LABORATORY INVESTIGATION

J Korean Neurosurg Soc 38 : 126-131, 2005

Cytotoxicities of Tumor-specific T Lymphocytes Primed by Glioma Apoptotic Body - or Glioma Cell Lysate-pulsed Dendritic Cells

Jong-Tae Kim, M.D., Dong-Sup Chung, M.D., Seung-Won Kwak, M.D., Young-Min Han, M.D., Young-Sup Park, M.D., Moon-Chan Kim, M.D.

Department of Neurosurgery, Our Lady of Mercy Hospital, College of Medicine, The Catholic University of Korea, Incheon, Korea

Objective : The choice of tumor antigen for dendritic cell(DC)-loading has still been an unresolved problem in the DC-based vaccine strategies against malignant gliomas that has not been found well-characterized tumor specific antigens. In this study, we compare tumor-specific T cell response induced by glioma apoptotic body(GAB)-pulsed DCs to response induced by glioma cell lysate-pulsed ones quantitatively.

Methods : DCs generated in the presence of granulocyte macrophage-colony stimulating factor and interleukin(IL)-4 from peripheral blood mononuclear cells(PBMCs) of HLA-A2 positive healthy donors were cultured. Each GABs and glioma cell lysate generated from HLA-A2 positive T98G glioblastoma cells were co-incubated with DCs. $CD8^+$ T lymphocytes isolated from PBMCs of same donors were cultured in media containing IL-2 and either stimulated by GAB- or lysate-pulsed DCs three times at a weekly interval. The interferon(IFN)-γ concentrations of each cell culture supernate were measured by enzyme immunoassay technique. Cytolytic activity of the generated cytotoxic $CD8^+$ T cells either stimulated with GAB- or lysate-pulsed DCs was determined by a standard 4-h ^{51}Cr-release assay.

Results : IFN-γ production and cytolytic activity of effector T cells stimulated by GAB-pulsed DCs were significantly higher than those of T cells stimulated by lysate-pulsed ones.

Conclusion : These results indicate the choice of antigen is a critical determinant in the induction of antitumor immunity against malignant glioma. Antigen preparations from GABs represent a promising alternative to glioma cell lysate in DC-based glioma vaccine strategies.

KEY WORDS : Dendritic cell · Interferon-γ · Tumor-specific cytotoxicity · Glioma apoptotic body · Glioma cell lysate.

Introduction

The major goal of tumor vaccine strategies using dendritic cells(DCs) is the induction of strong cytotoxic T lymphocyte(CTL) responses against tumor antigens to trigger an immune response against tumors. This strategy is quite attractive because it offers the potential for high tumor-specific toxicity[15]. To induce a CTL response against tumor cells, the antigenic peptide must be presented to naive T cells in the context of costimulatory molecules usually provided by professional antigen presenting cells(APCs)[4]. DCs are the most potent professional APCs that have a unique potency for activating T cells[18]. The therapeutic efficacy of DC-based anti-tumor vaccination against malignant tumors including malignant glioma has been widely investigated[3,12,13,19,20,23].

Priming of DCs with tumor antigens for generation of a tumor-specific CTL response requires prior identification of tumor-derived antigens. A critical issue in optimizing DC-based tumor vaccines is the choice of tumor antigen for DC loading. Pure peptides that are expressed in all tumor cells but not in normal cells are the most favorable antigens theoretically. Unfortunately, such peptides have not yet been found in malignant glioma. But this limitation can be overcome considerably by the use of unfractionated glioma-derived antigens such as apoptotic bodies or glioma cell lysates. Effective cross-priming of tumor specific CTLs by DCs pulsed with

• Received : February 15, 2005 • Accepted : April 4, 2005

• Address for reprints : Dong-Sup Chung, M.D., Department of Neurosurgery, Our Lady of Mercy Hospital, College of Medicine, The Catholic University of Korea, 665 Bupyeong 6-dong, Bupyeong-gu, Incheon 403-720, Korea Tel : +82-32-510-5500, Fax : +82-32-511-2370, E-mail : dschung@olmh.cuk.ac.kr

glioma apoptotic bodies(GABs)[10], or glioma cell lysates[22] has been demonstrated, but little quantitative comparison has been undertaken[14].

In this study we compared tumor-specific T cell responses induced by GAB-pulsed DCs to responses induced by glioma cell lysate-pulsed ones. To this end, DCs and $CD8^+$ T cells were obtained from mononuclear cells of HLA-A2 positive healthy donors. Either apoptotic bodies or tumor cell lysate from the HLA-A2 positive T98G glioma cells were used in activation of tumor-specific CTLs as an antigen source. The interferon(IFN)-γ concentrations of each CTLs culture supernate and cytolytic activities of the generated cytotoxic $CD8^+$ T cells stimulated by GAB-pulsed DCs(DCs/GABs), as well as lysate-pulsed ones(DCs/lysates) were evaluated.

