의학석사 학위논문

사람세포거대바이러스에 감염된 뇌신경종양세포에 의한 IE-1특이 세포독성 T림프구의 기능 감소

Human cytomegalovirus (HCMV)-infected astrocytoma cells decrease the function of HCMV IE-1-specific cytotoxic T lymphocytes

2015년 1월

서울대학교 대학원

협동과정 종양생물학전공

김 지 연

A thesis of the Master's degree

사람세포거대바이러스에 감염된 뇌신경종양세포에 의한 IE-1 특이 세포독성 T림프구의 기능 감소

Human cytomegalovirus (HCMV)-infected astrocytoma cells decrease the function of HCMV IE-1-specific cytotoxic T lymphocytes.

January, 2015

Interdisciplinary Program in Cancer Biology,
Seoul National University College of Medicine
Graduate School

Jiyeon Kim

사람세포거대바이러스에 감염된 뇌신경종양세포에 의한 IE-1 특이 세포독성 T 림프구의 기능 감소

지도교수 이 원 우

이 논문을 의학석사 학위논문으로 제출함

2014년 10월

서울대학교 대학원

협동과정 종양생물학 전공

김 지 연

김 지 연의 의학석사 학위논문을 인준함

2015 년 1 월

위 원 장 ____ (인)

부위원장 ____ (인)

위 원 ____ (인)

Human cytomegalovirus (HCMV)-infected astrocytoma cells decrease the function of HCMV IE-1-specific cytotoxic T lymphocytes

by

Jiyeon Kim

(Supervised by Prof. Won-Woo Lee)

A thesis submitted to Interdisciplinary Program in Cancer Biology in partial fulfillment of the requirement of the Degree of Master of Interdisciplinary Program in Cancer Biology at Seoul National University College of Medicine

Jan, 2015

Approved by Thesis Committees:

Professor Eung Soo Hwang Chairman

Professor Dong Le Vice Chairman

Professor

ABSTRACT

Jiyeon Kim

Department of Cancer Research Institute
The Graduate School
Seoul National University College of Medicine

Background Glioblastoma multiforme (GBM) is a rapid growing and invading tumor of glial origin with a poor prognosis. The level of human cytomegalovirus (HCMV) infection in GBM correlates with a prognosis and HCMV may involve the pathogenesis of GMB. Here I sought to verify a role of HCMV-infected astrocytoma cells on impairing the activity of cytotoxic T lymphocytes (CTLs) specific to HCMV immediate early (IE)-1.

Methods CTLs specific to HCMV IE-1 were prepared by the stimulation of the purified CD8+ cells from donors with U373MG expressing HCMV IE-1. Death rate of the target cells was determined by the total counting of the remaining target cells after

the interaction of the effector and the fluorescent dye-tagged target cells. Death rate of the effector cells was assayed by Annexi n V or TUNEL staining.

Results Cell death rate of the target cells by CTLs increased HLA-restrictedly and depending on effector:target(E:T) ratio. The death rate of effector cells in the culture of HCMV-infected U373MG were 37.1% at day 4 postinfection, and $4.5x10^4$ PFUs/ml were detected at that time. Removal of the culture supernatant from HCMV-infected U373MG at day 4 postinfection before adding effector cells enhanced the target death from 12.3% to 39.1% at E:T=1:1, but not at E:T=3:1. When the effector cells from 24-hour co-cultured HCMV-infected U373MG with CTLs were transferred to U373MG expressing HCMV IE-1 and cultured for another 24 hours, cell death rate of the target cells decreased from 31.5% to 14.3% at E:T=1:1, but increased from 41.5% to 65.3% at E:T=3:1.

Conclusion Productively infected U373MG with HCMV decreases the activity of CTLs specific to HCMV in case of the low number of the effector cells. These results suggest that HCMV could impair CTL activity and help glioblastoma unchecked by CTLs.

Key words: glioblastoma multiforme, human cytomegalovirus, cytotoxic T lymphocyte, astrocytoma cell, immediate early protein-1

Student ID:2013-21749

LIST OF FIGURES

Fig.1. FACS profiles of HLA expression on cells..... 13

Fig.2. Identification of HCMV IE-1-specific cytotoxic T lymphocytes..... 16

Fig.3. Preparation of HCMV IE-1-specific cytotoxic T lymphocytes from CD8+ T lymphocytes..... 17

Fig.4. Cell death of the effector cells by HCMV-infected U373MG..... 24

Fig.5. Changes of the expression of MHC I on U373MG infected by HCMV..... 28

Fig.6. Cell death of HCMV-infected U373MG by CTLs..... 31

Fig.7. Schematic presentation of the reactivity or the impairment of CTLs specific to HCMV by HCMV-infected astrocytoma cells according to the microenvironment of HCMV infection..... 35

LIST OF ABBREBATION AND SYMBOL

BCA: Bicinchoninic Acid

BSA: Bovine serum albumin

CPE: Cytopathic effect

CTL: Cytotoxic T cell

DMEM: Dulbecco's modified Eagles medium

FACS: Flow cytometry

FBS: Fetal bovine serum

FITC: Fluorescein isothiocyanate

GBM: Glioblastoma multiforme

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

HBSS: Hank's balanced salt solution

HCMV: Human cytomegalovirus

HRP: Horseradish Peroxidase

IE: Immediate early

IFN-γ : Interferon gamma

IgG: Immunoglobulin G

kDa: Kilo dalton

MFI: Mean flouroscene intensity

MHC: Major histocompatibility complex

m.o.i: Multiplicity of infection

NK cell: Natural killer cell

PBMC: Peripheral blood mononuclear cell

PBS: Phosphate-buffered saline

PBST: PBS-Tween 20

PCR: Polymerase chain reaction

PE: Phycoerythrin

PFU: Plaque forming unit

PVDF: Polyvinylidene fluoride

RT: Room temperature

SEM: Standard errors of the mean

TUNEL: Terminal deoxynucleotidyl transferase-dUTP nick end labeling

CONTENTS

ABSTRACT i

LIST OF FIGURES iv

LIST OF ABBREBATION AND SYMBOL v

CONTENTS vii

INTRODUCTION 1

MATERIALS AND METHODS 5

RESULTS 12

1. Characteristics of cells used in experiments

2. Preparation of HCMV IE-1-specific cytotoxic T lymphocytes

3. Effector cell death induced by HCMV-infected U373MG

4. Changes of cell surface MHC I antigen by HCMV infection

5. Cell death of HCMV-infected U373MG by CTLs

DISCUSSION 37

REFERENCES 42

ABSTRACT IN KOREAN 46

INTRODUCTION

The primary brain tumor, glioblastoma multiforme (GBM), is a rapid growing and invading tumor of glial origin with a poor prognosis (1). The mean survival time of patients with GBM is approximately one year, while that with anaplastic astrocytoma is two to three years (2). Many determinants have been suggested as causative factors to the development of GBM, but the exact mechanism of the tumorigenesis is not known.

There are three types of neuroglial cells in the central nervous system: astrocytes, oligodendrocytes, and microglial cells. Astrocytes, which are present in the brain and spinal cord, have local processes with a starlike appearance. The gliomas are graded from grade I to IV based on their pathology. Low-grade gliomas are well-differentiated and exhibit benign tendencies with a better prognosis for the patient. High-grade gliomas are undifferentiated or anaplastic and are malignant with a worse prognosis.

The level of human cytomegalovirus (HCMV) infection in GBM correlates with a prognosis and HCMV may involve the pathogenesis of GMB.

Some research teams have reported a potential correlation between

GBM and HCMV. Some findings support these correlations. HCMV sequences and proteins have been found in most GBMs but not healthy brain (3). Clinical trial of immunotherapy of dendritic cells pulsed with autologous glioblastoma lysate revealed the generation of cytotoxic T lymphocytes(CTLs) specific to HCMV pp65 (4). It has big hurdles for any pathogen or immune component to enter central nervous system. Brain has been known as one of the immunologically privileged sites and tolerates the antigenic challenges without any immune response (5). It has the very tight barrier structure known as blood-brain-barrier.

But the recent clinical trials with antibodies (6) and the experimental animal observations such as experimental autoimmune encephalitis (7) can explain the possibilities of the penetration of immune components to brain.

HCMV, a member of the *Herpesviridae*, is a double stranded DNA virus with genome size of 230 kb, which encodes over 200 proteins. During productive infection the replication cycle of HCMV is characterized by the sequential expression of immediate early (IE), early (E), and late (L) gene regions. Especially IE gene can be expressed prior to the synthesis of the viral protein after the viral infection. After the expression of IE gene products the E and L genes are successfully produced. HCMV infection is common and the seropositivity of HCMV is 50 - 100% world-widely (8).

HCMV proteins are immunodominant antigens and evoke the strong immune responses in human after HCMV infection (9). Around 10% of peripheral CD8+ memory T lymphocytes are reactive to HCMV proteins. The number of CD8+ T lymphocytes reactive to HCMV proteins increases with aging, and comprises over 45% of total CD8+ T lymphocytes (10).

HCMV has neurotrophic properties. Fetus can be infected with HCMV through transplacental transmission from mother, and reveal the damages in brain such as microcephaly, deafness and mental retardation. Recent study of the congenital infection with mouse cytomegalovirus (MCMV) in animal model broadened the understanding of CNS infection (11). The recruitment of immune cells and the expression of pro-inflammatory cytokines are involved in brain impairment.

The perivascular infiltration of mononuclear cells was found in GBM and is correlated with the prognosis (12). HCMV IE-1 was also detected in 93-100% of GBM (3).

In these aspect of the correlation of viral infection and tumor expansion I prepared the CTLs specific to HCMV IE-1, and found that they could lyse HCMV-infected astrocytoma cells with the dose-dependency, but be not functioning in case of the presence of small number of the effector cells.

Here I sought to verify a role of HCMV-infected astrocytoma

cells on impairing the activity of CTLs specific to HCMV IE-1.

