

Long-term expression of rAAV2-hIL15 enhances immunoglobulin production and lymphokine-activated killer cell-mediated human glioblastoma cell death

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Abstract. Glioblastoma is the most aggressive primary brain tumor and its prognosis remains poor despite different treatment modalities including surgery, radiotherapy and chemotherapy. Therefore, more useful treatments for glioblastoma patients are required. Human interleukin 15 (hIL15) is an immunomodulator that has antitumor activities. hIL15 combined with gene therapy method is also currently under consideration as a treatment option. Since recombinant adeno-associated virus serotype 2 (rAAV2) with low immunogenicity and long-term gene expression in human clinical trials has been demonstrated, rAAV2 encoding hIL15 (rAAV2-hIL15) were used to inhibit human glioblastoma growth in the present study. rAAV2-hIL15, which is able to express IL15 protein with bioactivity, was successfully produced and purified. Data of this study demonstrated that the long-term expression of rAAV2-hIL15 enhances immunoglobulin (Ig) production and the cytotoxic activity of lymphokine-activated killer (LAK) cells. In addition, results of the present study showed that rAAV2-hIL15 delays tumor growth on a xenografted human glioblastoma mice model. Taken together, these results indicated that rAAV2-hIL15 constitutes a powerful and potent gene immunotherapy method for human glioblastoma treatment.

Introduction

Human glioblastoma is one of the most malignant brain tumors. Currently, the traditional methods used to treat glioblastoma include surgery, chemotherapy, radiotherapy and a combination of these methods (1). However, the mean survival time of glioblastoma patients is one year and most patients do not survive more than two years in the clinical course (1). Therefore, more useful treatments for glioblastoma patients are required, thus a number of gene therapies for tumor treatment have been suggested (2-4).

Human interleukin 15 (hIL15) is a 14-15 kDa immunostimulatory cytokine that is able to induce cell proliferation and T-cell differentiation (5-7). A previous study demonstrated that immunotherapy with lymphokine-activated killer (LAK) cell cytotoxic activity has been widely used for breast, lung, ovary and pancreatic tumor treatment in phase II clinical trials (8). Due to the short half-life of IL15, repeated daily injection is required when employing IL15 administration, which is not convenient for human cancer treatments (9-11). Therefore, authors of previous studies suggested that replacing daily administration with gene therapy may be useful for tumor immunotherapy (12-14).

Type 2 recombinant AAV (rAAV2) infects dividing and non-dividing cells and maintains long-term gene expression in a number of tissues, including the liver, brain, retina and muscle (15,16). Previous studies demonstrated that rAAVs are non-pathogenic in humans and exhibit low immunogenicity compared with other viral delivery systems (17,18). rAAV2 viral vector expressing the glutamic acid decarboxylase (GAD) gene was successfully used to treat Parkinson's disease (19,20). Those results demonstrated that rAAV2 viral vector is safer and more efficacious for human gene therapy. Therefore, rAAV2 viral vector is used to carry the human IL15 (hIL15) gene for glioblastoma therapy in this study.

Gene immunotherapy using rAAV2-bearing human IL15 gene has recently been applied in the treatment of tumors (21-23). Those studies demonstrated that rAAV2-hIL15 is

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able to express IL15 proteins and to inhibit cervical tumor and JC breast tumor growth in mice models. Previously, we determined whether rAAV2-hIL15 was able to inhibit glioblastoma growth. In the present study, rAAV2-hIL15 was successfully produced and purified. Our results showed that rAAV2-hIL15 possesses bioactivities and is capable of delaying glioblastoma growth. Our studies may therefore provide a potential immunotherapy method for glioblastoma treatment in the future.

Materials and methods

Cell culture. DBTRG (human glioblastoma cells), HEK293 (human embryonic kidney cells), HT1080 (human fibrosarcoma cells), HT2 (murine IL2/IL15-dependent cells) and YAC-1 (murine cell line sensitive to LAK cells) cells were obtained from the Bioresource Collection and Research Center (BCRC, Shinchu, Taiwan). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 IU/ml penicillin/streptomycin. Moreover, since HT2 cells are IL15-dependent cells, IL15 protein (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) supplementation was required for maintaining survival. DMEM, RPMI, fetal bovine serum, L-glutamine, penicillin/streptomycin, sodium pyruvate and non-essential amino acids were purchased from Invitrogen (Carlsbad, CA, USA).