Materials and Methods

Preparation of peripheral blood mononuclear cells(PBMCs)

T98G malignant glioma cells with HLA-A*0201 were used as a source of apoptotic body and lysate. Two healthy donors who had HLA-A*0201 were selected to donate DCs and $CD8^+$ T cells. 100ml of peripheral blood were drawn from each donor. Mononuclear cells were isolated from whole blood by Ficoll-Hypaque centrifugation(Sigma, St. Louis, MO, USA) and plated in complete medium consisting of RPMI 1640(GibcoBRL, Grand Island, NY, USA) with 10% human serum(BioWhittaker, Walkerville, MD, USA) and 1% penicillin/streptomycin(GibcoBRL, Grand Island, NY, USA).

Isolation of $CD8^+$ T cells

$CD8^+$ T cells were isolated from non-adherent PBMCs using magnetic bead-conjugated mouse anti-human $CD8^+$ mAb(Miltenyi Biotec, Bergisch Gladbach, Germany), a MACS column for positive selection, and a vario-MACS magnet, according to the manufacturer instructions. The purified cells contained 96 to 99% $CD8^+$ T cells were confirmed by flow cytometry. Cells were cryopreserved before use.

DC culture

PBMCs were plated at a concentration of 2×10^7 cells/ml in a T75 flask containing 12ml of RPMI 1640 with 10% human serum. After 2hr-incubation at 37°C non-adherent cells were removed by washing with warm complete medium. Then adherent cells were cultured with complete medium for 5days in the presence of recombinant human granulocyte macrophage-colony stimulating factor(GM-CSF)(R&D systems, Minneapolis, MN, USA) 800U/ml, and interleukin (IL)-4(R&D systems, Minneapolis, MN, USA) 1,000U/ml. Cell morphologies were examined by microscope.

Phenotypic evaluation of DCs

DCs were evaluated phenotypically as described[10]. In brief, harvested DCs were washed twice with phosphate buffer solution(PBS) containing 1% bovine serum albumin(Sigma, St Louis, MO, USA). They were incubated on ice for 30minutes with monoclonal antibodies(mAbs) labeled with fluorescein isothiocyanate(FITC) or phycoerythrin(PE) and then washed twice. After staining but before flow cytometry, the cells were fixed with 1% paraformaldehyde(Sigma, St Louis, MO, USA) in PBS at room temperature. Flow cytometry analysis was performed using a FACScan(Becton Dickinson, Franklin Lakes, NJ, USA), and the results were processed using CellQuest software(Becton Dickinson, Franklin Lakes, NJ, USA). The antibodies used for cell staining included HLA ABC-FITC, HLA DR-FITC, CD1a-FITC, CD14-FITC, CD80-FITC, and CD86-PE(BD Pharmingen, San Diego, CA, USA).

Induction of apoptosis in T98G glioma cells and Annexin V staining

To induce apoptosis, 2×10^6 T98G glioma cells were incubated in RPMI 1640 with 10% bovine fetal serum and treated with actinomycin D(Sigma, St Louis, MO, USA). To confirm the *in vitro* condition that induces apoptosis, T98G cells were incubated for 18hours after treatment with various concentrations of actinomycin D. GABs were stained by Annexin V-FITC(Roche, Mannheim, Germany) together with propidium iodide(Roche, Mannheim, Germany) as described[10]. Cells were analyzed by FACScan(Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuest software(Becton Dickinson, Franklin Lakes, NJ, USA).

Preparation of glioma cell lysate

Tumor cell lysates were generated by rapid freeze-thaw cycles[6]. Confluent cultures of T98G glioma cell lines were incubated with 0.01% EDTA solution for 10 min, carefully detached with a cell scraper, washed twice in PBS, and resuspended at a density of 5×10^6/ml in serum-free medium. The tumor cell lysates were prepared by five rapid freeze-thaw cycles in liquid nitrogen and in a 37°C water bath until the cell membrane integrity was lost. For the removal of crude debris, the lysate was centrifuged for 30 min at 1,500×g. The supernatant was collected and passed through a 0.2㎛ filter. The protein concentration of the lysate was measured by a Bio-Rad protein assay(Bio-Rad Laboratories, Hercules, CA, USA). Aliquots of the lysate (1mg/ml) were used to pulse DCs.

DC pulsing with GABs or lysates

DCs were divided into three groups at a concentration of 5×10^6cells/ml in T25 flask. Group A was cultured without any tumor antigen, group B with GABs and group C with

lysates overnight. These cells were then cultured in 6ml complete medium containing 800U/ml of GM-CSF and 1000U/ml of IL-4 for 3days to allow DC digestion of antigens. DCs were then allowed to mature by culturing with 500U/ml of tumor necrosis factor(TNF)-α(R & D Systems, Minneapolis, MN, USA) and 1ug/ml of prostaglandin(PG) E2(Sigma, St. Louis, MO, USA) for 2days.