MATERIALS AND METHODS

Cells and virus

U373MG cells constitutively expressing HCMV IE-1, UMG1-2 (13), were used as the stimulating cells and the target cells for CTL to HCMV IE-1. U373MG transfected with empty LNCX vector were used as control cells. (14) Cells were cultured in Dulbecco's modified Eagles medium (GIBCO, Grand Island, NY , U.S.A.) with 10% fetal bovine serum (FBS, GIBCO), 100 U/mL penicillin and 100 U/mL streptomycin (Life Technologies, Carlsbad, CA, U.S.A.) in a 37℃ incubator with 5% CO_2. HCMV Towne strain (ATCC VR-977) was used to infect cells at a multiplicity of infection of 4.

The experimental protocol with human materials was reviewed and approved by Seoul National University Hospital Institutional Review Board (C-1306-021-494). Human peripheral blood was collected from healthy donors after voluntary consent. Peripheral blood mononuclear cells (PBMCs) were collected at the interface on Ficoll-Paque (GE Healthcare, Little Chalfront, United Kingdom) after centrifugation at 700 g for 30 minutes. CD8+ T lymphocytes were isolated with CD8+ T cell isolation kit (Miltenyi Biotech,

Surrey, UK) and MACS LS column (Miltenyi Biotech) according to the manufacturer's manual. PBMCs and CD8+ T lymphocytes were cultured in RPMI1640 (Thermo Scientific, Waltham, MA, U.S.A) containing 10% FBS in 5% CO_2 atmosphere at 37° C.

Antibodies and reagents

Monoclonal anti-HCMV IE-1 antibody (clone 6IE1)(gift from E-S Huang) and FITC-conjugated goat anti-mouse IgG antibody (Life Technologies) were used to detect HCMV IE-1 antigen. Phycoerythrin (PE)-conjugated anti-HLA-A*0201 antibody (BD Biosciences, San Jose, CA, U.S.A) was used to detect the expression of HLA antigen on the cells.

HCMV IE-1 overlapping peptides with 11 amino acids from IE-1 protein of HCMV AD169 (Miltenyi Biotech) were used to generate CTLs specific to HCMV IE-1. All reagents were purchased from Sigma Chemical (St. Louise, MO, USA) unless otherwise stated.

Preparation of CTLs specific to HCMV IE-1

PBMCs or CD8+ T lymphocytes from donors were added onto

cultured UMG1-2 cells, cultured for 48 hours with the intermittent gentle shaking. Cells in the supernatant were harvested, washed with media containing 10% FBS, and used as HCMV IE-1-specific CTLs.

Flow cytometry analysis

To analyze the surface expression of MHC I, HLA-A*0201, on cells, cells were collected, washed in FACS buffer (PBS with 0.5% BSA, 0.01% NaN), and reacted with PE-conjugated anti-HLA-A*0201 antibody (BD) on ice for 30 min. After washing with FACS buffer, fluorescent signals were analyzed with FACS LSR (BD). In case of the detection of HCMV IE-1, the collected cells were permeabilized with 70% ethanol on ice for 30 min, and reacted with anti-HCMV IE-1 antibody for 30 min, followed by FITC-conjugated with goat anti-mouse IgG antibody for 30 min.

Western blot analysis

Western blot analysis of HCMV IE-1 was performed at different time points after HCMV infection. Protein concentration in cell

lysates was determined with BCA protein assay kit (Thermo) as described in the manufacturer's instruction manual. Samples with 100 μg/lane were subjected on a denaturing SDS 10% polyacrylamide gel under reducing conditions by electrophoresis. Separated proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF; Millipore) membranes. After the appropriately diluted anti-HCMV IE-1 antibody was reacted for 24 hours at 4℃, horseradish peroxidase(HRP)-conjugated goat anti-mouse IgG antibody (Calbiochem) was used as a secondary antibody. Signal was developed using an enhanced chemiluminescence reagent (Thermo) as described in the manufacturer's instruction manual, and visualization was achieved by LAS-4000(Fujifilm).

Assays for cytotoxic activity

The cytotoxic activity of CTLs was assayed by a viable cell counting of target cells. Target cells pre-stained with cell proliferation dye 670 (eBioscience, San Diego, U.S.A) were incubated with effector cells for 24 hours, harvested and analyzed with flow cytometry. The total number of target cells without the effector cells was used as a control. Cell death rate was calculated

with the formula of (number of control − number of target cells after the incubation with effectors)/number of control X 100. All experiments were repeated three times independently and the result was expressed as mean + standard error.

TUNEL assay

Cells undergoing death was determined by flow cytometry using terminal deoxynucleotidyl transferase(TdT)−dUTP nick end labeling (TUNEL) staining methods using APO−BrdU™ Kit (Millipore, Darmstadt, Germany) according to the manufacturer's instruction. Approximately $1x10^6$ cells were fixed with 70% ethanol and stored overnight at −20° C. The cells were washed twice with washing buffer, followed by incubation for at least 1 hour with staining solution at room temperature. Staining solution contained 1 mg/mL RNase A and TdT reaction reagent and antibody solution containing BrdU−fluorescein isothiocyanate (FITC). DNA content or DNA fragmentation was measured in cells with a FACS Fortessa (BD). Data of cell cycle were analyzed using FlowJo cytometry analysis software (Tree star, Ashland, OR, USA). The cell death experiments were repeated three times and the results were expressed as mean + standard error.

Quantitation of HCMV

The copy number of HCMV was assayed by real-time polymerase chain reaction (PCR). Sample DNA was prepared with Media kit (Qiagen, Valencia, CA, USA) from culture supernatant according to the manufacturer's recommendation. Real-time PCR reactions were performed using a Taqman MasterMix (Applied Biosystems, Foster City, CA, USA) with 10 pmol HCMV US17 primer pairs, (5'- GCGT GCTTTTTAGCCTCTGCA-3') and (5'-AAAAGTTTGTGCCCCAA CGGTA-3'), 10 pmol probe, FAM-5'-TGATCGGCGTTATCG CGTTCTTGATC-3'-TAMRA, and sample DNA in a 20㎕ reaction, and run on an ABI PRISM 7900 sequence detection system (Applied Biosystems). Primers and Taqman probes were synthesized commercially at Bioneer (Daejeon, Korea). Reactions were performed under standard universal reaction conditions; hot start cycle of 10 minutes at 95° C, and 40 cycles of denaturation for 10 seconds at 95° C and annealing and extension for 60 seconds at 60° C.

Statistical analysis

Statistical analysis was performed using the Chi-square test or

Fisher's exact test where appropriate. A level of $p< 0.05$ was accepted as statistically significant.

RESULTS

1. Characteristics of cells used in experiments

HLA expression on the cells was determined by FACS analysis. U373MG, UMG 1-2 and PBMCs from three donors, Donor A1, A2 and A3, expressed HLA-A*0201, but PBMCs from another three donors, Donor C1, C2 and C3, did not (Figure 1).

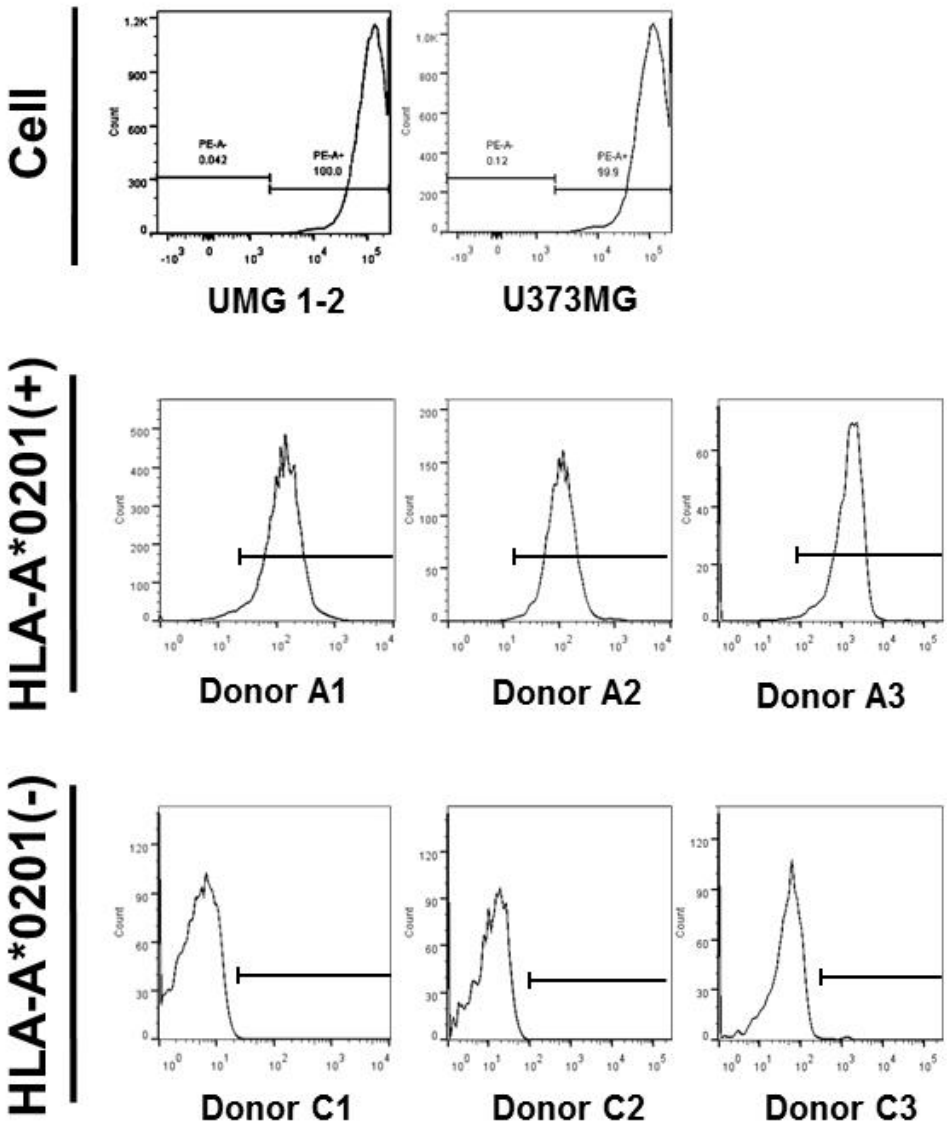


Figure 1. FACS profiles of HLA expression on cells. U373MG, UMG1-2 and PBMCs from Donor A1, A2, and A3 expressed HLA-A*0201, but PBMCs from donors, C1, C2 and C3, did not.

