The Analysis of Peri-Tumor Necrosis Following the Subcutaneous Implantation of Autologous Tumor Cells Transfected with an Episome Transcribing an Antisense Insulin-Like Growth Factor 1 RNA in a Glioblastoma Multiforme Subject

ADISAK WONGKAJORNSILP, M.D., Ph.D.*,
MONGKOL OUYPRASERTKUL, M.D.***,
TUMTIP SANGRUCHI, M.D.***,
SUKIT HUABPRASERT, Cert. in M.S.T.*,
YUEXIN PAN, Ph.D.*****,
NANTASAK TISAVIPAT, M.D.**,
VORAVUT CHANYAVANICH, M.D.**,
SUPATRA SANGRUCHI, M.D.****,
KARUNA CHOMSAMUT, B.Sc.**,
DONALD D. ANTHONY, M.D.*****

Abstract

A subject inflicted with glioblastoma multiforme who received partial tumor resection and radiotherapy was recruited for an *ex vivo* gene therapy protocol using irradiated autologous tumor cells that had been engineered to suppress the expression of insulin-like growth factor I as the tumor vaccine. After subcutaneous injection for 8 weeks, the subject developed peri-tumor necrosis with mass effect. The authors wondered whether this event could have resulted from the tumor vaccine. The tissue section bordering the necrotic tumor tissue to the viable normal tissue was examined for nature of any infiltrated cells and their activities. Lymphocytes, macrophages, and a small number of neutrophils diffused into the necrotic tumor tissue were found. The infiltrated lymphocytes consisted of both $CD4^+$ and $CD8^+$ T cells. The functional activity of these lymphocytes was demonstrated by the active production of interferon γ and tumor necrosis factor α based on the respective immunofluorescent staining localized to these cells. This finding is compatible with the proposed mechanism underlying the tumor vaccination. However, the contribution of radiation treatment to this event cannot be clearly ruled out.

Key word : Peri-Tumor Necrosis, Insulin-Like Growth Factor 1, Glioblastoma Multiforme, Gene Therapy

WONGKAJORNSILP A, TISAVIPAT N, OUYPRASERTKUL M, et al
J Med Assoc Thai 2001; 84 (Suppl 3): S740-S747

* Department of Pharmacology,
** Department of Surgery,
*** Department of Pathology,
**** Department of Radiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.
***** Department of Pharmacology, Case Western Reserve University, Cleveland, Ohio 44106-4965, USA.

Enhanced expression of IGF-I and IGF-II has been demonstrated in gliomas, meniningiomas, and other tumors over the respective normal tissues (1). This over-expression of selective growth factors including IGF-I and II has been implicated in uncontrolled growth and maintenance of the tumor phenotype(2). Epidemiological data indicate that cancer risk is associated with high serum IGF-I values(3). Circulating IGF-I is related to future colorectal cancer risk and may specifically predict adenoma progression(4). The signal resulted from IGF-I binding to IGF-I receptor on cancer cells has generated multiple cellular functions such as cell survival, motility, invasion, metastasis, the induction of angiogenesis, and the acquisition of multiple drug-resistance(5). IGF-I can block sensitization of human malignant glioma cells to FasL-induced-apoptosis by wild-type PTEN(6). Therefore, disruption of IGF-I and its receptor binding may be an effective strategy for the treatment of a wide spectrum of human cancers. The treatment of rat C6 glioma cells using antisense RNA of IGF-I resulted in loss of tumorigenicity and induced a tumor specific immune response involving $CD8^+$ T cells(7). The treatment by antisense IGF-I engineered with triple helix technologies has been reported to drive human glioma cells to enter apoptosis(8) and to increase the expression of both MHC class I and B7 molecules both in human glioma cells(9) and PCC-3 murine embryonic carcinoma cells(10). Alternatively, antisense IGF-I receptor was employed in human malignant glioma(11).