Effector T cell activation

$CD8^+$ T cells were also divided into three groups. Cryopreserved $CD8^+$ T cells (1×10^6) were cocultured with normal DCs(group A), DCs/GABs(group B), or DCs/lysates(group C) in a 24-well plate with 2ml of RPMI 1640 containing 60U/ml of IL-2.

Every seven days the T cells in each group were restimulated with DCs(group A), DCs/GABs(group B), or DCs/lysates (group C). Low doses of IL-2 (10U/ml) were also added to the culture media. Fifty percent of the culture media was replaced with fresh media containing 25U/ml IL-2 on day 4 after every stimulation.

Quantification of IFN-γ secretion

Supernatants in each group on day 21 of coculture were harvested, and concentrations of human cytokine IFN-γ were quantified by enzyme-linked immunosorbent assay(ELISA)(R & D systems, Minneapolis, MN, USA) according to the manufac-turer instructions.

Cytotoxicity assay

The cytolytic activities of CTLs in each group were assessed by 4hr [^{51}Cr]-release assay. This method was performed at two effector to target (E:T) ratios. Briefly, T98G glioma cells were labeled with 100μCi $Na_2[^{51}Cr]O_4$ (Perkin Elmer, Boston, USA)/106 cells at 37°C for 1hr. After three washes, labeled cells were incubated for 4hr with graded numbers of effector cells in 96-well plates. Thereafter 100$\mu\ell$ of cell-free supernatants were then collected using a Skatron harvester and analyzed in a gamma counter(LKBWallac CliniGamma 1272, Wallac, Finland). The mean of triplicate samples was found, and the percent of specific lysis was calculated as follows : Percent specific [^{51}Cr] release = 100$\times$(experimental release-spontaneous release)/(maximum release-spontaneous release).

Data analysis

Statistical anlayses of data were performed using SPSS version 11 for Windows(SPSS Inc., Chicago, IL, USA). Means were compared using the Student's two-sample t test, with significance assumed at a value of $P\leq0.05$.

Results

Phenotypes of DCs

For generated DCs, 75 to 85% of mononuclear cells showed typical DC morphology as revealed by microscopic examination. Additionally, expression of major histocompatibility complex(MHC) class I, MHC class II, CD1a, and costimulation molecules, CD80(B7.1) and CD86(B7.2) were increased as in typical DCs. Their endocytotic activity was also increased(Fig. 1).

Apoptosis induction of glioma cells

T98G malignant glioma cells were treated with actinomycin D at various concentrations. Following treatment, the apoptotic tumor cells showed positive Annexin V staining(Fig. 2, 3). The highest apoptotic rate (53%) was shown for an actinomycin D concentration of 5×10^2pg/ml.