2. Preparation of HCMV IE-1-specific cytotoxic T lymphocytes

I screened the presence of HCMV IE-1-specific CTL clones in PBMCs of donors. When PBMCs from HLA-A*0201(+) Donor A1 were stimulated with HCMV IE-1 overlapping peptides CD8+ T lymphocytes secreting IFN-γ were detected with 1.99%, but not without the stimulation with HCMV IE-1 overlapping peptides. These results mean the presence of HCMV IE-1-specific CTLs in donor A1 (Figure 2 (A)).

For a better production of HCMV IE-1-specific CTLs CD8+ T lymphocytes were purified from whole PBMCs and the cell line UMG1-2, which expresses HCMV IE-1 constitutively, was used as a stimulator. The purity of CD8+ T lymphocytes were 71.8% (Figure 2(B)). Expression of HCMV IE-1 was detected in UMG1-2, but not in U373MG as a negative control cell (Figure 3 (A)). Expression of HCMV IE-1 in U373MG after HCMV infection was determined by western blot analysis and FACS. HCMV IE-1 increased in U373MG infected with HCMV from day 1, reached at maximum in 2 to 3 days postinfection, and declined thereafter in both analytical methods. U373MG used as a negative control cell. CTLs were generated by the stimulation of purified CD8+ T lymphocytes from HLA-A*0201(+) donor with UMG1-2 for 2 days. Increased morphological changes in UMG1-2 as compared in

U373MG were observed under microscope when each cell was co-cultured with the increasing numbers of CTLs (Figure 3 (B)). Cell death rate of UMG1-2 was 19.0% at E:T=1:1, and 73.9% at E:T=3:1 after the incubation of the target cells, UMG1-2, with the increasing number of effector cells generated by the stimulation of the purified CD8+ T lymphocytes from HLA-A*0201-positive donor with UMG1-2 (Figure 3(C)). In case of effector cells from HLA-A*0201-negative donor cell the death rate of UMG1-2 was 4.6% and 22.4% at E:T=1:1 and 3:1, respectively. When the target cell was U373MG cell death rate was below 15.3% in all the assays (Figure 3 (C)).

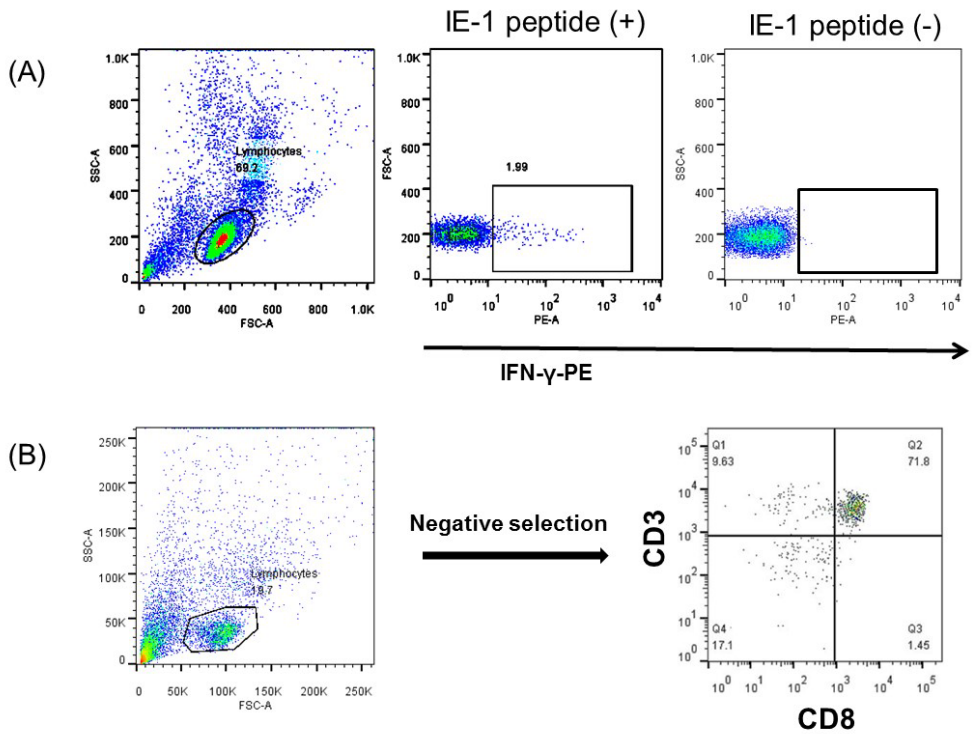
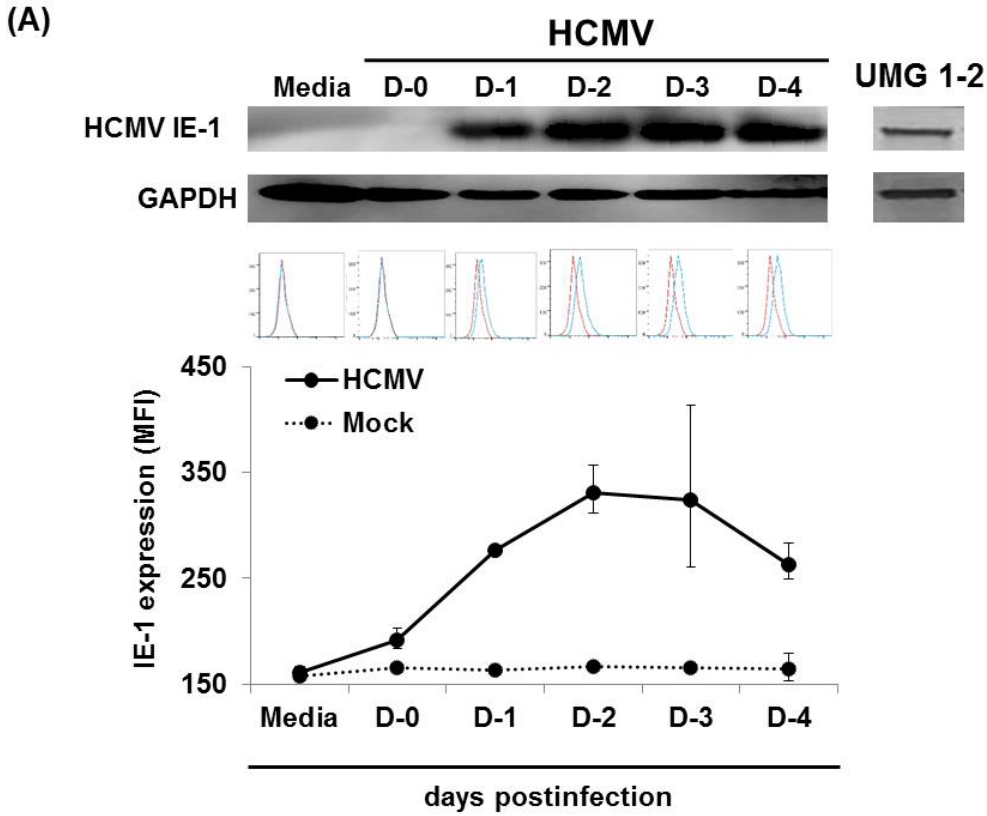


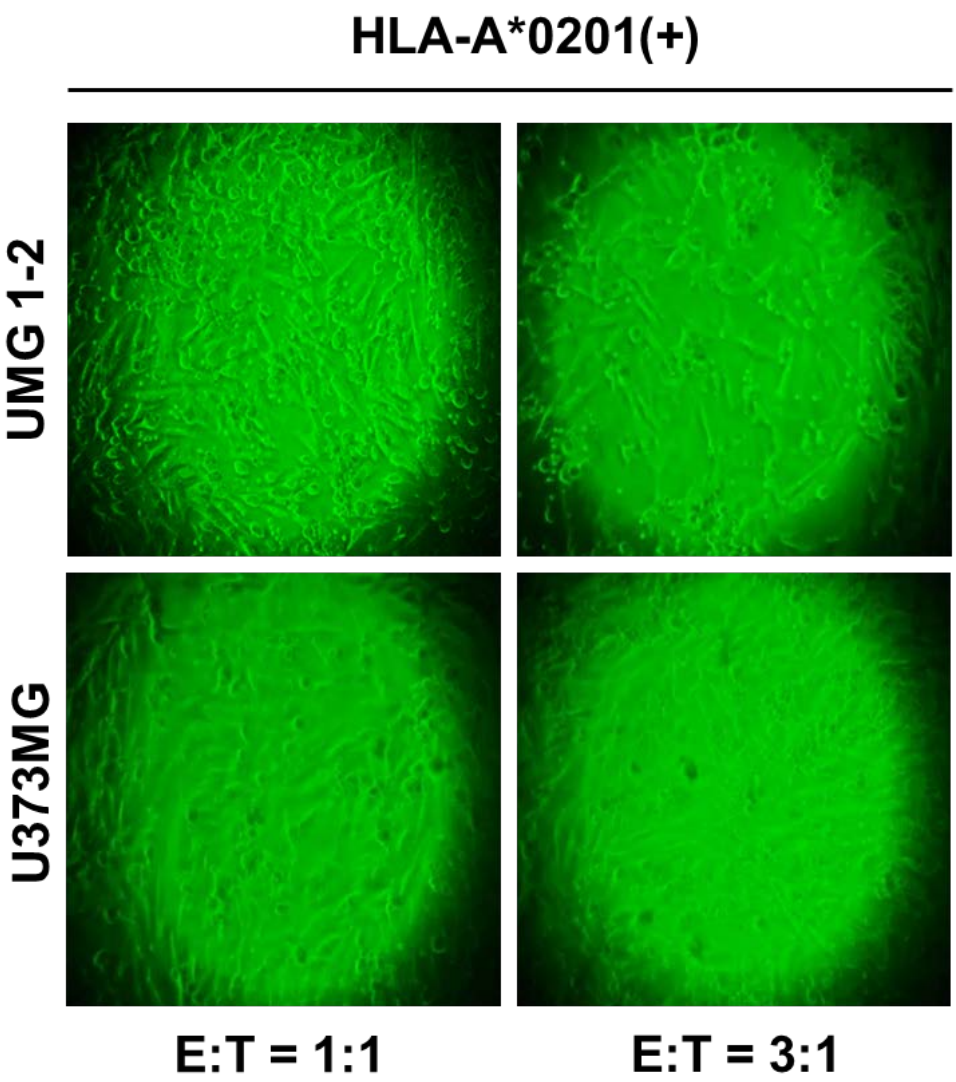
Figure 2. Identification of HCMV IE-1-specific cytotoxic T lymphocytes.