The efficacy of treatment using an episome-based antisense cDNA transcription of IGF-1 was demonstrated in rats bearing C6 glioma cells(7) and later in mice bearing PCC3 murine teratocarcinoma cells(12). The subcutaneous injection of these transfected tumor cells into syngeneic animals resulted in the induction of an immune response against the transfected tumor cells as well as the wild-type tumor cells that had earlier been implanted at a different site. The mechanism of tumor cytotoxicity includes the infiltration of $CD8^+$ T cell within the tumor mass. The upregulation of MHC class I and B7 molecules on the transfected human glioma cells may improve the immune recognition of these cells (9). A clinical trial protocol of human malignant glioma using this strategy was approved by the then Recombinant DNA Advisory Committee (RAC) of the US Food and Drug Administration (FDA) on June 8, 1993 with the first injection held at Case Western Reserve University in March 1994. An extension of this project in Thailand has been held at Siriraj Hospital with the first injection in October 1999. The purpose of this trial was for safety and efficacy studies with a possible expansion of this approach to a spectrum of other cancers with high IGF-I expression. These candidate tumors include non-small cell lung cancer and cholangiocarcinoma of which the primary cultured cells have been established in similar fashion. The authors expected an enhanced T cell mediated immune response similar to the rat model, and ultimately tumor regression or delayed tumor progression.

MATERIAL AND METHOD

The episome-pAnti-IGF-I(13) contains Epstein-Barr virus replicative signals and a hygromycin resistant gene (a gift from Dr. Anthony, CWRU, Cleveland, OH, USA). This episome incorporates the $ZnSO_4$-inducible metallothionein I (MT-I) transcriptional promoter driving the antisense transcript of IGF-I. The transfected cells with high copy numbers of intracellular pAnti-IGF-I were enriched by gradually increasing the concentration of hygromycin as a selective pressure. The suppression of IGF-I expression was determined by FACS analysis using a mouse monoclonal antibody against human IGF-I.

Subject

A 53-year old male subject inflicted with glioblastoma multiforme in the left frontal lobe was admitted for tumor resection (bifrontal craniotomy with partial left frontal lobectomy and subtotal tumor removal) on June 28, 1999 at the Division of Neurosurgery. Informed consent for the study was given. The subject had received radiotherapy for a total dose of 56 Gy between July 16, 1999 - August 31, 1999, and was subcutaneously injected with irradiated pAnti-IGF-I-transfected on October 7, 1999, November 5, 1999, and December 30, 1999. This study proceeded according to the same guidelines proposed originally at CWRU and approved for domestic trial by the Institutional Review Board for ethical consideration of the Faculty of Medicine Siriraj Hospital in July 1999. The subject did not have active Epstein-Barr virus infection nor any evidence of HIV infection through the screening for

a panel of antibodies. Throughout the study the subject always had a Karnofsky Score above 60 except during December 1999 when tumor necrosis took place.

Study Procedures

1. Tumor cells were obtained from the patient at the time of open surgery on July 16, 1999.

2. Cultured cells were demonstrated for IGF-1 production and were transfected with the episome containing antisense cDNA to IGF-1. The transfected cells were tested for down regulation of IGF-1 by immunohistochemistry.

3. The transfected cells were irradiated with 50 Gy and subcutaneously injected to the subject on October 7, 1999.

4. Booster injections of transfected and irradiated cells were given at 4 weeks (November 5, 1999) and at 12 weeks (December 30, 1999).

Acquisition of Human Tissue and Primary Cell Culture

The resected tissues were obtained from the operating room at Siriraj Hospital using standard surgical techniques and transferred to a tissue culture facility at the Department of Pharmacology. Tumor cells were released by digestion with 0.025 per cent trypsin in Dulbecco's modified Eagle medium (DMEM), and seeded at a density of 500-1000 cells/mm^2 of tissue culture flask in growth medium (DMEM, 15% fetal bovine serum, 1 mM sodium pyruvate, 1 mg/mL insulin, 0.55 mg/mL transferrin, 0.67 μg/mL sodium selenite, 0.1 mM non-essential amino acid solution, 2 mM *l*-glutamine, 50 unit/mL penicillin, 50 μg/mL streptomycin). Cultured cells were maintained in growth medium incubated at 37°C, 5 per cent CO_2.