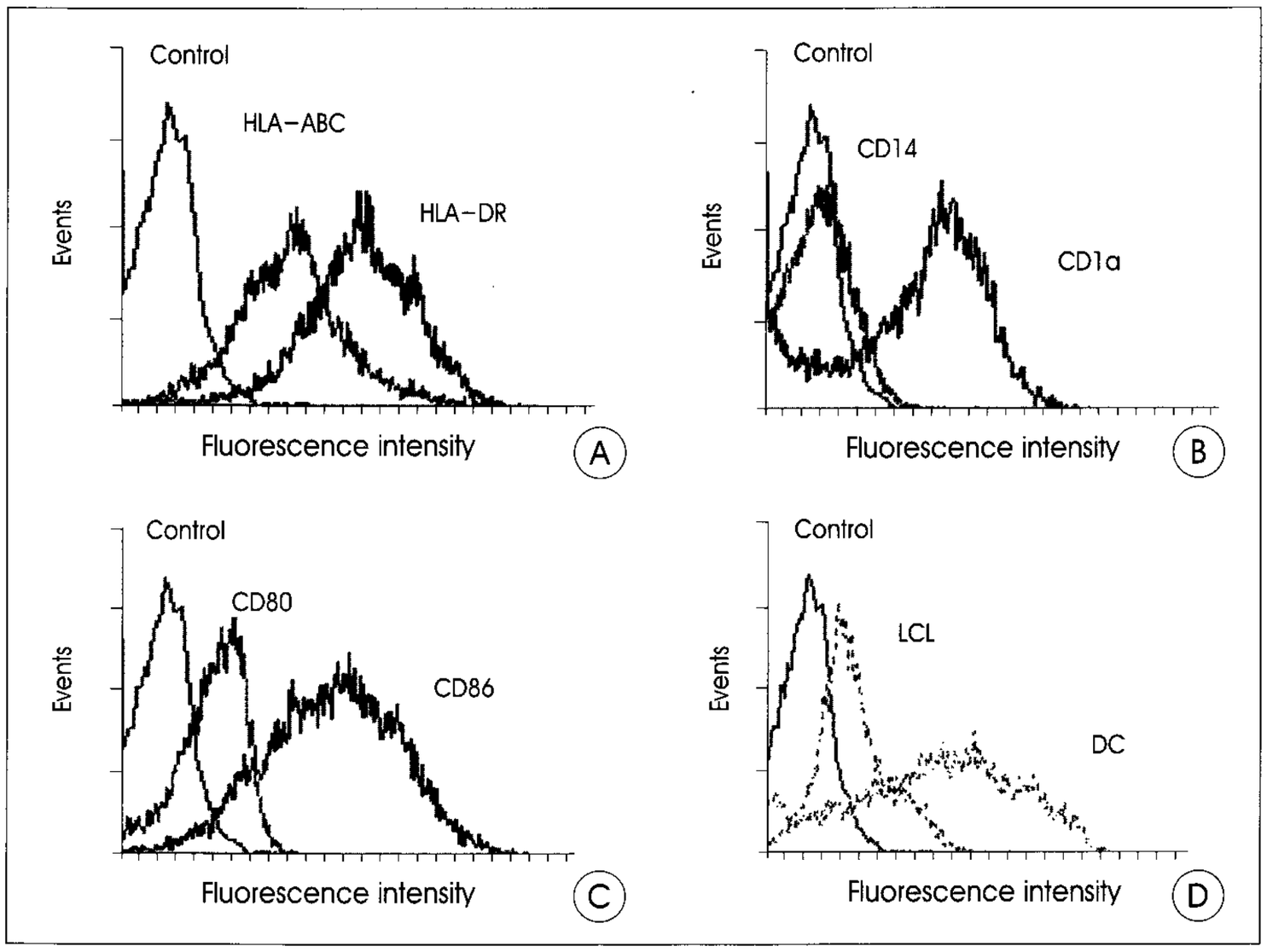

Fig. 1. Phenotypic analysis of surface markers of dendritic cells (A to C) and endocytotic activity of dendritic cells (D). Dendritic cells were derived from peripheral blood monocytes in media supplemented with GM-CSF(800ng/ml) and IL-4(1,000U/ml). Dendritic cells shows highly expressed major histocompatibility complex(MHC) class I, class II, CD1a, CD80, and CD86 molecules. They also have higher endocytotic activity than Epstein barr virus(EBV)-transformed B lymphoblastoid cell lines(LCLs).

IFN-γ production detected by ELISA

Level of IFN-γ was measured by ELISA in culture supernates from DCs, DCs/GABs and DCs/lysates. As shown in table 1, the intensity of IFN-γ production was higher in T cells stimulated by DCs/GABs and DCs/lysates than that in T cells stimulated by normal DCs(control). IFN-γ production of effector T cells stimulated by DCs/GABs was also significantly higher than that of T cells stimulated by DCs/lysates ($P<0.001$)(table 1).

Table 1. Production of interferon-γ by cytotoxic T cells of 3 groups determined by immunoassay

Group	Interferon-γ
Group A (DCs)	75.1±20 pg/ml
Group B (DCs/GABs)	12,798.4±1676 pg/ml
Group C (DCs/lysate)	2,317±320 pg/ml

DCs : dendritic cells, GAB : glioma apoptotic body

Cytotoxicities of induced tumor-specific T lymphocytes

Cytolytic activities of the generated cytotoxic $CD8^+$ T cells against T98G glioblastoma cells were determined by a standard 4hr [^{51}Cr]-release assay. Cross-primed CTLs with DCs/GABs or DCs/lysates were able to lyse T98G cells at E:T ratios of 40:1 and 20:1, but control CTLs were not as shown in Fig. 4 ($P<0.05$). Average percentage of T98G cell lysis of CTLs stimulated by DCs/GABs and DCs/lysates was 38.2% and 14.2% at an E:T cell ratio of 40:1, and 21.0% and 8.4% at an E:T ratio of 20:1 respectively. The average lysis of controls was 2.8% at an E:T cell ratio of 40:1 and 1.7% at an E:T ratio of 20:1. The average lysis of CTLs stimulated by DCs/GABs was higher than that of CTLs stimulated by DCs/lysates. The difference between % cytotoxicities of DCs/GABs group and DCs/lysates group was statistically significant at an E:T cell ratio of 40:1 as well as 20:1 ($P<0.05$).