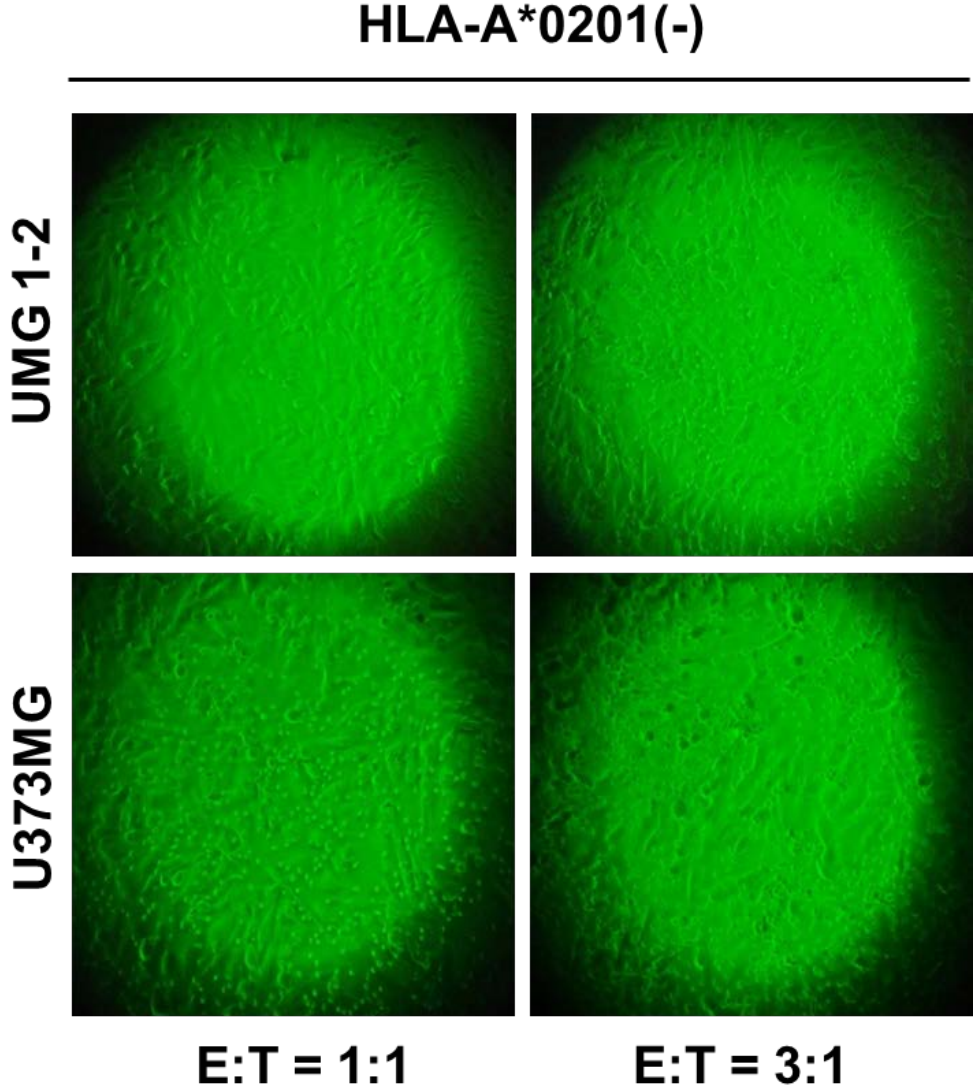
(A) FACS profiles of IFN-γ expression in PBMCs of HLA-A*0201-positive donor stimulated with HCMV IE-1 overlapping peptides. Cell population with IFN-γ expression was found only in the stimulation with HCMV IE-1 overlapping peptides.

(B) Representative demonstration of FACS analysis of purified CD8+ T lymphocytes from PBMCs of HLA-A*0201-positive donor.


(A)
HCMV
Media
D-0
D-1
D-2
D-3
D-4
UMG 1-2
HCMV IE-1
GAPDH
HCMV
Mock
IE-1 expression (MFI)
450
350
250
150
Media
D-0
D-1
D-2
D-3
D-4
days postinfection

(B)




HLA-A*0201(-)
UMG 1-2
U373MG
E:T = 1:1
E:T = 3:1

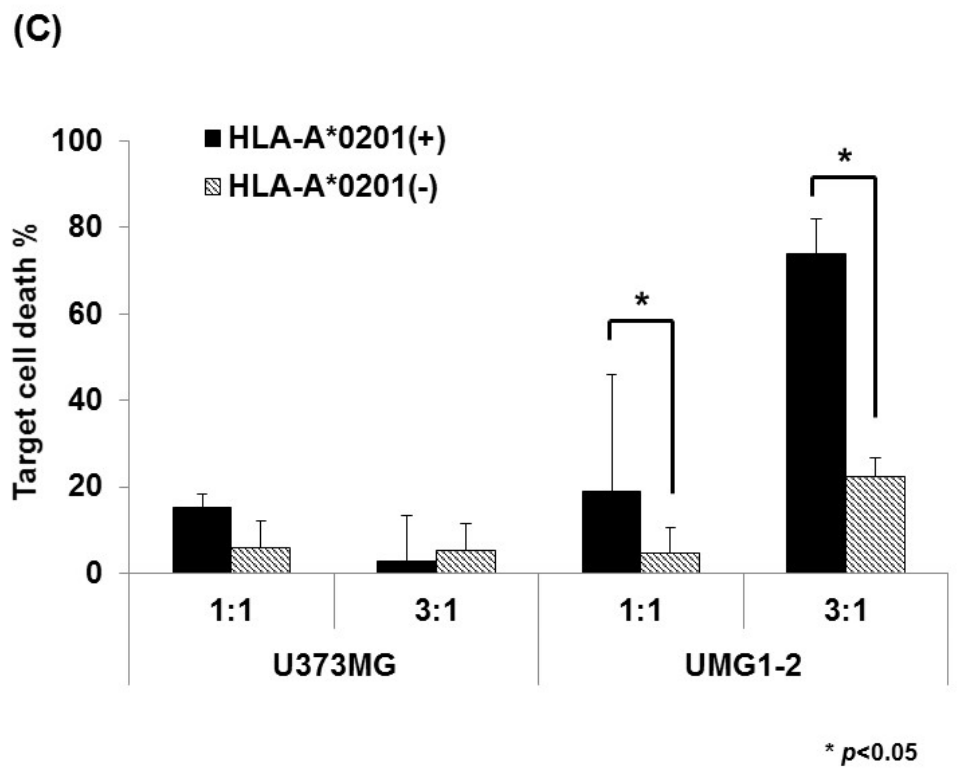


Figure 3. Preparation of HCMV IE-1-specific cytotoxic T lymphocytes from CD8+ T lymphocytes

(A) Expression profile of HCMV IE-1 determined by western blot analysis and FACS. HCMV IE-1 was detected in U373MG infected with HCMV in the indicated time points. UMG1-2 used as a positive control cell.

(B) Representative photographs of UMG1-2 and U373MG cells co-

cultured with CTLs. The morphological changes increased in UMG1-2 by the adding of the increasing number of CTLs generated by the stimulation of purified CD8+ T lymphocytes from HLA-A*0201-positive PBMCs with UMG1-2.

(C) Cell death rate of the target cells by CTLs. Cell death of UMG1-2 increased after the incubation of the target cells with the increasing number of effector cells generated by the stimulation of purified CD8+ T lymphocytes from HLA-A*0201-positive donor with UMG1-2.

3. Effector cell death induced by HCMV-infected U373MG

U373MG cells were infected with HCMV Jurkat cells were added at the indicated day and maintained the culture for 4 hours. The harvested Jurkat cells were stained with Annexin V, and the cell death was analyzed with flow cytometry. From day 0 to day 1 postinfection cell death was not found in Jurkat cells, but from day 2 postinfection cell death increased to 30% and rapidly upto 80-90% thereafter. There was no death of Jurkat cells throughout the experiment in case of mock infection (Figure 4 (A)).

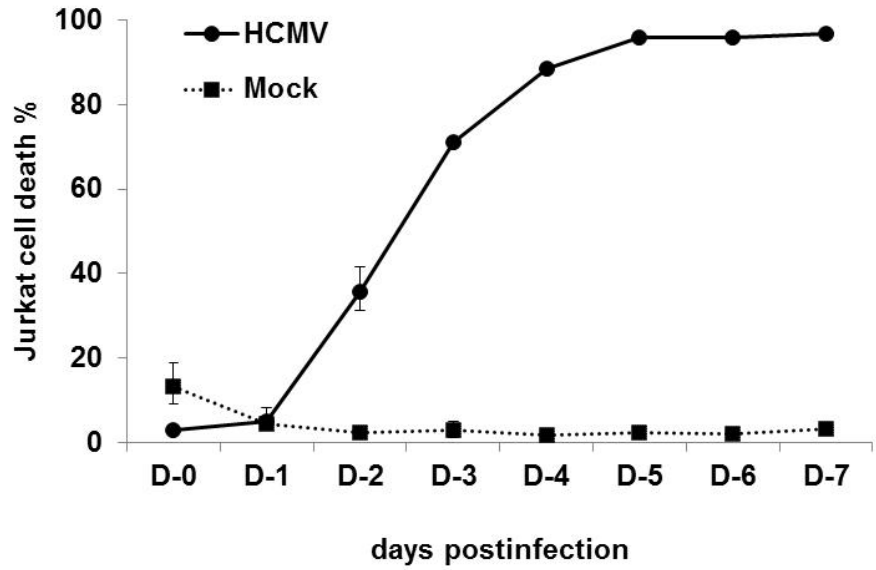
For the determination of the death-inducing capacity of HCMV-infected U373MG to the effector cells the cytotoxic assay was performed with the varying ratio of Jurkat cells to HCMV-infected U373MG. Variable number of Jurkat cells was added into U373MG infected with HCMV for 4 days. After co-culture for 24 hours cytotoxic assay was performed with TUNEL assay. The cell death rate of Jurkat cell with the ratio of 1:4, 1:2 and 1:1 was 79.2%, 68.7% and 75.7%, respectively, but that of 2:1, 4:1 and 8:1 was 58.1%, 40.1% and 32.5%, respectively (Figure 4(B)). These results suggested that the more the effector cells were present in the culture of HCMV-infected U373MG, the less the death rate of effector cells was.