Transfection of Cultured Cells

Cells were transfected with pAnti-IGF-I using the lipofectamine reagent kit (Gibco BRL). The transfected cells were enriched for high copy numbers of episome by adding hygromycin starting at 0.5 μg/mL for 1-2 weeks. The hygromycin concentration was gradually raised by 0.5 - 1 μg/mL every 1 - 2 weeks until at least a minimum concentration of 2 μg/mL was reached. The activation of MT-I promoter that drove the expression of antisense IGF-I RNA was achieved by incubation with 0.3 mM $ZnSO_4$ in serum-free growth medium for 16 h. The suppression of IGF-I expression was determined by immunohistochemical staining with the mouse primary monoclonal antibodies against human IGF-I (Serotec, Oxford, UK). The goat secondary antibody was conjugated with biotin and directed against the Fc fragment of the mouse primary antibody (Dako, Japan). The streptavidin conjugated with horse radish peroxidase (Dako, Japan) would be attracted to the biotin group of the secondary antibodies. The conjugated peroxidase activity would catalyze the appearance of brown color upon exposure to DAB substrate (Sigma, USA).

Preparation for Injection

The transfected cells were incubated with 0.3 mM $ZnSO_4$ in serum free growth medium for 16 h, transferred to fresh serum-free growth medium, irradiated for 50 Gy with ^{60}Co source, washed and finally resuspended in preservative-free normal saline for injection at a cell density of 1×10^6 cells/ 2 mL. The 2-mL cell suspension was subcutaneously injected into the deltoid region.

Immunohistochemistry and Immunofluorescent Staining

Tumor samples in paraffin blocks were sliced into thin pieces with a thickness of 2-3 μm. They were immunohistochemically stained with the mouse primary monoclonal antibodies against either human CD4, or CD8 (Becton Dickinson). The sections were further processed in the same way as in IGF-I staining. Finally, the sections were counterstained with hematoxylin prior to viewing. For immunofluorescent staining, the sections were directly stained with the primary mouse anti-human interferon γ conjugated with PE or the primary mouse anti-human tumor necrosis factor α conjugated with FITC. The stained sections were viewed under a fluorescent microscope.

RESULTS

Quality Control of Cultured Cells

The cultured cells were plated onto a chambered slide and immunohistochemically stained for IGF-I protein (Fig. 1A). Dark brown staining appeared in the cytoplasm of almost all cells. After the transfection with pAnti-IGF-I followed by the maintenance of the transfected cells in growth medium containing up to 2 μg/mL hygromycin, there was still cytoplasmic staining, but of a slightly lighter color (Fig. 1B). When 0.3 mM $ZnSO_4$ was