pAAV-hIL15 construction. The rAAV2 helper-free packing system (pAAV-MCS, pAAV-RC and pHelper plasmids) was purchased from Strategene (La Jolla, CA, USA). The human IL15 gene (hIL15) with the IL2 secretory peptide gene were kindly provided by Dr K.W. Liao (National Chiao Tung University, Hsin-Chu, Taiwan) and were amplified using the polymerase chain reaction (PCR) method. Primers containing *EcoRI* and *BamHI* sites were indicated as follows: sense primer, 5'-GAATTCAAAGAATTCATGTACAGGATGCAACTCCT and anti-sense, 3'-GGATCCAAAGGATCCTTAAGAAGTGTGATGAACATTTGG. The amplified hIL15 cDNA was cloned into pAAV-MCS to yield the pAAV-hIL15 plasmid.

Production and purification of rAAV2. The rAAV2 helper-free packing system was used according to the manufacturer's instructions in order to produce rAAV2-hIL15 and rAAV2-vector. Briefly, for the production of rAAV2-hIL15, HEK293 cells were cultured in fifty 15-cm dishes and co-transfected with 2 mg pAAV-hIL15, 2 mg pAAV-RC and 2 mg pAAV-helper plasmids using the CaCl_2 method. For rAAV2-vector production, HEK293 cells were cultured on fifty 15-cm dishes and co-transfected with 2 mg pAAV-hIL15, 2 mg pAAV-RC and 2 mg pAAV-helper plasmids using the CaCl_2 method. After 72 h of transfection, rAAV2-hIL15 and rAAV2-vector were produced in HEK293 cells. rAAV2-hIL15 and rAAV2-vector were purified with heparin column using a single-step column purification method and concentrated with an Amicon Ultra-15 centrifugal filter (Millipore, Billerica, MA, USA). The titer of rAAV2-hIL15 and rAAV2-vector was then measured by real-time PCR. rAAV2-hIL15 and rAAV2-vector were stored at -80°C until further use.

IL15 expression assay. In order to determine IL15 protein expression, the culture media obtained from 10^{13} viral particles/ml rAAV2-hIL15 and rAAV2-vector-infected HT1080 cells (1.5×10^5 cells in a 6-well plate) for 3 days were collected and determined using the ELISA assay (Biosource International, Camarillo, CA, USA). In brief, the culture media were added in 96-well plates which were coated with the hIL15 antibody. After a 4-h reaction, the plates were measured under an ELISA reader at an optical density (OD) of 450 nm.

Bioactivity assay of rAAV2-hIL15-expressed IL15. The viability of HT2 cells was determined for the bioactivity assay of rAAV2-hIL15-expressed IL15. HT2 cells are IL15-dependent cells and IL15 is required for their survival. The culture media obtained from rAAV2-hIL15- or rAAV2- vector-infected HT1080 cells were added to HT2 cell culture. After 16 h, the viability of HT2 cells was determined using the MTS assay (Promega, Madison, WI, USA). Media containing purified recombinant hIL15 ($1 \mu\text{g/ml}$) (Santa Cruz Biotechnology, Inc.) were added to HT2 cells which served as the positive control.

Cytotoxicity activity assay. Mice were infected with rAAV2-hIL15- or rAAV2-vector for >4 weeks, respectively. After the experiment mice were sacrificed, their spleens were collected and LAK cells were separated from dead cells and red blood cells by using ACK lysis buffer. YAC-1 cells are the target cells that can be killed by LAK cells. For the LAK cell cytotoxic activity assay, YAC-1 cells (target) and LAK cells (effector) were cocultured in a total volume of $200 \mu\text{l}$ in RPMI-1640 containing 10% FCS in 96-well plates at various cell densities to achieve effector-to-target (E/T) ratios (12.5:1, 25:1 and 50:1) for 4 h at 37°C . Target cell lysis was determined by using the lactate dehydrogenase (LDH) release assay using CytoTox 96® Non-Radioactive Cytotoxicity assay kit (Promega) and was calculated as: $(\text{OD}_{490 \text{ nm of sample}} - \text{OD}_{490 \text{ nm with spontaneous release of LDH from target cells}} - \text{OD}_{490 \text{ nm with spontaneous release of LDH from effector cells}}) \times 100 / (\text{OD}_{490 \text{ nm with maximum release of LDH from target cells}} - \text{OD}_{490 \text{ nm with spontaneous release of LDH from effector cells}})$.