Two time points, day 0 and day 4 postinfection, were selected to

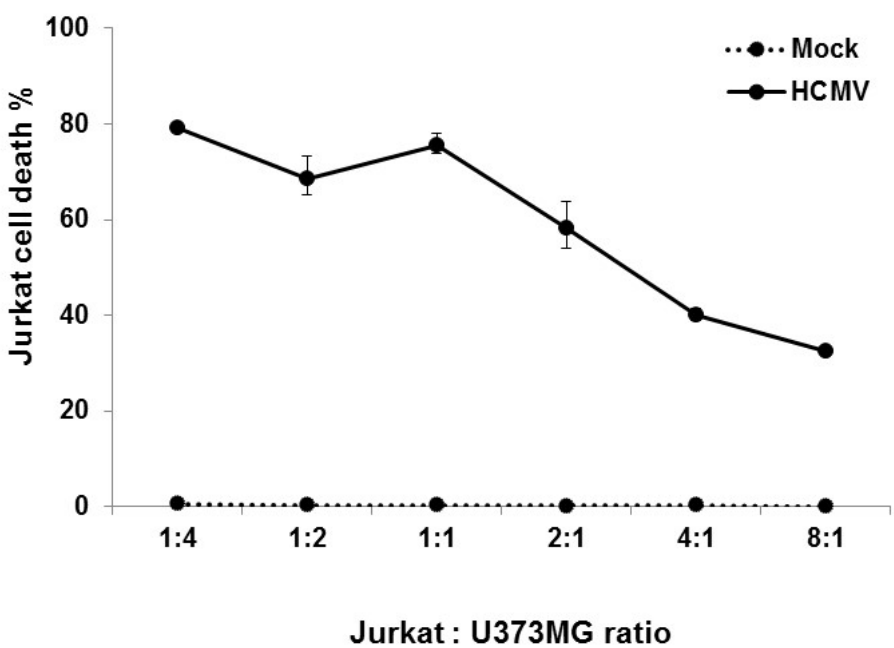
assay the death of CTL by the co-culture with HCMV-infected U373MG. When CTLs generated from CD8+ T cells from Donor A1 were added into the culture, cell death rates of CTLs were 9.4% at day 0, and 37.1% at day 4 postinfection. In case of the cultures of mock infection and UMG1-2 the cell death rate was below 8.8% in all the assays (Figure 4 (C)).

HCMV was detected in the culture supernatant from HCMV-infected U373MG at day 4 postinfection by real-time PCR. The mean quantity of HCMV was 4.5×10^4 PFUs/ml. The data were from three independent experiments.

(A)


HCMV
Mock
Jurkat cell death %
100
80
60
40
20
0
D-0
D-1
D-2
D-3
D-4
D-5
D-6
D-7
days postinfection

(B)


Mock
HCMV
Jurkat cell death %
100
80
60
40
20
0
1:4
1:2
1:1
2:1
4:1
8:1
Jurkat : U373MG ratio

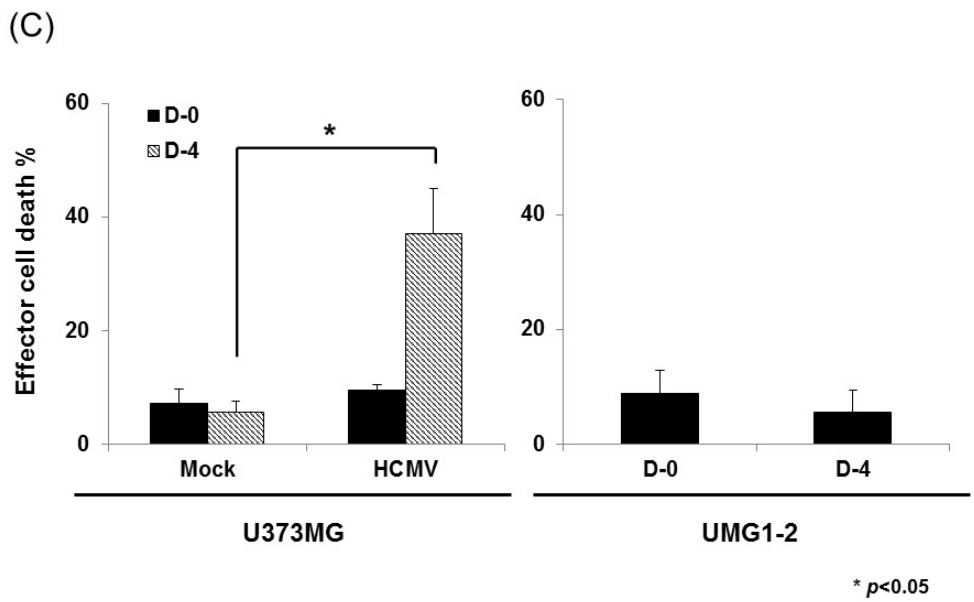


Figure 4. Cell death of the effector cells by HCMV-infected U373MG.

(A) Death of Jurkat cells by HCMV-infected U373MG depending on the duration of infection. Jurkat cells were added into U373MG infected with HCMV on the indicated day, maintained for 4 hours, and the death of the harvested Jurkat cells were determined by Annexin V staining assay. Cell death increased from 2 days postinfection and reached at maximum at 4 days postinfection.

(B) Death of Jurkat cells by HCMV-infected U373MG depending on the number of Jurkat cells were added into U373MG infected with

HCMV on day 4 postinfection, maintained for 24 hours, and the death of the harvested Jurkat cells were determined by TUNEL assay. Cell death of Jurkat cells maintained at 68.7% - 79.2% in the cultures of 1:4 to 1:1, but decreased to 58.1% - 32.5% in the cultures of 2:1 to 8:1.

(C) Death of effector cells by HCMV-infected U373MG.

CTLs made of the purified CD8+ T lymphocytes were added into HCMV-infected U373MG at day 0 and 4 postinfection, maintained for 24 hours, and the death of the harvested CTLs were determined by TUNEL assay. Increased death of effector cells was found in the cultures of HCMV-infected U373MG, not in the cultures of U373MG and UMG1-2.

4. Changes of cell surface MHC I antigen after HCMV infection

Expression level of MHC I on U373MG after HCMV infection was monitored with anti-HLA-A*0201 antibody by FACS analysis. At day 0 postinfection the expression level decreased to 94.1% and maintained at 87.3 - 97.4% until 3 days postinfection. The expression level decreased to 61.3 - 67.6% at day 4 to 5 postinfection, and 35.1% at day 6 postinfection. The expression level on mock-infected U373MG did not change significantly throughout the experiment time (Figure 5).

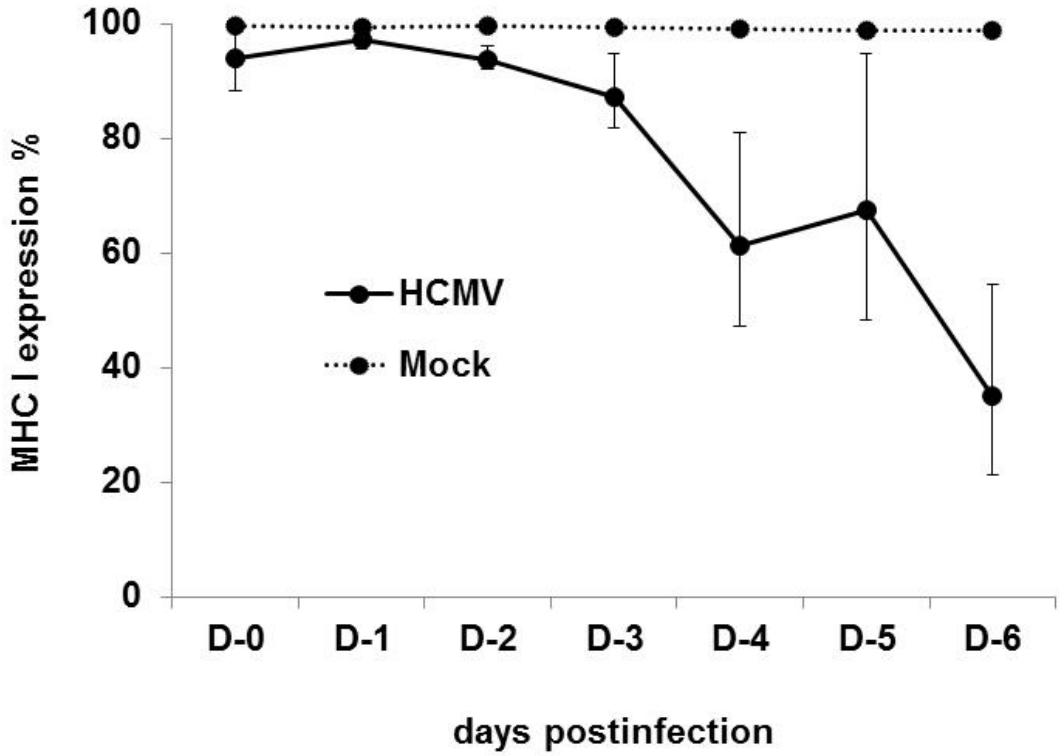


Figure 5. Changes of the expression of MHC I on U373MG infected with HCMV.

Expression of MHC I on U373MG was assayed with the specific anti-HLA-A*0201 antibody and FACS analysis at the indicated time points. MHC I on U373MG after HCMV infection decreased significantly after 4 days infection.

