Cytotoxic activity of allogeneic natural killer cells on U251 glioma cells *in vitro*

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Received May 11, 2015; Accepted March 16, 2016

DOI: 10.3892/mmr.2016.5220

Abstract. The present study aimed to observe the cytotoxic activity of allogeneic natural killer (NK) cells on U251 glioma cells and to investigate their mechanism of action to establish an effective treatment strategy for neuroglioma. Cell survival curves, colony formation assays and karyotype analysis were performed to investigate the characteristics of U251 glioma cells. The present study demonstrated that natural killer group 2, member D (NKG2D)-major histocompatibility complex class I-related chain A/B (MICA/B) interactions contributed to the cytotoxic effect of NK cells on K562 and U251 cells. In antibody-blocking assays to inhibit NKG2D ligands, the cytotoxic activity was not completely attenuated, which suggested that other signaling pathways contribute to the cytotoxic activity of NK cells on tumor cells in addition to the NKG2D-mediated activity. The present study identified that the expression levels of NKG2D ligands on the surface of target cells influenced the strength of the NK cell immune response. Furthermore, allogeneic NK cells were observed to kill glioma cells *in vitro*, and this anticancer activity is associated with the rate of NKG2D expression on the surface of glioma cells.

Introduction

Glioma is the most common primary malignant tumor of the central nervous system. Neuroectodermal tumors account for ~40-60% of central nervous system tumors (1). Complete surgical excision of lesions is difficult, as the boundaries between the glioma and surrounding brain tissue are unclear due to the active infiltrative growth along nerve fibers, which is observed in the majority of gliomas. The poor tolerance of brain tissue to radiation reduced sensitivity of malignant gliomas to radiotherapy. Systemic chemotherapy is associated with high whole-body toxicity and the blood-cerebrospinal fluid barrier restricts the penetration of anti-cancer therapeutic agents. Although these methods produce a limited curative effect, peripheral tumors cannot be effectively eradicated. Clinical and imaging studies have demonstrated that neurogliomas, particularly malignant gliomas, are prone to recurrence, with the majority recurring within 0.5-3.0 cm of the primary tumor (2). Only 5.5% of patients diagnosed with glioblastoma in the United States in 1980 exhibited a survival time >5 years (3). Surgical resection, radiotherapy and chemotherapy are ineffective in controlling disease progression, and do not markedly improve the prognosis, spontaneous generation or development of glioma. Thus, it is necessary to develop novel auxiliary treatments for glioma, to control the growth and recurrence of tumors, and to prolong survival time.

As the current comprehensive treatment includes surgical resection, radiotherapy and chemotherapy, it is difficult to treat malignant gliomas. To prevent tumor recurrence, therapeutic agents are administered directly to the tumor, to the lumen of the tumor or into surgically created resection cavities. Therapeutic strategies include injection of killer cells activated by lymphokines, interleukin-2 (IL-2), chemotherapeutics, embedded slow-release chemotherapy pills and monoclonal antibodies marked with radioisotopes (4). Although these methods are currently in clinical use, their effects are unsatisfactory. With advances in molecular biology and tumor immunology, certain novel therapeutic strategies have been proposed. In particular, biological therapy is garnering increasing attention due to its low toxicity and few side effects. The anti-carcinoma effect mediated by killer cell immunoglobulin-like receptor (KIR) ligand-mismatch in natural killer (NK) cell surface receptors has been successfully applied in allogeneic bone marrow transplantation (5,6). Certain previous studies have demonstrated that this effect can be applied in transplants for the treatment of leukemia and for solid tumors (5,7).

However, few studies have focused on the use of allogeneic NK cells to treat neuroglioma. As neuroglioma is associated with frequent recurrence and treatment is difficult, the present study aimed to identify an effective method for the biological treatment of neuroglioma by observing the cytotoxic activity of allogeneic NK cells on U251 glioma cells and to investigate the mechanism of action *in vitro*.

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Key words: natural killer cells, U251 glioma cell, natural killer group 2, member D

Materials and methods

Subjects and cell culture. The U251 glioma and K562 human chronic myeloid leukemia cell lines were donated by the Institute of Genetics at Xiangya Medical College of South Central University (Changsha, China). Peripheral blood samples were obtained from 7 healthy volunteers (4 women, 3 men; age, 25-36 years; median age, 32 years). The current study was conducted in accordance with the Declaration of Helsinki and with approval from the Ethics Committee of South Central University. Written informed consent was obtained from all participants.

U251 and K562 cells were cultured in complete RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in an incubator at 37˚C with 5% CO_2 and sufficient moisture. The medium was replaced every 2 days and the cellular morphology was observed daily using an inverted DM IL microscope (Leica Microsystems, Inc., Buffalo Grove, IL, USA).

Cell cloning and observation. The number of cloned U251 cells (>50 cells) was counted using Giemsa staining (Sigma-Aldrich, St. Louis, MO, USA) under a microscope (Nikon TMS; Nikon, Tokyo, Japan). Briefly, cells were washed with phosphate buffered saline, fixed with formaldehyde and washed again prior to incubation with Giemsa staining solution. After 30 min of staining, cells were washed and allowed to dry. Colony formation efficiency was calculated as follows: Colony formation efficiency (%) = colony no. / inoculated cell no. x 100.