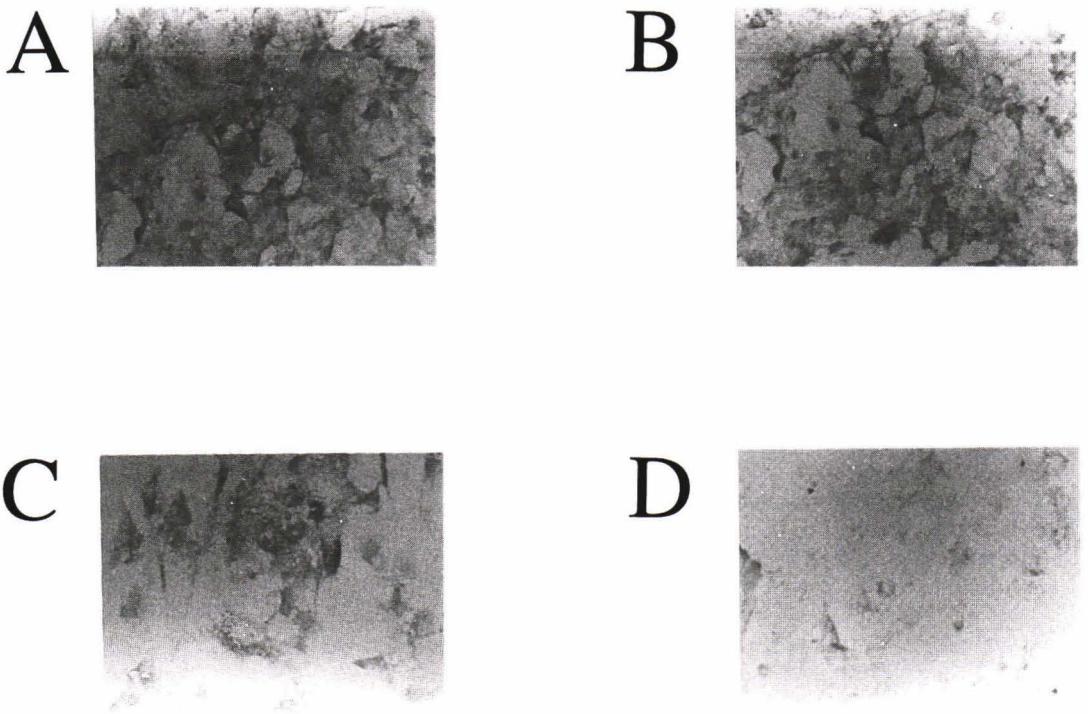

Fig. 1. The immunohistochemical staining of IGF-I in the cytoplasm of primary cultured glioblastoma multiforme. The unmanipulated cultured cells (A) attached to a chambered slide were fixed in acetone, stained with the mouse primary monoclonal antibody against human IGF-I followed by the goat anti-mouse secondary antibody conjugated with biotin and the streptavidin conjugated with horse radish peroxidase. The brown color appeared after the exposure of the conjugated peroxidase to DAB substrate. The pAnti-IGF-I transfected cultured cells in the presence of at least 2 μg/mL hygromycin (B) also express IGF-I in the cytoplasm. The exposure of unmanipulated cells to 0.3 mM $ZnSO_4$ (C) did not significantly alter cytoplasmic IGF-I staining, whereas, the exposure of the transfected cells to $ZnSO_4$ (D) clearly diminished cytoplasmic IGF-I staining.

inoculated with the unmanipulated tumor cells for 16 h, there was no significant change in the staining (Fig. 1C). But after the transfected cells were exposed to $ZnSO_4$, the staining was extensively decreased, although it did not completely disappear (Fig. 1D). The cultured cells were confirmed for their glial origin using immunohistochemical staining for glial fibrillary acidic protein (GFAP) in the cytoplasm (data not shown).

Blood Chemistry

There was no significant alteration in CBC, urinalysis, CD4/CD8 ratio, PT/PTT, serum immunoglobulin levels and a routine chemistry panel including blood electrolytes, BUN, creatinine, total bilirubin, alkaline phosphatase, serum transaminases, and liver function studies that had been periodically checked throughout the 6-month study schedule. The tuberculin skin test that represents delayed type hypersensitivity before the treatment was negative but was later positive at the end of 6 months.

Tumor Necrosis with Brain Edema

Approximately 8 weeks after entering the experimental treatment (4-5 months after the radiation treatment), the subject developed tumor necro-

Fig. 2. The immunoperoxidase staining of CD4+ cells at the border of tumor necrotic tissue. The tissue was counter-stained with hematoxylin.

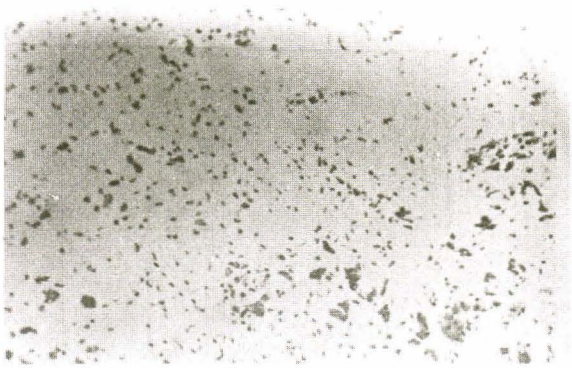

Fig. 3. The immunoperoxidase staining of CD8+ cells at the border of tumor necrotic tissue.

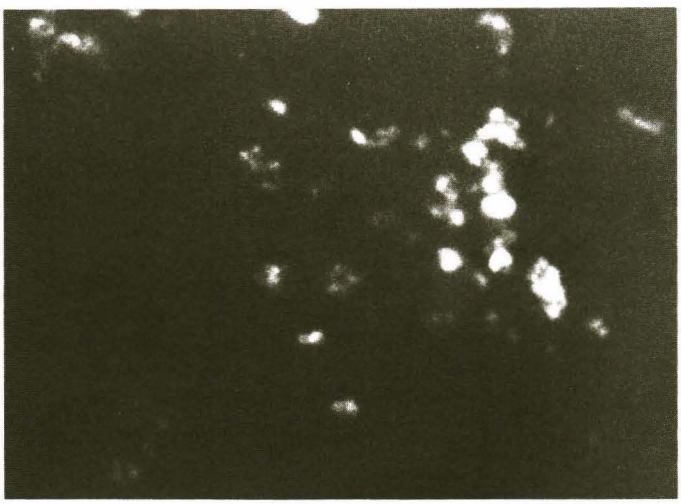

Fig. 4. The immunofluorescent staining of interferon γ of lymphocytes infiltrating the tumor necrotic tissue using mouse anti-human interferon γ conjugated with PE.