5. Cell death of HCMV-infected U373MG by CTLs

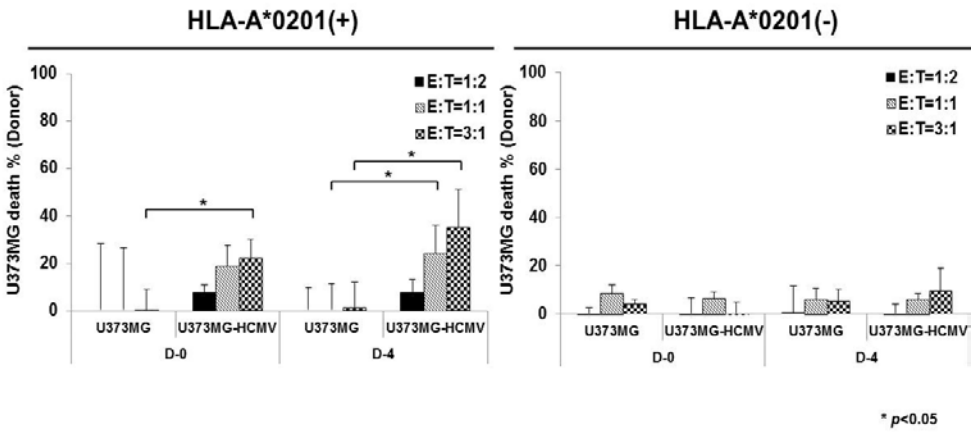
Cell death rate in HCMV-infected U373MG was measured after the treatment of CTLs generated from the stimulation of purified CD8+ T lymphocytes of HLA-A*0201-positive or -negative donors with UMG1-2. Cell death rate by CTLs increased 18.7% to 24.3% at day 0 and 22.1% to 34.7% at 4 days postinfection with the increasing E:T ratio from 1:1 to 3:1 in HLA-A*0201 (+) donors. It was below 9.4% in all assays when the CTLs from HLA-A*0201 (-) donors. The data were expressed as mean from three independent assays in three donors (Figure 6 (A)).

The cell death rates in UMG1-2 by CTLs generated from the stimulation of purified CD8+ T lymphocytes of HLA-A* 0201-positive donor with UMG1-2 were 8.7%, 32.3% and 48.2% at E:T=1:2, 1:1 and 3:1, respectively. When CTLs were generated from HLA-A* 0201-negative donor they were under 3.8% at any E:T ratio (Figure 6 (B)). These results demonstrated that the generated CTLs kill target cells HLA-restrictedly and effector cell dose-dependently.

Removal of the culture supernatant from HCMV-infected U373MG before adding the effector cells to HCMV-infected U373MG enhanced the target death from 8.4% to 35.5% at E:T=1:1 ($p<0.05$), but not at E:T=3:1 (33.9% - 41.9%) ($p>0.05$). The data

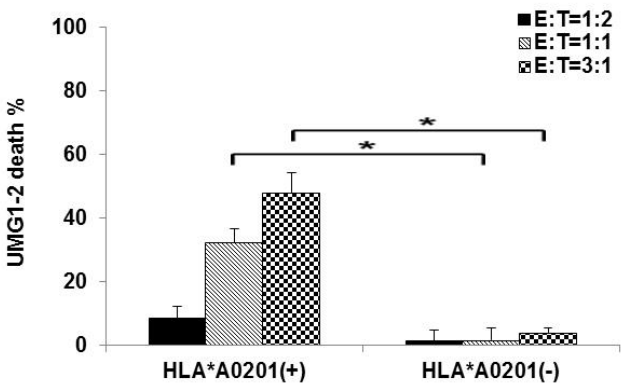
were expressed as mean from three independent assays in two donors (Figure 6 (C)). When the effector cells from 24-hour co-cultured HCMV-infected U373MG with CTLs were transferred to UMG1-2 and cultured for another 24 hours, cell death rate of the target cells, UMG1-2, decreased from 31.0% to 13.0% at E:T=1:1 ($p<0.05$), but increased from 38.0% to 50.9% at E:T=3:1 ($p>0.05$). The data were expressed as mean from three independent assays in two donors (Figure 6 (D)).

(A)

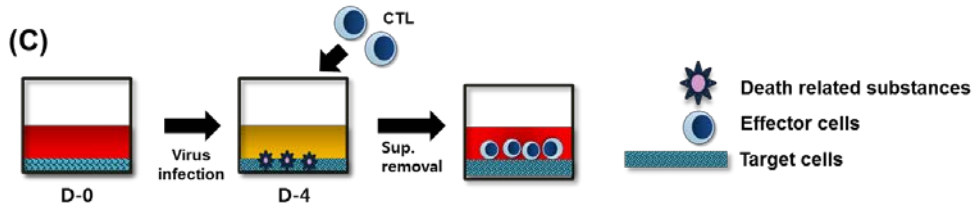

HLA-A*0201(+)
HLA-A*0201(-)
U373MG death % (Donor)
E:T=1:2
E:T=1:1
E:T=3:1
100
80
60
40
20
0
U373MG
U373MG-HCMV
D-0
D-4
*

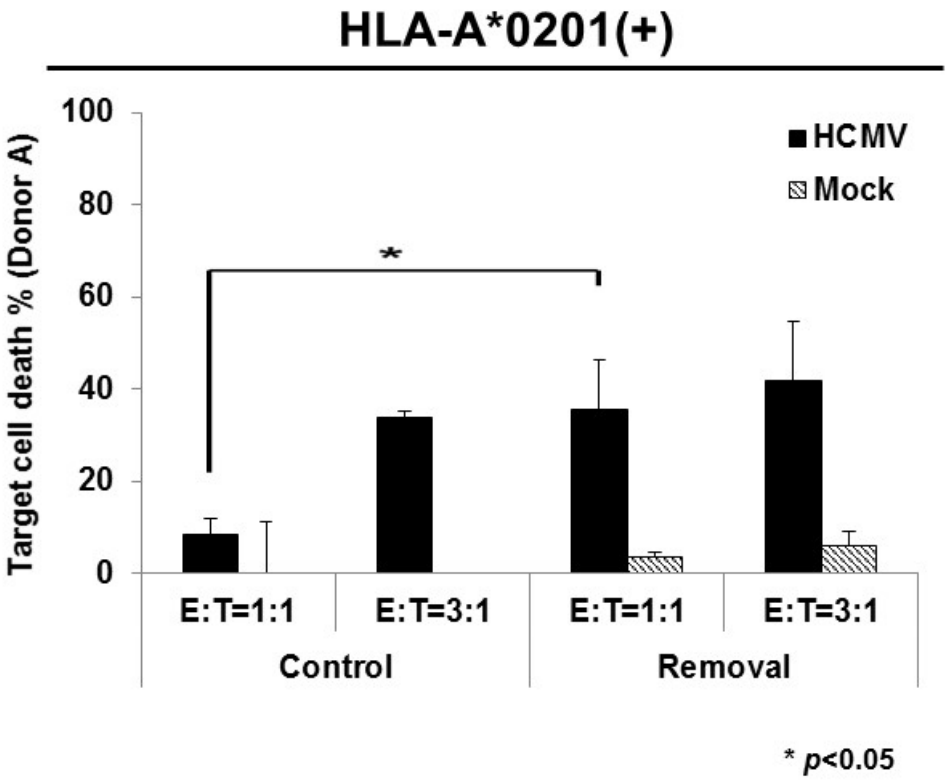
* $p<0.05$

(B)


UMG1-2 death %
E:T=1:2
E:T=1:1
E:T=3:1
100
80
60
40
20
0
HLA*A0201(+)
HLA*A0201(-)
*

* $p<0.05$


(C)
CTL
D-0
Virus infection
D-4
Sup. removal
Death related substances
Effector cells
Target cells


HLA-A*0201(+)
Target cell death % (Donor A)
100
80
60
40
20
0
HCMV
Mock
*
E:T=1:1
E:T=3:1
E:T=1:1
E:T=3:1
Control
Removal

* $p<0.05$

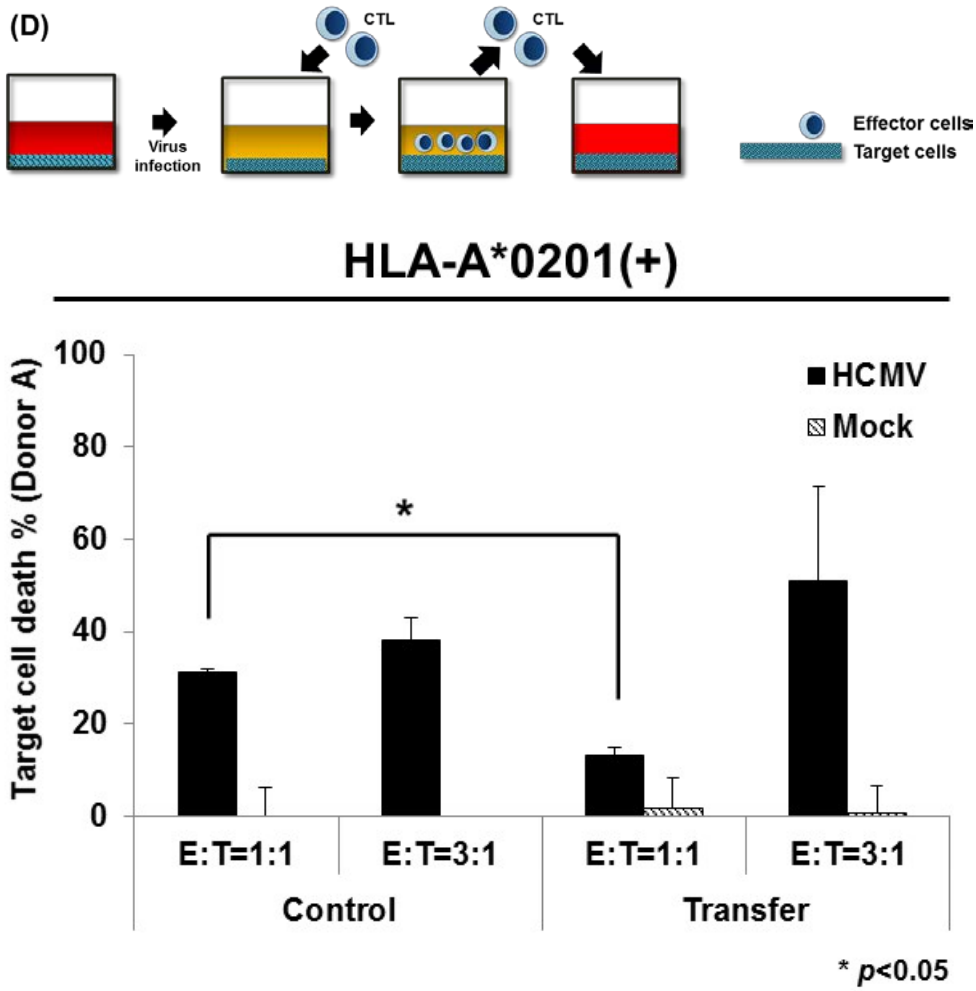


Figure 6. Cell death of HCMV-infected U373MG by CTLs.