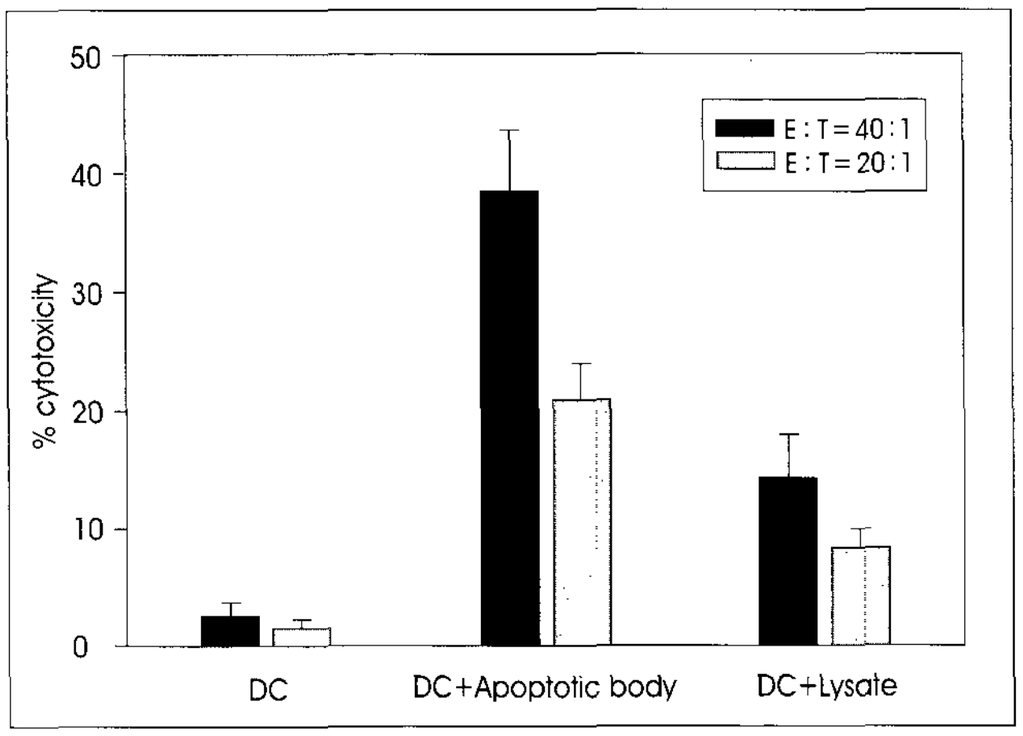

Fig. 4. Cytotoxicities of $CD8^+$ T cells stimulated by DCs, DCs/GABs, and DCs/lysates against T98G glioblastoma cells determined by a standard 4hr [^{51}Cr]-release assay. Cross-primed CTLs with DCs/GABs and DCs/lysates were able to lyse T98G cells, but control CTLs were not ($P<0.05$). The average lysis of DCs/GABs was significantly higher than that of DCs/lysates at E:T cell ratios of 40:1 as well as 20:1 ($P<0.05$).

