non transduced wild type cells did not expressed BAI1 protein (Figure 3).

In vivo transduced AdBAI1 after 48hour U373MG cell were immunochamistry stained with the antibody for anti BAI1 antigen as an expressed the cell marker. The staining revealed that MOCK and AdLacZ transduced cell was negative result(Figuer8 A and B). and BAI1 transduced cell is positive(Figuer8 C, D, E and F)., demonstrated the inserted BAI1gene expressed protein .

Angiogenesis inhibition effect

Transplanted BAI1 transduced U373MG cells were observed to have significantly inhibited tumor cell-induced angiogenesis in vivo with DSFTC. Angiogenesis network was observed in mice transplanted with in wildtype and AdLacZ transduced cells mice over all tumor area at day 12. But angiogenesis was completely inhibited in dorsal skinfold chamber of SCID mice transplanted with AdBAI1 transduced cells at Day12 (Figure 4) .

In injected DMEM and AdLacZ tumors showed blood vessel infiltration (Fig8 H and I), immunohistochemical staining showed normal capillaries in AdLacZ injected mice tumor(16.0+_3.2 blood vessels per 200X field) but stained vessels in the AdBAI1 injected tumor (1.6+_2.5 blood vessels per 200x frame) displayed very few and extremely small bloodvessels. (Fig8 G),

Tumor inhibition effect

The U373MG tumor bearing SCID mice mean tumor sizes of WT and Adeno were statistically larger than those of the other 3 groups of P53, BAI1 or P53+BAI1 35days after injection ($P<0.05$, Figure 6) tumor.

The suppressed tumor growth was observed in mice group transplanted with AdBAI1 transduced cells in contrast to controls(*$p<0.05$,Figure 6).

The in vivo antitumoral effects of intratumorolly injected AdLacZ , AdBAI1 or DMEM were analyed in SCID mice bearing U-373 MG tumors. Tumolals injected with AdBAI1 regressed significantly compared with the tumors injected with AdLacZ (Fig.5;N=20;P=0.003)and DMEM(Fig.5 ;N=18;P=0.003). The experiments were repeated twice, and the results were reproducible. Representative data are included the Fig 8.

Combination Therapy

Combined use of AdBAI1 and AdP53 did not potentiate U373MG tumor growth nhibition by AdBAI1 or AdP53. the mean tumor sizes of DMEM and Adeno were statistically larger than

those of the other 3 groups of P53, BAI1 or P53+BAI1 35 days after injection ($p<0.05$, Figure6)

Survival

The survival of mice after in vivo transduction of AdBAI1 vector or control vector at U373MG tumor was shown in Fig7. Mean survival time of mice transplanted with U373MG ranged between 42 and 70 day and the survival rate was 20%. Mice transplanted with U373MG cells and treated with AdBAI1 survived much longer in 80 day in 80%($p<0.05$). Mice treated with AdLacZ and DMEM survived similarly.The U373MG tumor bearing SCID mice survival curves of animals injected with AdLacZ or DMEM($p<0.05$,Figure 6).

Discussion

angiogenesis is crucial for the growth of tumors and metastasis diffusion. The beginning of tumor angiogenesis is caused by the selection of a cancer cell clone which induces an unbalance between inducers and inhibitors of angiogenesis.

In this study, we examined the efficacy of the novel p53-inducible genes BAI1 alon or BAI1 and p53 combination for antitumor response. Both genes were intrgrated into adenoviral vectors and transferred alone or in combination directly into human glioblastoma, Analysis the antiangiogenesis and antitumor effect.

The BAI1 protein are a family of three related protein, each gene designated BAI1, BAI2 and BAI3, were specifically expressed in brain, and are likely to be expressed in the same type of cells, members of this novel gene family considered to be a member of the secretin receptor family (Shiratuchi,1998). This novel gene family may play important roles in suppression of glioblastoma Progression of a glioma to its more malignant form, glioblastoma, is associated with dramatic neovascularization. The most frequently altered gene in human glioblastomas is p53. Neovascularization might be a direct consequence of inactivation of p53, possibly due to loss of transactivation of one or more target genes regulated by p53 (Van,1994). p53 produced a secretory inhibitor of capillary endothelial cell migration designated glioma-derived angiogenesis inhibitory factor Isolated a novel brain-specific gene BAI1(Nishimori,1997). BAI1 contained at least one "fuctional" p53-binding site within an intron, and its expression was shown to be induced by wild type p53. The BAI1 protein includes a 7-span transmembrane region similar to that of the secretin receptor. The axtracellular region of BAI1 possesses a single arg-gly-asp motif recognized by integrins, and 5 sequences corresponding to the thrombospondin type I repeats that can inhibit experimental angiogenesis induced by basic fibroblast growth factor. in spite of similar

tissue specificity among the 3 BAI gene, only BAI1 was transcriptionally regulated by p53. this novel gene may play important roles in supperession of glioblastoma by fluorescence in situ hybridization, (shiratruchi et al.1997).

we used BAI1 have evaluated the potential of gene therapy of human glioblastoma cells, using viral vectors encoding human BAI1 cDNA.