(A) Cell death rate in HCMV-infected U373MG by CTLs generated from the stimulation of purified CD8+ T lymphocytes of HLA-A*0201-positive or negative donor with UMG1-2. Cell death rate increased HLA-restrictedly and effector dose-dependently. The

data were expressed as mean + standard error from three independent assays in Donor A1, two in Donors A2, A3 and C1-3.

(B) Cell death rate in UMG1-2 by CTLs generated from the stimulation of purified CD8+ T lymphocytes of HLA-A*0201-positive or negative donor with UMG1-2. Cell death rate increased HLA-restrictedly and effector dose-dependently.

(C) Effect of the removal of the cultured supernatant from HCMV-infected U373MG before adding the effector cells to HCMV-infected U373MG on the killing of target cells. Removal of the culture supernatant from HCMV-infected U373MG enhanced the target death at E:T=1:1, but not at E:T=3:1. The result was from two donors, and the data were expressed as mean + standard error from three independent assays.

(D) Effect of the transfer of effector cell from 24-hour co-cultured HCMV-infected U373MG with CTLs to UMG1-2 on the killing of target cells. Transfer of the effector cells to UMG1-2 decreased the target death at E:T=1:1, but not at E:T=3:1. The result was from two donors, and the data were expressed as mean + standard error from three independent assays.

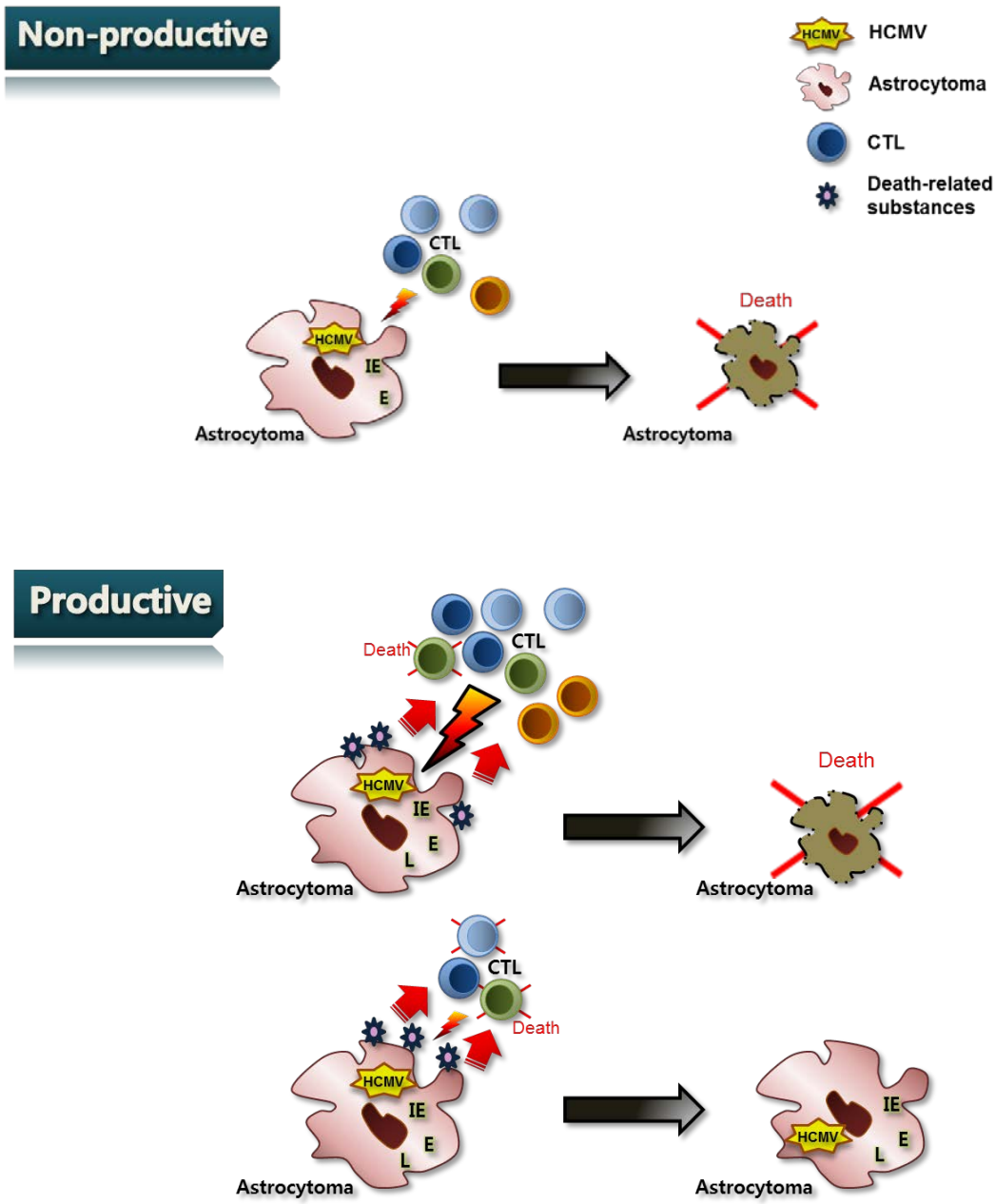


Figure 7. Schematic presentation of the reactivity or the impairment of CTLs specific to HCMV by HCMV-infected astrocytoma cells

according to the microenvironment of HCMV infection.

DISSCUSSION

I demonstrated that CTLs prepared in this study could lyse HCMV-infected astrocytoma cells with the dose-dependency, but have limited lytic activity to target cells in case of the small number of the effector cells.

HCMV pp65 and IE-1 have been found to be major targets of the cytotoxic T cell response (15, 16). HCMV pp65 may be more suitable than HCMV IE-1 for the candidate molecules of the vaccine or immunotherapy because both CD4+ and CD8+ T cell reactive to HCMV pp65 were detected in many cases while only CD8+ T cell to HCMV IE-1 were detected (17). But the situation is complicate in the immune response to GBM.

Once HCMV genome is introduced into the cell, major immediate early promoter (MIEP) in HCMV can be activated, IE genes are transcribed, and IE proteins are produced in almost cells. The susceptibility and permissiveness to HCMV infection are different from cell line to cell line of the same origin of tissue, and even from cell to cell of the same tissue. HCMV IE-1 was detected in all GBM (18). HCMV pp65 and HCMV IE-1 was present in fresh isolated GBM but HCMV pp65 was less ubiquitous than HCMV IE-1 (3)

HCMV late antigen, pp65, cannot be produced in the cell if viral replication does not occur in any reason. I chose HCMV IE-1 as a target molecule to produce the specific CTLs for the study of the reactivity to astrocytoma cells infected with HCMV. I showed that CTLs to HCMV IE-1 could react to cells expressing HCMV IE-1 throughout the whole period of viral infection, the early time (Figure 6(A), D-0) and the late time (Figure 6(A), D-4).

I found that UMG1-2, but not U373MG, showed the slow growth and the slight morphological changes in the presence of human AB serum (data not shown). I used FBS instead of human AB serum in the culture of U373MG, UMG1-2 and PBMCs throughout the experiment.

The presence of CTL clones reactive to HCMV IE-1 was demonstrated by the stimulation of PBMCs from HLA-A*0201(+) donor with HCMV IE-1 overlapping peptides and probing IFN-r-secreting cells (Figure 2 (A)). For the effective production of CTLs to HCMV IE-1, UMG1-2 was used as stimulator cells. When donors were HLA-A*0201(+) the specific CTLs generated with UMG1-2 have the specific lytic activity to UMG1-2 cells dose-dependently, but not to U373MG (Figure 3 (B) and (C)).

HCMV has many strategies of immune evasion such as the decreased expression of MHC I molecules on the infected cells (19). But the effective CTLs reactive to HCMV-infected cells were

produced in healthy persons. This means that immune system of host overcomes the evasion attempt of HCMV. There is no report on the study of the death of the effector cells in CTL assays. I demonstrated that the effector cells in the cultures of CTLs and HCMV-infected U373MG at day 4 postinfection for 24 hours were dead up to 37.1%, while in case of the cultures of CTLs and U373MG the death rate was below 8.8% (Figure 4 (C)). This result suggested that HCMV-infected U373MG at day 4 postinfection produced the impairing factors of the function of CTLs. The exact mechanism of the death of effector cells by HCMV infection should be elucidated in the future study.

I monitored the expression of MHC I molecules on U373MG after HCMV infection with anti-HLA-A*0201 antibody instead of antibody to common MHC I or beta2 microglobulin. Many tumors expressed variant MHC molecules(20, 21), and it is feasible to use the antibody to the exact polymorphic MHC epitope as a probe. MHC I molecules maintained above 80% until 3 days postinfection, and decreased to 40% - 60% thereafter (Figure 5). The decreased level of MHC I on U373MG after HCMV infection is different from other report (22). It may be from the use of the different antibody. Cell death rate by CTLs generated from HLA-A*0201 (+) donor with UMG1-2 to HCMV-infected U373MG increased dose-dependently up to 41.9% at E:T=3:1 and day 4 postinfection

although the expression of MHC I decreased to 61.3%. These data suggest that the immune reaction to tumor cells infected with HCMV might be different from the normal cells.

I could not recruit the HCMV-sero-negative donor because almost all persons were sero-positive in Korea(23).