sis with mass effect that eventually resulted in blindness to both eyes. The subject was operated on December 16, 1999 to relieve the pressure and removal of the necrotic tissue. The resected tissue as well as the margin to adjacent normal tissue was diffusely infiltrated with lymphocyte, neutrophil, and macrophage. The immunofluorescent staining of CD4 (Fig. 2) and CD8 (Fig. 3) was localized to the infiltrated lymphocytes. The immunofluorescent staining of intracellular interferon γ (IFN-γ; Fig. 4) and tumor necrosis factor α (TNF-α; Fig. 5) were also localized to the infiltrating lymphocytes.

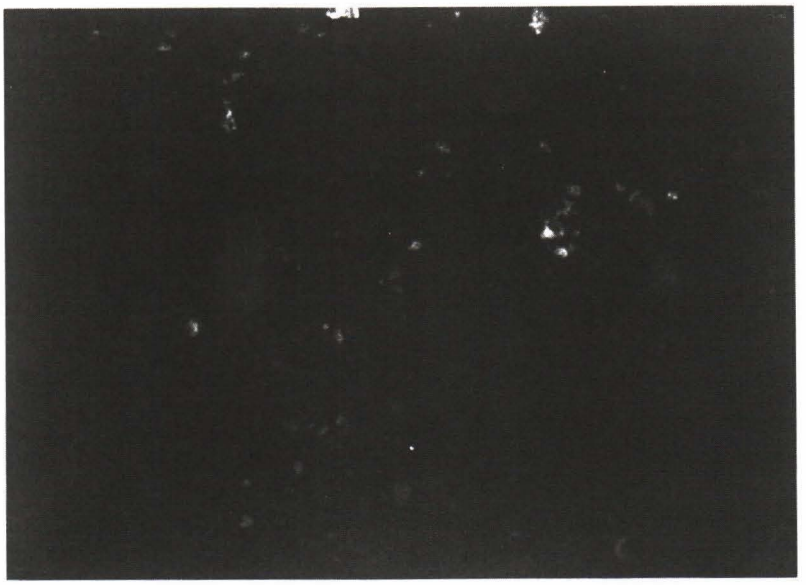

Fig. 5. The immunofluorescent staining of tumor necrosis factor α of lymphocytes infiltrating the tumor necrotic tissue using mouse anti-human tumor necrosis factor α conjugated with FITC.

DISCUSSION

The only observed effect during the 6-month period was the development of tumor necrosis with mass effect. This necrosis could have resulted from either radiotherapy or from the tumor vaccine. The incidence of tumor necrosis following radiation was documented as early as 1975[14]. However, only an article reported the involvement of inflammatory cells and cytokines in this event. In radiation-induced delayed brain injury, diffusely infiltrated cells included $CD4^+$ T cells, $CD8^+$ T, and macrophages with the high expression of TNF-α, IL-1α, and IL-6 predominantly localized to infiltrated macrophage in malignant glioma[15]. Generally the development of radiation-induced tumor necrosis occurred very late (several months to years) after the treatment with 0-9 per cent of patients receiving less than a total dose of 60 Gy[16]. The development of tumor necrosis as early as 4-5 months after the radiation treatment as well as the absence of vasculitis (Fig. 2, 3) suggested that the tumor vaccination could have played a causal role. The authors then investigated whether the infiltrated lymphocytes that had been proposed as the mediator for the tumor vaccination were active in agreement with the animal model. Since the T cell function tests such as cytotoxicity test and proliferation assay could not be performed on these infiltrated cells, the authors therefore investigated for the production of cytokines (IFN-γ and TNF-α) as the only feasible alternative T cell function test[17]. As expected, the infiltrated lymphocytes actively produced these cytokines (Fig. 4, 5). The favorable response included the reduction of tumor mass viewed with MRI after the trial for 4 months (February 2, 2000).