First was BAI1 transduction rate in glioblastoma cell line. Fortunately, 5 human glioblastoma cell lines all was high level expressed BAI1 gene. Futher analysis transduced BAI1 gene cell lines BAI1 protein expresse function also performed 4glioblastoma cell lines was detected. In curture transduced BAI1 cell lines, BAI1 protein were partially released solubility protein, moreover multi of infection (MOI) dependent increased this release in perfomed between MOI 1to 20.

BAI1 in vivo have a anti angiogenesis effect. In this study, transparent chamber model and implanted intradermally to the right flank of SCID mice model were used to study the effect of BAI1 on antiangiogenesis and antitumor function in U373MG glioblastoma. Transparent chamber is a model particularly useful to study the temporal sequence of events occurring during early stages of angiogenesis. In the present studies, BAI1 suppressed tumor growth apparently by preventing efficient vascularization of U373MG inoculation within the dorsal skinfold chamber of SCID mice. Two week after tumor inoculation, functionl vascular networks, that consistently covered all tumor areas, developed in animals treated with AdLacZ and DMEM as vehicle. Showed significantly lager vessles, but not showed in Ad BAI1.

BAI1 have a direct antiangiogenesis effect and through this ability inhibit tumor growth. In this study A substantial reduction of tumor volume was present in mice injected with the AdBAI1. exogenous human BAI1 can be efficiently transduced and expressed in human glioblastoma cells by an Ad-vector. High level of wild-type BAI1 protein detected at least 3 days after infection in vitro. and significantly inhibited growth of AdBAI1 injected tumor compared to MOCK injected mice in vivo tumor, suggesting that the growth-inhibiting effect is not mediated by the virus itself and this tumor inhibition effect from anti angiogenesis result.

BAI1 have a extension of life effect in carried tumor mice. Mean survival time of mice transplanted with U373MG ranged between 42 and 70 day, the survival rate was 10% in day 80 . Mice transplanted with U373MG cells and treated with AdBAI1 survived much longer in 80 day in 80%. Mice treated with AdLacZ and DMEM survived similarly. Suggest BAI transduced tumor carried mice extensied of life.

BAI1 antitumor effect was direct producted from the lower reaches(下流), and not induced by p53. We study observed in vivo a same to invitro of lesult. Introduction of BAI1 or p53

alone each was induce growth retardation in the majority of mice. the combination of Adp53 with AdBAI1 expressing Ad-vector induced a greater antitumor response. But, where as it was not showed combination group than either BAI1 or p53 vector alone more stronger a syngenic effect. We considered that was because in vivo between p53 and BAI1 in the express process be extent feedback mechanism, when the BAI1 starting express or concentration arrive at high level, conversely inhibition p53 to keep up appearance tumor inhibition function. It was different with physiological phenomenon, BAI1 induced by upper reacher p53. BAI1 was direct display antiangiogenesis and antitumor effect in the lower reaches. Therefore in p53 and BAI1 combination treated tumor effect keep with any BAI1 or p53 alone treated tumor antitumor effect level.

A previous study has shown that p53 inhibition of human tumors closely correlates with a reduction of blood vessel density in the tumor, suggesting that p53 effect are mediated, at least in part, by an antiangiogenesis mechanism (Xu,1997). Maybe BAI1 was one of among this madia of p53 antiangiogenesis more over antitumor.

these findings and specifically discuss their implications for brain tumor genesis, molecular diagnosis and prognosis. Of clinical importance are the findings that brain tumors with wild type (wt) or mutant p53 status may respond differently to radiation therapy and that novel therapeutic strategies using TP53 gene transfer or specifically targeting tumor cells with mutated p53 are being evaluated in clinical trials. (Fulci G, 1998). We result suggested BAI1 directly have a antiangiogenesis and antitumor effect. And in p53 antibodies were detected case, BAI1 may substitute P53 treatment. p53 antibodies were detected in the serum in 2 of 14 patients but never in the cerebrospinal fluid (CSF). Soluble p53 protein was detected neither in serum nor in CSF of the glioma patients. CSF levels of the immunosuppressive cytokine, transforming growth factor (TGF)-beta, were elevated in the glioma patients, including those with a humoral response to p53. These preliminary findings raise the possibility of systemic humoral immune responses to antigens, including mutant p53, expressed by glioma cells in the central nervous system (Weller M, 1998). Hypoxia-mediated selection for p53 mutant cells with diminished apoptotic potential in solid tumors may account for the high prevalence of p53 mutations in human cancers. Our increasing understanding of the role of p53 mutations and apoptosis in human cancers has also provided some insights into strategies for anticancer therapy. Studies reconstituting the wild-type p53 through gene therapy have been encouraging. More importantly, further elucidation of the mechanisms of therapy-induced p53-independent apoptosis in cancer cells will facilitate the development of more efficient, less toxic anticancer therapy. (Wang TH,. 1996)。 The BAI1 gene may be important new media for antiangiogenic gene therapy. As stated above, simply add BAI1 protein or AdBAI1 treatment seems to have a profound

effect on the ability of tumors to growth and/or metastasize. This molecule inhibitors might therefore be useful as anti-angiogenic drugs or targets gene in the treatment of human cancers or angiogenesis disease as with many new anti angiogenesis methods (Lyden,1999.,Napoleone,1999). In this case need diagnosis by association experiment, especially check p53 gene mutation in glioblastoma patient.