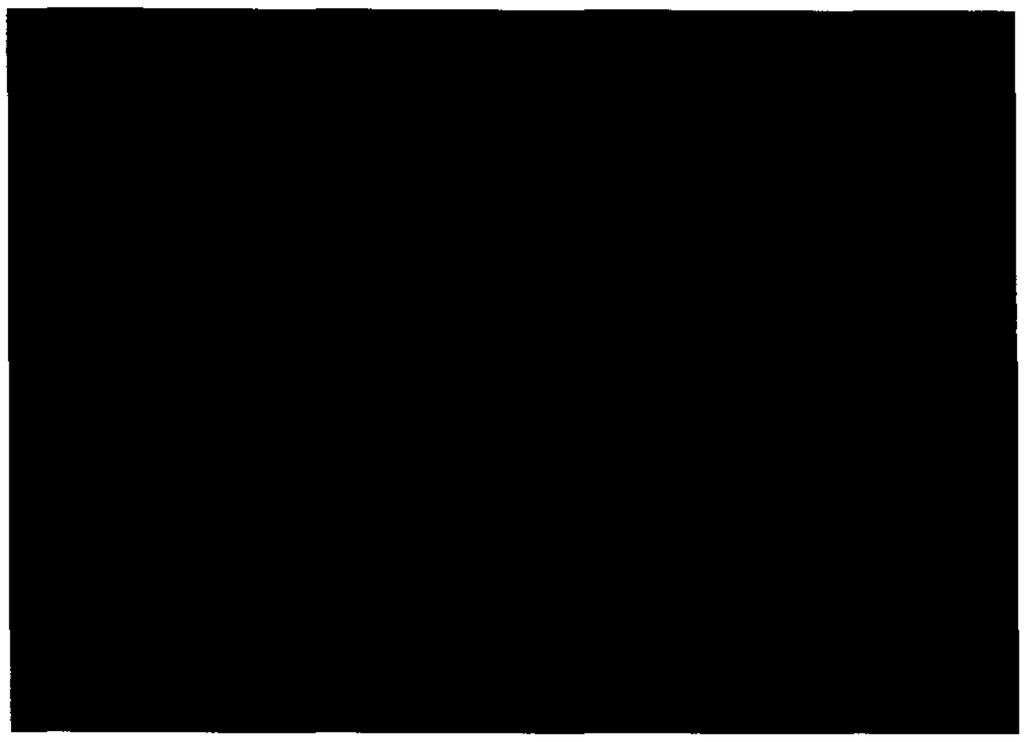

Fig. 2. Fluorescence microphotograph of Annexin V-fluorescein/propidium iodide-stained T98G glioblastoma cells after actinomycin D treatment. The cells that have green colored cytoplasm are apoptotic cells(X400).

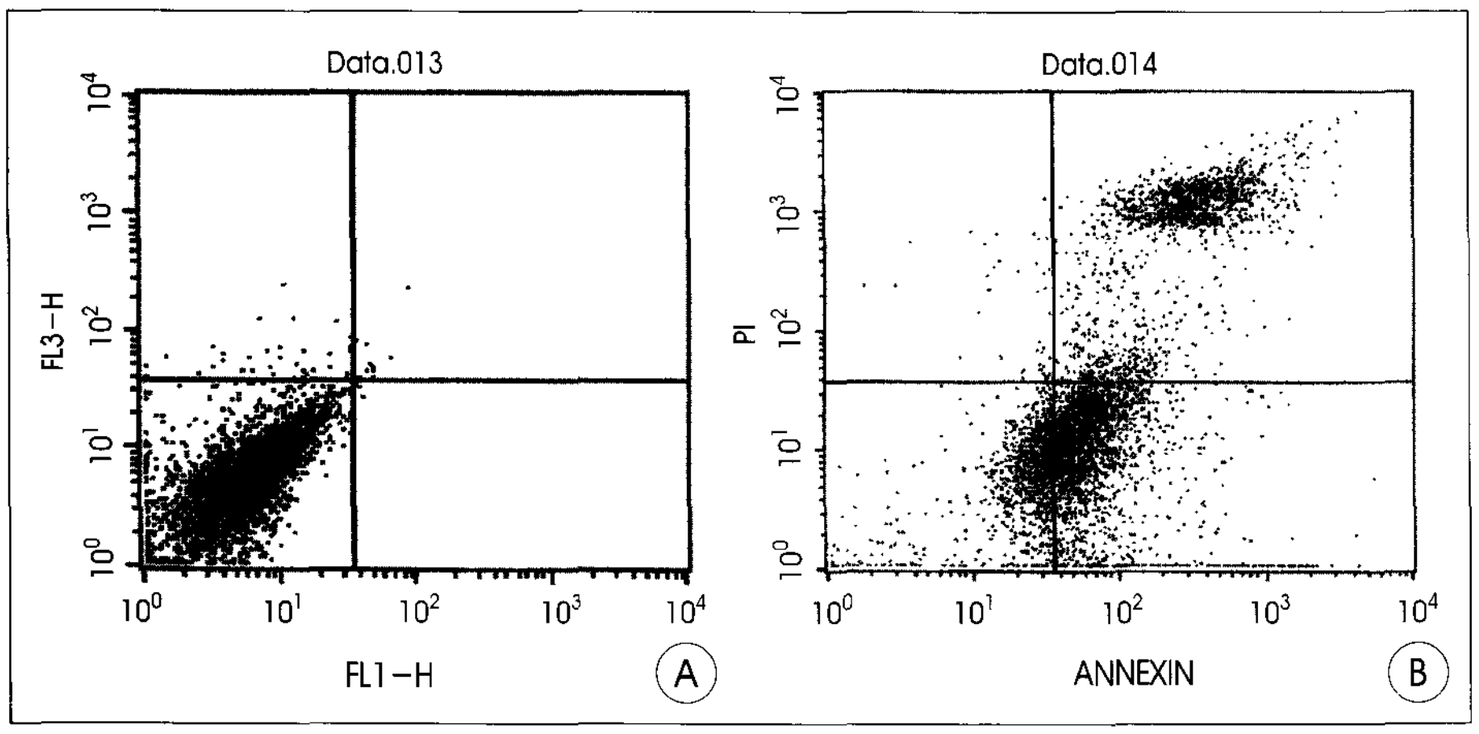

Fig. 3. Dot blot analysis of Annexin V-fluorescein/propidium iodide flow cytometry of T98G glioblastoma cells before(A) and after(B) treatment of $5\times10^{-4}\mu g$/ml actinomycin D for 18hr. The rate of cells undergoing apoptosis(lower right quadrant) was changed from 0.1% to 53% after actinomycin D treatment.