By the end of the 6-month study (April 4, 2000), the tumor mass had enlarged. The subject got his final tumor resection on May 2, 2000, and expired in July 2000. The subject at least developed partial response to tumor vaccination early in the study with tumor necrosis and regression. There was no other serious observed adverse effect derived from the vaccination. The recurrent tumor by the end of the study could have been due to the selection of clones that escaped the cross reaction with the clone grown in culture condition. In this case, another cycle of fresh primary culture with transfection could have been of therapeutic benefit.

ACKNOWLEDGEMENT

The funding of this study came from grant number 75-348-261 of the Siriraj Grant for Research Development, Faculty of Medicine Siriraj Hospital, Mahidol University awarded to Adisak Wongkajornsilp.

(Received for publication on November 22, 2001)

REFERENCES

1. Zumkeller W, Westphal M. The IGF/IGFBP system in CNS malignancy. Mol Pathol 2001; 54: 227-9.
2. Merrill MJ, Edwards NA. Insulin-like growth factor-I receptors in human glial tumors. J Clin Endocrinol Metab 1990; 71: 199-209.
3. Zumkeller W. IGFs and IGFBPs : Surrogate markers for diagnosis and surveillance of tumour growth? Mol Pathol 2001; 54: 285-8.
4. Renehan AG, Painter JE, Atkin WS, Potten CS, Shalet SM, O'Dwyer ST. High-risk colorectal adenomas and serum insulin-like growth factors. Br J Surg 2001; 88: 107-13.
5. Samani AA, Brodt P. The receptor for the type I insulin-like growth factor and its ligands regulate multiple cellular functions that impact on metastasis. Surg Oncol Clin North America 2001; 10: 289-312, viii.
6. Wick W, Furnari FB, Naumann U, Cavenee WK, Weller M. PTEN gene transfer in human malignant glioma: Sensitization to irradiation and CD95L-induced apoptosis. Oncogene 1999; 18: 3936-43.
7. Trojan J, Johnson TR, Rudin SD, Ilan J, Tykocinski ML. Treatment and prevention of rat glioblastoma by immunogenic C6 cells expressing antisense insulin-like growth factor I RNA. Science 1993; 259: 94-7.
8. Ly A, Duc HT, Kalamarides M, et al. Human glioma cells transformed by IGF-I triple helix technology show immune and apoptotic characteristics determining cell selection for gene therapy of glioblastoma. Mol Pathol 2001; 54: 230-9.
9. Trojan J, Duc HT, Upegui-Gonzalez LC, et al. Presence of MHC-I and B-7 molecules in rat and human glioma cells expressing antisense IGF-I mRNA. Neurosci Lett 1996; 212: 9-12.
10. Ly A, Francois JC, Upegui-Gonzalez LC, et al. Alterations in tumorigenicity of embryonal carcinoma cells by IGF-I triple-helix induced changes in immunogenicity and apoptosis. Life Sci 2000; 68: 307-19.
11. Andrews DW, Resnicoff M, Flanders AE, et al. Results of a pilot study involving the use of an antisense oligodeoxynucleotide directed against the insulin-like growth factor type I receptor in malignant astrocytomas. J Clin Oncol 2001; 19: 2189-200.
12. Trojan J, Johnson TR, Rudin SD, et al. Gene therapy of murine teratocarcinoma: Separate functions for insulin-like growth factors I and II in immunogenicity and differentiation. Proc Natl Acad Sci USA 1994; 91: 6088-92.
13. Trojan J, Blossey BK, Johnson TR, Rudin SD, Tykocinski M, Ilan J. Loss of tumorigenicity of rat glioblastoma directed by episome-based antisense cDNA transcription of insulin-like growth factor I. Proc Natl Acad Sci USA 1992; 89: 4874-8.
14. Graffman S, Haymaker W, Hugosson R, Jung B. High-energy protons in the post-operative treatment of malignant glioma. Acta Radiol Ther Phys Biol 1975; 14: 443-61.
15. Kureshi SA, Hofman FM, Schneider JH, Chin LS, Apuzzo ML, Hinton DR. Cytokine expression in radiation-induced delayed cerebral injury. Neurosurgery 1994; 35: 822-9; discussion 829-30.
16. Levin VA, Leibel SA, Gutin PH. Section 2: Neoplasms of the central nervous system. In: Devita Jr. VT, Hellman SH, Rosenberg SA editors. Cancer: Principles & Practice of Oncology, 6 ed. Philadelphia: Lippincott Williams & Wilkins, 2001: 2100-60.
17. Hickling JK. Measuring T lymphocyte function. Expert Review in Molecular Medicine (http://www-ermm.cbcu.cam.ac.uk). Accession number: txt001jhc; Accession date: 13 October 1998.