In the future tumor clinical is quantification of microvessel density in tumor specimen correlates either metastasis or recurrence in many malignancies such as breast cancer and lung cancer. Therefore, assessment of tumor angiogenesis may serve as prognostic factors. Therapeutic applications include the development of new agents with antiangiogenic properties, vascular targeting drugs, antibody-based therapy, and BAI1gene therapy. Combination of antiangiogenic therapy with cytotoxic drugs may enhance antitumor activity. Moreover, the role of antiangiogenic therapy in adjuvant setting may provide and alternative approach to better cancer treatment in the near future.(Voravud N, 1999) therapy-induced independent factor "BAI1 "in cancer cells will facilitate the development of more efficient, less toxic anticancer therapy. (Wang TH,. 1996)

Fig.1. Constructs of BAI1 Adenovirus vector. BAI1 cDNA was inserted to Swa1 site of pAdex1Cawt.

Fig.2. Northern blot analysis of BAI1 transduced glioblastoma cell. Five glioblastoma cell lines(YKG1, U87MG, U373MG, SW1783 and DBTRG05MG) were transduced with AdBAI1 vector and Northern blot analysis was performed All of the BAI1 transduced glioblastoma cells expressed BAI1 mRNA. But non transduced wild type cells did not.
(+) means transduced BAI1 adeno vector, (-) means untransduced BAI1 adeno vector

Fig.3. Western blot analysis of BAI1 transduced glioblastoma cells. Four glioblastoma cell lines(YKG1 , U87MG, U373MG and SW1783 were transduced with AdBAI1 vector and Western blot analysis was perfoemed. All of the BAI1 transduced glioblastoma cells expressed BAI1 protein band a placed in 17Kd. But non transduced wild type cells did not expressed BAI1 protein. (+) means transduced BAI1 adeno vector, (-) means untransduced BAI1 adeno vector

Fig.4. The observation of in vivo angiogenetic effects AdLacZ or BAI1 transduced U373MG

tumor cell using DSFTC in SCID mice. $2x10^5$ of transduced or non tranduced U373MG tumor cells were inoculated. Functional angiogenesis network was seen in all of the mice transplanted with WT U373MG cell(n =5) and AdLacZ transduced cells(n =5) at day 12. Angiogenesis inhibition was observed in all (n=6)mice transplanted with AdBAI1 transduced cells at Day12.

Fig.5. The in vivo inhibit or effect of AdBAI1 to growth of U373MG tumor. $1x10^7$ of U373MG cells were intradermally implanted. To the right flank of SCID mouse. AdBAI1 vector, AdLacZ or DMEM were injected every three days for 5 times then every 7 days for 3 times. Each group consisted of 10 mice. The diameters of tumors were determined palpation by every 3-4 days. The AdBAI1 group showed remarkable reduction of tumor growth inhibition in contrast to controls groups($P<0.05$)

Fig.6. Comparison of the tumor forming effects of BAI1,P53 and BAI1+P53 transduced U373MG cell. $1x10^7$ of BAI1, P53 and BAI1+P53 transduced U373MG cells were implanted to the right flank of SCID mouse. The product of bisecting diameters of U373MG cells was estimated by palpation every 3-4 days. The mean tumor sizes of WT and Adeno groups were statistically lager than those of the other p53, BAI1 and P53+BAI1 3 groups in 35days after the observation period ($p<0.05$).

Fig.7. The BAI1 gene therapeutic effects on SCID mice established with U373MG tumor. Survival was compared among the SCID mice treated with AdBAI1 virus, AdLacZ virus or DMEM. The SCID mice with BAI1 significantly ($p<0.05$) showed longe while survival than those treated with LacZ or medium. While injection with LacZ did not length then the survival time compared to that of DMEM control.

Fig.8. in vitro transduced AdBAI1, AdLacZ and MOCK with U373MG cell after 48Hr stained by anti BAI1 rabbit antibody and anti rabbit HRPgoat antibody. A and B was MOCK and AdLacZ transduced U373MG cell, C,D,Eand F was BAI1 transduced U373MG cell.A,B and C was 80X folder,D,E and F was 400X folder , G,H and I was 40X folder.
Decreased Vascularization of xenografts in AdBAI1 compared with AdLacZ and DMEM treated U373MG with SCID mouse. G was injected AdBAI1 U373MG formed tumor. H and I was injected DMEM and AdLacZ injected U373MG formed tumor section.

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