Discussion

DCs play a pivotal role in the initiation of T cell mediated immune responses[18] and can be obtained by culturing PBMCs in the presence of GM-CSF and IL-4[16]. A critical issue in optimizing DC-based anti-tumor vaccine is which tumor antigens for DC loading are effective to induce the CTL response. The vaccination against a single antigen can induce tumor-

specific CTLs but may give rise to promote tumor antigen escape variants[19]. Actually the generation of CTLs against three or more tumor antigens correlates with clinical response[3]. Antigen loading can be performed by pulsing DCs with synthetic immunodominant peptides from identified tumor antigens for prostate cancer[20], carcinoembryonic antigen expressing tumors[12], and malignant melanoma[19]. But this approach has some disadvantages including the unavailability of peptides for all HLA subtypes, the lack of CD4 helper T cell-related epitopes for most antigens, and the uncertainty regarding the maintenance of antigen expression[2]. So unfractionated tumor-derived antigens such as tumor cell lysates or apoptotic bodies can be the alternative to tumor specific antigens especially in DC-based vaccine strategies against malignant glioma that has not been found well characterized tumor specific antigens. These tumor-derived materials contain multiple known and unknown glioma associated antigens that can be presented to T cells. DCs stimulated by these antigens are, therefore, more likely to induce a polyclonal expansion of T cells. One possible drawback of this approach is the potentially low concentration of effective tumor antigens in the mixture. Antigenic tumor peptides may conceivably be diluted by relatively non-antigenic proteins, which can downregulate the effectiveness of the antitumor immune response.

In this study we investigated tumor specific T cell responses induced by DCs pulsed with different antigen preparations, glioma cell lysates and apoptotic bodies, from glioblastoma cell line T98G. This study showed DCs pulsed with GABs were more potent than those with glioma cell lysates to induce T cell priming and activation. This was evidenced by enhanced secretion of IFN-γ, Th1 cytokine, which is related with cytotoxic activity[7], as well as a higher rate of MHC class I-restricted tumor cell lysis. This result is in accord with recent studies demonstrated that DCs which had ingested apoptotic cancer cells were more effective in generating $CD8^+$ CTLs than DCs pulsed with tumor cell lysates in squamous cell carcinoma of the head and neck[6], malignant melanoma[9], and pancreatic tumor[17]. Enhanced CTL activation by antigens from apoptotic cells may be attributed to several mechanisms. After phagocytosis, DCs digest the cellular fragments of the apoptotic cells into peptides and efficiently present to helper T cells in the MHC class-II pathway[8]. This is believed to be same as the processing pathway of cell lysates in DCs. But phagocytosis of apoptotic tumor cells by DCs allows antigens to gain access to MHC class-I molecules, resulting in cross-presentation of the antigens to CTLs[1]. Exogenous antigens from apoptotic tumor cells endocytosed by DCs are just presented on major histocompatibility complex(MHC) class I molecules as well as on class II molecules, a process called cross-presentation. This cross-presentation might be mediated by heat shock proteins(HSPs) expressed by stress-induced apoptotic tumor cells[5]. Induction of HSPs expression has been shown to provide the danger signals required for generation of a more effective immune response and to improve uptake of antigens by DCs[21]. Moreover, high dose antigens are preferentially presented by the cross-presenting APCs and destruction of cells such as induction of apoptosis leads to enhanced access of antigens to the cross-presentation pathway[11]. On the basis of this theoretical background and our own observations, we concluded that antigen preparations from GABs present a promising alternative to glioma cell lysates in DC-based glioma vaccines.

Conclusion

We compared tumor-specific T cell response induced by GAB-pulsed DCs to that by glioma cell lysate-pulsed ones in vitro. This study showed DCs pulsed with GABs were more potent than glioma cell lysates in inducing cytotoxic T cell priming and activation. These results indicate the choice of antigen is a critical determinant in the induction of antitumor immunity against malignant gliomas. Antigen preparations from GABs represent a promising alternative to glioma cell lysate in DC-based glioma vaccines.

• Acknowledgement
The authors wish to acknowledge the financial support of the Catholic Medical Center Research Foundation made in the program year of 2002.