การศึกษาภาวะ peri-tumor necrosis ที่เกิดขึ้นในผู้ป่วย glioblastoma multiforme หลังจากได้ฉีดวัคซีนที่ผลิตจากเซลล์มะเร็งที่ผ่านกระบวนการทางพันธุวิศวกรรม เพื่อยับยั้งการสังเคราะห์ insulin-like growth factor I

อดิศักดิ์ วงศ์ขจรศิลป์, พ.บ., ปร.ด., นันทศักดิ์ ทิศาวิภาต, พ.บ.**, มงคล อุยประเสริฐกุล, พ.บ.***, วรวุฒิ จรรยาวณิชย์, พ.บ.**, ดุ้มทิพย์ แสงรุจิ, พ.บ.***, สุพัตรา แสงรุจิ, พ.บ.****, สุกิจ ฮวบประเสริฐ, ประกาศนียบัตร พ.ว.ก.*, กรุณา ชมสมุทร วท.บ.**, Yuexin Pan, Ph.D.*****, Donald D. Anthony, M.D.******

ผู้ป่วย glioblastoma multiforme ที่ได้รับการผ่าตัดขจัดเนื้องอกไปบางส่วนร่วมกับการฉายรังสีรักษาเรียบร้อยแล้ว ได้เข้าสู่การทดลองรักษา *ex vivo* gene therapy โดยใช้เซลล์มะเร็งของตนเองที่ผ่านกระบวนการทางพันธุวิศวกรรม เพื่อขจัดการผลิต insulin-like growth factor I แล้วจึงนำมาฉายแสงก่อนใช้เป็นวัคซีน หลังจากผ่านการทดลองรักษาเป็นเวลา 8 สัปดาห์ผู้ป่วยเกิดมีภาวะ peri-tumor necrosis with mass effect จึงมีการศึกษาเพื่อสืบค้นว่าภาวะนี้เกิดจากการทดลองรักษาหรือจากรังสีรักษา โดยศึกษาชิ้นเนื้อที่รอยต่อระหว่าง necrotic tumor tissue กับเนื้อเยื่อปกติเพื่อหาเซลล์เม็ดเลือดขาวที่แทรกซึมอยู่ ได้ตรวจพบว่ามี lymphocytes, macrophages, และ neutrophils อีกจำนวนเล็กน้อยกระจายแทรกใน necrotic tumor tissue การศึกษาแยกแยะหาชนิดของ lymphocytes ได้พบว่ามีทั้ง $CD4^+$ และ $CD8^+$ T cells เมื่อศึกษาความสามารถในการทำงานของ lymphocytes เหล่านี้โดยตรวจหาการผลิต interferon γ และ tumor necrosis factor α โดยวิธี immunofluorescent staining ก็พบว่ามีอยู่มากภายใน lymphocytes เหล่านี้ ผลการศึกษานี้จึงเข้าได้กับกลไกการออกฤทธิ์ของ tumor vaccine แต่ทว่าอาจมีผลจากรังสีรักษาที่ร่วมก่อให้เกิด peri-tumor necrosis ครั้งนี้

คำสำคัญ : Peri-Tumor Necrosis, Insulin-Like Growth Factor 1, Glioblastoma Multiforme, Gene Therapy

อดิศักดิ์ วงศ์ขจรศิลป์, นันทศักดิ์ ทิศาวิภาต, มงคล อุยประเสริฐกุล, และคณะ จดหมายเหตุทางแพทย์ ฯ 2544; 84 (ฉบับพิเศษ 3): S740-S747

* ภาควิชาเภสัชวิทยา,
** ภาควิชาศัลยศาสตร์,
*** ภาควิชาพยาธิวิทยา,
**** ภาควิชารังสีวิทยา, คณะแพทยศาสตร์ศิริราชพยาบาล, มหาวิทยาลัยมหิดล, กรุงเทพฯ 10700
***** Department of Pharmacology, Case Western Reserve University, Cleveland, Ohio 44106-4965, USA