Animal studies. Female nude mice (4 weeks old) were obtained from the National Animal Laboratory Center (Taipei, Taiwan). Every 8 nude mouse was infected on one site of the quadriceps muscles with rAAV2-hIL15 (5×10^{12} viral particles), rAAV2-vector (5×10^{12} viral particles), and PBS (mock), respectively. After 1 month, 10^7 DBTRG glioblastoma cells were transplanted on the lower abdominal region of the mice. Tumor growth was observed every 4 days, the tumor volume was measured with the caliper and calculated as $1/2 \times \text{length} \times \text{width}^2$. Animals were sacrificed when tumor size was $1,500 \text{ mm}^3$.

Statistical analysis. Data are presented as the mean \pm SEM. Statistical significance was analyzed using the Student's t-test. Survival analysis was performed using the Kaplan-Meier method. $P < 0.05$ was considered statistically significant.

Results

rAAV2-hIL15 expresses hIL15 protein with bioactivity in vitro. HT1080 cells were treated with rAAV2-hIL15, rAAV2-vector

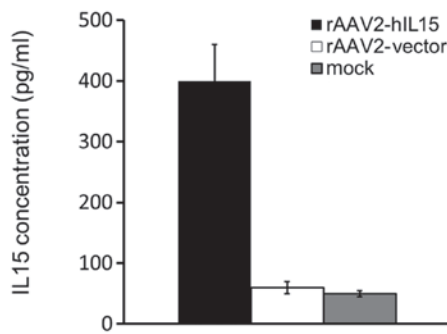


Figure 1. hIL15 protein expression. Culture media obtained from rAAV2-hIL15, rAAV2-vector and non-infected (PBS-treated mock) HT1080 cells were measured using ELISA. Notably, only the culture media obtained from rAAV2-hIL15-infected HT1080 cells exhibited high hIL15 level. hIL15, human interleukin 15; rAAV2, recombinant adeno-associated virus serotype 2; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

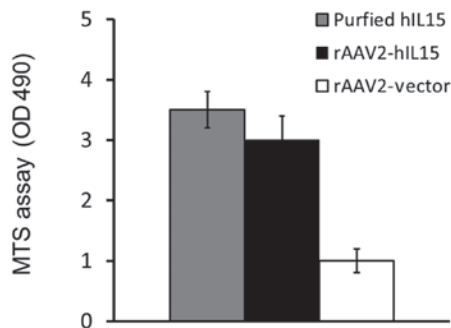


Figure 2. Bioactivity of hIL15 *in vitro*. Culture media obtained from the rAAV2-hIL15 and rAAV2-vector-infected HT1080 cells were added to HT2 cell culture for the bioactivity assay. Purified hIL15 was added to HT2 cell culture as the positive control. Bioactivity was determined by HT2 cell viability, measured using the MTS assay. Notably, the rAAV2-hIL15 group had high cell viability, similar to the purified hIL15 group. hIL15, human interleukin 15; rAAV2, recombinant adeno-associated virus serotype 2.

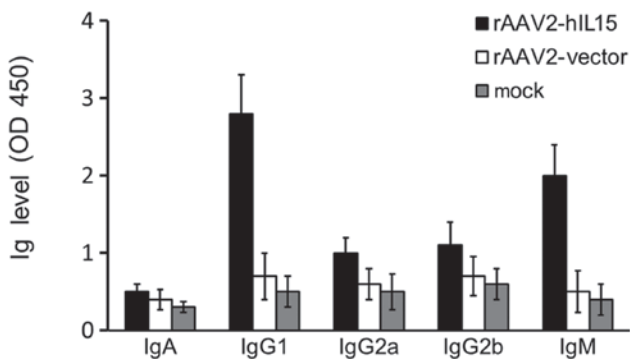


Figure 3. Immunoglobulin (Ig) level was analyzed among rAAV2-hIL15-treated, rAAV2-vector-treated and mock groups. Sera obtained from these groups were examined, respectively. Quantification of immunoglobulin levels was measured via ELISA. hIL15, human interleukin 15; rAAV2, recombinant adeno-associated virus serotype 2; ELISA, enzyme-linked immunosorbent assay.

and PBS (mock), respectively. After 72 h, the media obtained from the above groups were determined for the hIL15 level using ELISA. Our data showed that rAAV2-hIL15-treated HT1080