References

1. Albert ML, Sauter B, Bhardwaj N : Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. **Nature 392** : 86-89, 1998
2. Amoscato AA, Prenovitz DA, Lotze MT : Rapid extracellular degradation of synthetic class I peptides by human dendritic cells. **J Immunol 161** : 4023-4032, 1998
3. Banchereau J, Palucka AK, Dhodapkar M, Burkeholder S, Taquet N, Rolland A, et al : Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. **Cancer Res 61** : 6451-6458, 2001
4. Banchereau J, Steinman RM : Dendritic cells and the control of immunity. **Nature 392** : 245-252, 1998
5. Feng H, Zeng Y, Whitesell L, Katsanis E : Stressed apoptotic tumor cells express heat shock proteins and elicit tumor-specific immunity. **Blood 97** : 3505-3512, 2001
6. Hoffmann TK, Meidenbauer N, Dworachi G, Kanaya H, Whiteside TL : Generation of tumor-specific T lymphocytes by cross-priming with human dendritic cells ingesting apoptotic tumor cells. **Cancer Res 60** : 3542-3549, 2000
7. Hunter CA, Reiner SL : Cytokines and T cells in host defense. **Curr Opin Immunol 12** : 413-418, 2000
8. Inaba K, Turley S, Yamaide F, Iyoda T, Mahnke K, Inaba M, et al : Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products of dendritic cells. **J Exp Med 188** : 2163-2173, 1998
9. Jenne L, Arrighi JF, Jonuleit H, Saurat JH, Hauser C : Dendritic cells containing apoptotic melanoma cells prime human $CD8^+$ T cells for efficient tumor cell lysis. **Cancer Res 60** : 4446-4452, 2000
10. Kim IS, Kim JT, Cho HI, Park YS, Kim MC, Chung DS : Induction

of cellular immune response by dendritic cells pulsed with glioma apoptotic bodies. **J Korean Neurosurg Soc 34** : 360-365, 2003
11. Kurts C, Miller JFAP, Subramanium R, Carbone FR, Heath WR : Major histocompatibility complex class I-restricted cross-presentation is biased towards hight dose antigens and those released during cellular destruction. **J Exp Med 188** : 409-414, 1998
12. Morse MA, Deng Y, Coleman D, Hull S, Kitrell-Fisher E, Nair S, et al : A phase I study of active immunotherapy with carcinoembryonic antigen peptide (CAP-1)-pulsed, autologous human cultured dendritic cells in patients with metastatic malignancies expressing carcinoembryonic antigen. **Clin Cancer Res 5** : 1331-1228, 1999
13. Liau LM, Black KL, Prins RM, Sykes SN, DiPatre PL, Cloughesy TF, et al. : Treatment of intracranial gliomas with bone marrow-derived dendritic cells pulsed with tumor antigens. **J Neurosurg 90** : 1115-1124, 1999
14. Parajuli P, Mathupala S, Sloan AE : Systemic comparison of dendritic cell-based immunotherapeutic strategies for malignant gliomas : in vitro induction of cytolytic and natural killer-like T cells. **Neurosurgery 55** : 1194-1204, 2004
15. Rubin J, Lotze M : Adoptive cellular immunotherapy of cancer in Mitchel M (ed) : **Biological approaches to cancer treatment : biomodulation**, New York, McGraw-Hill, Inc., 1993, pp379-410
16. Sallusto F, Nicolo C, De Maria R, Corinti S, Testi R : Ceramide inhibits antigen uptake and presentation by dendritic cells. **J Exp Med 184** : 2411-2416, 1996
17. Schnurr M, Scholz C, Rothenfusser S, Galambos P, Dauer M, Robe J, et al : Apoptotic pancreatic tumor cells are superior to cell lysates in promoting cross-priming of cytotoxic T cells and activate NK and gammadelta T cells. **Cancer Res 62** : 2347-2352, 2002
18. Steinman RM : The dendritic cell system and its role in immunogenicity [Review]. **Annu Rev Immunol 9** : 271-296, 1991
19. Thurner B, Haendle I, Roder C, Dieckmann D, Keikavoussi P, Jonuleit H, et al : Vaccination with mage-3A1 peptide-pulsed mature, monocyte-derived dendritic cells expands specific cytotoxic T cells and induces regression of some metastases in advanced stage IV melanoma. **J Exp Med 190** : 1669-1678, 1999
20. Tjoa BA : Evaluation of phase I/II clinical trials in prostate cancer with dendritic cells and PSMA peptides. **Prostate 36** : 39-44, 1998
21. Todryk S, Melcher AA, Hardwick N, Linardkis E, Bateman A, Colombo MP, et al : Heat shock protein 70 induced during tumor cell killing induces Th1 cytokines and targets immature dendritic cell precursors to enhance antigen uptake. **J Immunol 163** : 1398-1408, 1999
22. Yoshida S, Morii K, Watanabe M, Saito T, Yamamoto K, Tanaka R : The generation of anti-tumoral cells using dendritic cells from the peripheral blood of patients with malignant brain tumors. **Cancer Immunol Immunother 50** : 321-327, 2001
23. Yu JS, Wheeler CJ, Zeltzer PM, Ying H, Finger DN, Lee PK, et al : Vaccination of malignant glioma patients with peptide-pulsed dendritic cells elicits systemic cytotoxicity and intracranial T-cell infiltration. **Cancer Res 61** : 842-847, 2001