I performed the removal of the supernatant from the cultures of HCMV-infected U373MG before adding the effector cells to the cultures of HCMV-infected U373MG to see the effect of the supernatant on the death of the effector cells. As expected from the death of the effector in Figure 4 (C), cell death rate of the target cells increased from 8.4% to 35.5% at E:T=1:1 after the removal of the culture supernatant, but not changed at E:T=3:1 (Figure 6(C)) Similarly when the transfer of the effector cells after the co-culture of CTLs and HCMV-infected U373MG for 24 hours to UMG1-2, the cell death rate decreased from 31.0% to 13.0% at E:T=1:1, but not at E:T=3:1 (Figure 6(D)). These results implied that CTLs specific to HCMV IE-1 could lose their function near HCMV-infected astrocytoma cells when the number of CTLs is relatively low. The capacity of HCMV-infected astrocytoma cells to induce the death of nearby target cells seems to be limited. When the relatively high number of Jurkat cells, which may be the equivalent cells to effector cells, the death rate of Jurkat cells by HCMV-infected astrocytoma cells decreased as compared to the

experiment with the low number of Jurkat cells (Figure 4(B)). Therefor it is recommended the high number of CTLs specific to HCMV for the treatment of GBM infected with HCMV.

Conclusively HCMV-infected U373MG decreases the activity of CTLs specific to HCMV in case of the low number of the effector cells. These results suggest that HCMV impairs CTL function and helps glioblastoma unchecked by CTLs.

REFERENCES

1. Dolecek TA, Propp JM, Stroup NE, Kruchko C. CBTRUS statistical report: primary brain and central nervous system tumors diagnosed in the United States in 2005-2009. Neuro-oncology. 2012;14 Suppl 5:v1-49.

2. Garside R, Pitt M, Anderson R, Rogers G, Dyer M, Mealing S, et al. The effectiveness and cost-effectiveness of carmustine implants and temozolomide for the treatment of newly diagnosed high-grade glioma: a systematic review and economic evaluation. Health Technology Assessment. 2007;11(45):iii-iv, ix-221.

3. Mitchell DA, Xie W, Schmittling R, Learn C, Friedman A, McLendon RE, et al. Sensitive detection of human cytomegalovirus in tumors and peripheral blood of patients diagnosed with glioblastoma. Neuro-oncology. 2008;10(1):10-8.

4. Prins RM, Cloughesy TF, Liau LM. Cytomegalovirus immunity after vaccination with autologous glioblastoma lysate. The New England Journal of Medicine. 2008;359(5):539-41.

5. Hong S, Van Kaer L. Immune privilege: keeping an eye on natural killer T cells. The Journal of Experimental Medicine. 1999;190(9):1197-200.

6. Mitchell DA, Fecci PE, Sampson JH. Immunotherapy of malignant brain tumors. Immunological Reviews. 2008;222:70-100.

7. Wekerle H, Sun D, Oropeza-Wekerle RL, Meyermann R. Immune reactivity in the nervous system: modulation of T-lymphocyte activation by glial cells. The Journal of Experimental Biology. 1987;132:43-57.

8. Hertel L, Chou S, Mocarski ES. Viral and cell cycle-regulated kinases in cytomegalovirus-induced pseudomitosis and replication. PLoS Pathogens. 2007;3(1):e6.

9. Jackson SE, Mason GM, Wills MR. Human cytomegalovirus immunity and immune evasion. Virus Research. 2011;157(2):151-60.

10. Hadrup SR, Strindhall J, Kollgaard T, Seremet T, Johansson B, Pawelec G, et al. Longitudinal studies of clonally expanded CD8 T cells reveal a repertoire shrinkage predicting mortality and an increased number of dysfunctional cytomegalovirus-specific T cells in the very elderly. Journal of Immunology. 2006;176(4):2645-53.

11. Slavuljica I, Kvestak D, Csaba Huszthy P, Kosmac K, Britt WJ, Jonjic S. Immunobiology of congenital cytomegalovirus infection of the central nervous system-the murine cytomegalovirus model. Cellular & Molecular Immunology. 2014:doi:10.1038/cmi.2014.51.

12. Han S, Zhang C, Li Q, Dong J, Liu Y, Huang Y, et al. Tumour-infiltrating CD4(+) and CD8(+) lymphocytes as

predictors of clinical outcome in glioma. British Journal of Cancer. 2014;110(10):2560-8.

13. Kim J, Kwon YJ, Park ES, Sung B, Kim JH, Park CG, et al. Human cytomegalovirus (HCMV) IE1 plays role in resistance to apoptosis with etoposide in cancer cell line by Cdk2 accumulation. Microbiology and Immunology. 2003;47(12):959-67.

14. Shin KC, Park CG, Hwang ES, Cha CY. Human cytomegalovirus IE1 protein enhances herpes simplex virus type 1-induced syncytial formation in U373MG cells. Journal of Korean Medical Science. 2008;23(6):1046-52.

15. Kern F, Surel IP, Faulhaber N, Frommel C, Schneider-Mergener J, Schonemann C, et al. Target structures of the CD8(+)-T-cell response to human cytomegalovirus: the 72-kilodalton major immediate-early protein revisited. Journal of Virology. 1999;73(10):8179-84.

16. McLaughlin-Taylor E, Pande H, Forman SJ, Tanamachi B, Li CR, Zaia JA, et al. Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for CD8+ virus-specific cytotoxic T lymphocytes. Journal of Medical Virology. 1994;43(1):103-10.

17. Slezak SL, Bettinotti M, Selleri S, Adams S, Marincola FM, Stroncek DF. CMV pp65 and IE-1 T cell epitopes recognized by healthy subjects. Journal of Translational Medicine. 2007;5:17.

18. Scheurer ME, Bondy ML, Aldape KD, Albrecht T, El-Zein R. Detection of human cytomegalovirus in different histological types of gliomas. Acta Neuropathologica. 2008;116(1):79-86.

19. Michelson S. Human cytomegalovirus escape from immune detection. Intervirology. 1999;42(5-6):301-7.

20. Aptsiauri N, Cabrera T, Mendez R, Garcia-Lora A, Ruiz-Cabello F, Garrido F. Role of altered expression of HLA class I molecules in cancer progression. Advances in Experimental Medicine and Biology. 2007;601:123-31.

21. Goepel JR, Rees RC, Rogers K, Stoddard CJ, Thomas WE, Shepherd L. Loss of monomorphic and polymorphic HLA antigens in metastatic breast and colon carcinoma. British Journal of Cancer. 1991;64(5):880-3.

22. Fletcher JM, Prentice HG, Grundy JE. Natural killer cell lysis of cytomegalovirus (CMV)-infected cells correlates with virally induced changes in cell surface lymphocyte function-associated antigen-3 (LFA-3) expression and not with the CMV-induced down-regulation of cell surface class I HLA. Journal of Immunology. 1998;161(5):2365-74.

23. Sohn YM, Park KI, Lee C, Han DG, Lee WY. Congenital cytomegalovirus infection in Korean population with very high prevalence of maternal immunity. Journal of Korean Medical Science. 1992;7(1):47-51.

국문초록

다형성아교모세포종(glioblastoma multiforme, GBM)은 빠르게 증식하고 침윤하는 중추신경계 종양으로서 절제술을 포함한 방사선치료나 면역보조제 치료를 병행하여도 예후가 매우 나쁘다. GBM에 감염된 사람세포거대바이러스 (human cytomegalovirus, HCMV)의 감염정도에 따라 예후가 달라질 수 있는 등 HCMV에 의한 GBM의 병인과의 연관성이 주장되고 있다. 본 실험에서는 HCMV IE-1에 특이하게 반응하는 세포독성 T 림프구 (CTL)를 만들어서 HCMV에 감염된 별아교세포종(astrocytoma) 세포에 반응하는 사멸활성의 변화를 확인하고자 하였다.

HLA-A*0201(+) 공여자의 말초 CD8+ T 림프구를 분리하여 HCMV IE-1을 발현하는 U373MG세포로 자극하여 HCMV IE-1에 특이하게 반응하는 CTL을 만들었다. 표적세포의 사멸율은 전체 세포수에서 작동세포와 반응 후 살아남은 세포의 수를 뺀 후 전체 세포수를 나누어 계산하고 백분율로 산출하였다. 작동세포의 사멸율은 TUNEL분석으로 통해 같은 방법으로 산출하였다.

표적세포와 공여자 말초혈액림프구의 주요조직적합성 항원계가 동일하고, 생성된 CTL의 비율이 1:1에서 3:1로 높아질수록 표적세포의 사멸율이 0일과 4일에서 각각 18.7%에서 24.3%, 22.1%에서 34.7%로 증가하였

다. 별아교세포종 세포에 HCMV를 감염시킨 후 4일에 세포배양액을 제거하고 새로운 배지를 첨가하고 CTL을 반응시키면 HCMV에 4일간 감염된 별아교세포종 세포의 사멸율이 작동세포:표적세포(E:T)=1:1인 경우 8.4%에서 35.5%로 증가하였다. 또한 HCMV에 4일간 감염된 별아교세포종 세포와 CTL을 24시간 반응시킨 후 CTL을 회수하여 HCMV IE-1을 발현하는 별아교세포종 세포와 24시간 반응시켰을 경우에는 표적세포의 사멸율이 31.0%에서 13.0%로 감소하는 것을 확인하였다. E:T=3:1에서는 유의한 변화가 관찰되지 않았다.

결론적으로 HCMV IE-1에 특이성이 있는 CTL의 수가 적을 경우에는 HCMV에 감염되어 바이러스를 생산하는 별아교세포종세포에 대한 사멸율이 감소하며, 수가 많을 경우에는 사멸을 계속 유지하는 것을 확인하였다. 이로써 HCMV 감염은 주위의 CTL의 세포사멸 작용을 조절함으로써 종양세포으ㅏ 증식에 영향을 미칠 수 있는 것을 제시하였고, 면역세포를 이용한 GBM의 치료 방법을 제시할 수 있는 근거가 될 것이다.

주요어: 다형성아교모세포종, 사람세포거대바이러스, 세포독성 T 림프구, 별아교세포종, 극초기단백-1

학번: 2013-21749