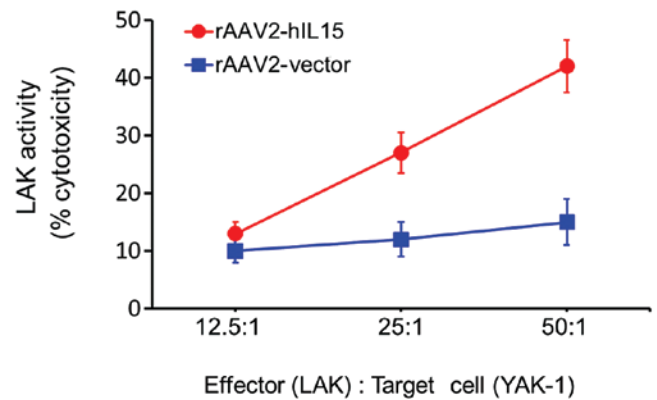


Figure 4. rAAV2-hIL15 induced cytotoxic activity of LAK cells. LAK cells obtained from rAAV2-hIL15-treated (red) and rAAV2-vector-treated groups (blue) were cocultured with their target cells (YAK-1 cells) at various ratios, respectively. Cytotoxic activity of LAK cells was measured using the LDH release assay. hIL15, human interleukin 15; rAAV2, recombinant adeno-associated virus serotype 2; LAK, lymphokine-activated killer.

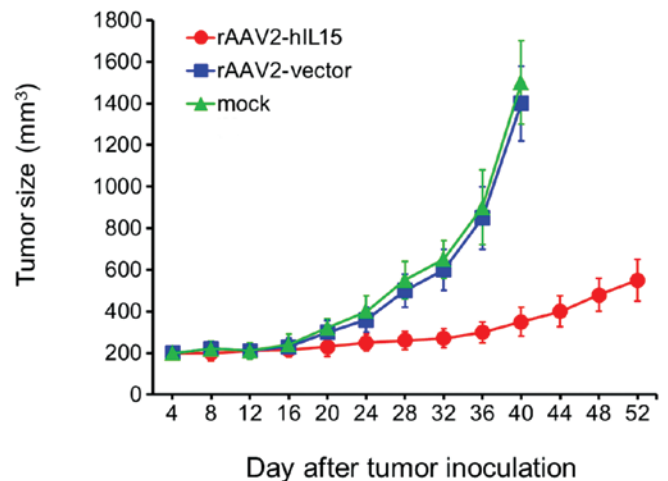


Figure 5. rAAV2-hIL15 effectively delayed glioblastoma tumor growth. Tumor growth was observed every 4 days. Tumor size was calculated as $1/2 \times \text{length} \times \text{width}^2$ and presented as the mean \pm SEM. Notably, tumor size was not significantly different between the rAAV2-vector (blue) and mock (green) groups. Tumor growth was significantly slow in the rAAV2-hIL15 group (red). hIL15, human interleukin 15; rAAV2, recombinant adeno-associated virus serotype 2; SEM, standard error of the mean.

cells expressed a 4- to 5-fold level of hIL15 protein as compared with mock or rAAV2-vector groups (Fig. 1). We further determined the bioactivity of rAAV2-hIL15-expressed hIL15 *in vitro* using a cell viability assay. HT2 cells are IL15-dependent cells that survive under IL15 supplementation. In this study, culture media obtained from the rAAV2-hIL15 and rAAV2-vector were added to HT2 cells for 48 h, and HT2 cell viability was determined by using the MTS assay. Our results demonstrated that the media obtained from the rAAV2-hIL15 group showed higher HT2 cell viability as compared with that of the rAAV2-vector group. The purified hIL15 protein served as the positive control (Fig. 2). Thus, results showed that rAAV2-hIL15 expressed hIL15 protein with bioactivity *in vitro*.

rAAV2-hIL15 induces the production of IgG1 and IgM in nude mice. Nude mice were injected with rAAV2-hIL15, rAAV2-vector and mock in the quadriceps muscle of the left thigh, respectively. After 28 days, the mice sera obtained from the above three groups were analyzed for immunoglobulin (Ig) production (IgG1, IgG2a, IgG2b, IgM and IgA). IgG1 and IgM levels were higher in the rAAV2-hIL15 group compared with the rAAV2-vector and mock groups (Fig. 3). However, the levels of IgG2a, IgG2b and IgA were not significantly different among the rAAV2-hIL15, rAAV2-vector and mock groups. Therefore, our data showed that rAAV2-hIL15 was capable of inducing the production of IgG1 and IgM in nude mice.

rAAV2-hIL15 activates cytotoxic activity of LAK cells. Mice were administered an intramuscular injection over the quadriceps muscle of the left thigh with rAAV2-hIL15 and rAAV2-vector, respectively, for 28 days. Consequently, the mice were sacrificed and their spleens collected. LAK cells were then separated and collected from dead cells and red blood cells. YAK-1 cells, the target cells of LAK cells, were used for the cytotoxic activity assay of LAK cells. YAK-1 and LAK cells were cocultured according to different ratios and the cytotoxic activity of LAK cells was studied using CytoTox 96® Non-Radioactive Cytotoxicity assay (LDH release assay). The cytotoxic activity of LAK cells was significantly induced in the rAAV2-hIL15 group (Fig. 4). At an effector/target cell ratio of 50:1, the cytotoxic activity level of LAK cells was induced with rAAV2-hIL15 up to 3-fold as compared with the rAAV2-vector group. This result suggested that rAAV2-hIL15 was able to activate the cytotoxic activity of LAK cells.

rAAV2-hIL15 delays efficient glioblastoma growth. After nude mice were intramuscularly injected with rAAV2-hIL15, rAAV2-vector and mock, respectively, for 28 days, mice were with human glioblastoma DBTRG cells subcutaneously injected. Tumor size was determined every 4 days. The tumor growth rate was slower in the rAAV2-hIL15 group compared with that in the rAAV2-vector and mock groups (Fig. 5). Although there was still a slow-paced tumor growth in the rAAV2-hIL15 group, rAAV2-hIL15 delayed tumor growth as compared with the control groups.

Discussion

Treatment of human malignant brain tumor remains a challenge and the average survival time of brain tumor patients is ~12-24 months when traditional therapies are employed (1,24,25). Thus, new therapies for brain tumor patients have been suggested and studied (26-28). Currently, gene therapy is also used in the treatment of brain tumor (28-30). Adeno-associated virus (AAV), lentivirus, herpes virus and adenovirus transfer are generally applied in gene therapy (31,32). Due to the non-pathogenic characteristics and the low immunogenicity of rAAV2, rAAV2 is considered safer than other viral delivery systems (33-36). However, rAAV2 is a small virus and it only carries small genes of ~3,000 nucleotides (37). Findings of previous studies have indicated that IL15 has a short half-life and repeated daily injection is required for immunotherapy in clinical course (9-11). Therefore, gene therapy is suggested for IL15 application.

Since the human IL15 gene is ~600-nucleotide long, it can be carried by rAAV2 viral delivery systems. In this study, we successfully produced rAAV2-hIL15 and revealed that rAAV2-hIL15 possesses antitumor bioactivities in a xenografted brain tumor mice model.

A previous study demonstrated that rAAV2-hIL15 was able to induce IgG1, IgG2b and IgM production in a nude mice model (23). However, IgG2b level was lower than that of IgG1 and IgM. Concerning our results, rAAV2-hIL15 was able to enhance IgG1 and IgM production, but there was no significant difference in the IgG2b level between the rAAV2-hIL15 group and control groups (Fig. 3). A comparison of our results with those of this previously published study suggests that rAAV2-hIL15 is capable of inducing IgG1 and IgM production although it does not induce substantial IgG2b production. Recently, it has been demonstrated that rAAV2-hIL15 is able to activate LAK cell cytotoxic activity (21-23). Our data also showed that rAAV2-hIL15 induces LAK cell cytotoxic activity in glioblastoma cells.

It has been suggested that rAAV2-hIL15 inhibits tumor growth on both human cervical tumors and mouse JC breast tumors (21-23). Additionally, rAAV2-hIL15 also inhibits the growth of human glioblastoma cells. Taken together, these studies suggest that rAAV2-hIL15 inhibits the tumor growth of various tumor types. Recently, human interleukin 12 (hIL12) was carried by rAAV2 for the treatment of brain tumor (38). This study has demonstrated that rAAV2-hIL12 induces Ig production and cytotoxic activity of LAK cells, and exerts antitumor activity on brain tumor. Ig production, the cytotoxic activity of LAK cells and the antitumor activity on human glioblastoma have also been demonstrated in this study by using rAAV2-hIL15. However, Ig production between rAAV2-hIL12 and rAAV2-hIL15 is different. IgG1 and IgG2a levels were significantly increased previously by rAAV2-hIL12 treatment (38). However, IgG1 and IgM levels were significantly increased by rAAV2-hIL15 treatment in our study. Although there are different Ig production levels between rAAV2-hIL12 and rAAV2-hIL15, they are able to inhibit brain tumor growth effectively. Therefore, we suggest that the rAAV2 gene transfer system is a useful method for tumor immunotherapy and a potential therapeutic method that may be combined with hIL12 and hIL15 for brain tumor immunotherapy in the future.

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