U251 cells in the logarithmic growth phase were passaged (1:2) and a cell suspension was created by adding a small quantity (2 ml) of fixation liquid (methanol) to the cells following pretreatment. The cells were Giemsa-stained and sealed, then the chromosome number was counted in 15 cells and changes in the chromosome structure were observed.

Cell separation, purification and culture. A total of 50 ml peripheral venous blood, containing heparin anticoagulant (Shanghai Chemical Reagent Co., Ltd., Shanghai, China), was obtained from the healthy volunteers. The precipitate was separated via centrifugation (1,000 x g) using a lymphocyte separation medium (relative ratio, 1.077 g/l; Litton Bionetics, Kensington, MD, USA). Cells were then suspended in phosphate-buffered saline and the number of cells was counted using a Countstar automatic cell counter (Biomen Biosystems Co., Ltd., Guangzhou, China). Purification of NK cells was performed using the method described by Xu *et al* (8).

NK cells were suspended in RPMI-1640 cell culture medium with 10% FBS, 100 μg/ml penicillin and 100 μg/ml streptomycin (both purchased from Gibco; Thermo Fisher Scientific, Inc.). The concentration of NK cells was adjusted to $1x10^6$ cells/ml and the cells were seeded in 24-well culture plates (1 ml/well) and 1,000 μg/ml recombinant human (rh)IL-2 (Shenyang Sunshine Pharmaceutical Co., Ltd., Shenyang, China) was added. The medium was changed every 2 days and simultaneously supplemented with an equal concentration of rhIL-2. Cellular morphology was observed daily under the inverted microscope.

Cell activity measurement. Cell activity was determined using a lactate dehydrogenase (LDH) detection kit (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. Briefly, samples (0.5-1 ml) were collected at regular time points between 12 and 24 h from the culture. Samples were then centrifuged at 250 x g and the supernatant was removed. Then, the samples were titrated in a microtiter plate in the appropriate culture medium by serial dilution with a final volume of 100 μl/well. A total of 100 μl/well Solution C (reaction mixture) was then added to each well and incubated for up to 30 min at room temperature. The absorbance of the samples at 490 or 492 nm was measured using a microplate reader (iMark; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). The primers used in the current study (Table I) were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) based on previously published sequences (8). Total RNA was isolated using TRIzol (Thermo Fisher Scientific, Inc.), and the concentration and purity of the RNA were determined based on the A260/A280 ratio by the NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). cDNA was synthesizes from 2 μg total RNA using an AMV Reverse Transcriptase First-strand cDNA Synthesis Kit (GE Healthcare Life Sciences, Chalfont, USA) according to the manufacturer's protocol, which was used to detect the mRNA expression levels of the MHC class I polypeptide-related sequence A and B ligands of natural killer group 2, member D (NKG2D) in U251 cells. LightCycler-Fast Start DNA SYBR Green I mix (Takara, Tokyo, Japan) and 500 nmol/l of each primer were used in a final volume of 20 μl. The reaction was initiated at 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 10 sec, annealing for 10 sec at 69˚C, and extension at 72˚C for 15 sec. A DNA ladder (cat. no. D514C; Takara) and ethidium bromide (cat. no. 1203-10; BioVision, Milpitas, CA, USA) were used in electrophoresis. The presence of amplified PCR products was confirmed using a 1% agarose gel followed by UV visualization following ethidium bromide staining.

Quantification of MICA/B and UL16 binding proteins 1-3 (ULBP1-3) on the cell surface. Expression levels of NKG2D ligands on the surface of U251 cells, including MICA/B and ULBP1-3, were determined using an EPICS XL flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) with an indirect immunofluorescence labeling method. Briefly, whole blood was labelled with combinations of monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin, and either peridinin chlorophyll protein (PerCP), cychrome, or Tricolour. Isotype-matched fluorochrome-conjugated antibodies served as controls (CD3 FITC, CD45RO FITC, CD27 FITC, CD19 phycoerythrin, CD27 phycoerythrin, CD4 phycoerythrin, CD8 phycoerythrin, CD45 PerCP, CD4 PerCP, CD8 cychrome, CD45RO cychrome, CD16+CD56 FITC phycoerythrin, IgM FITC, IgD FITC and CD19 Tricolour; BD Biosciences, Oxford, UK). The resulting three-colour cell staining was analyzed using an Epic XL flow cytometer (Beckman Coulter, Inc.). Cells were sorted with a Beckman Coulter Epics Altra with Expo32 software (verxion ADC 1.1C; Beckman Coulter, Inc.).

Table I. Primer sequences used in the present study.

Gene	Forward primer	Reverse primer	Product size (bp)
MICA	5'-GTGCCCCAGTCCTCCAGAGCTCAG-3'	5'-GTGGCATCCCTGTGGTCACTCGTC-3'	635
MICB	5'-GGCGTCAGGATGGGGTATCTTTGA-3'	5'-GGCAGGAGCAGTCGTGAGTTTGCC-3'	690
ULBP1	5'-CTGCAGGCCAGGATGTCTTGTGAG-3'	5'-TGAGGGTGGTGGCCATGGCCTTGG-3'	319
ULBP2	5'-CTGCAGGCAAGGATGTCTTGTGAG-3'	5'-TGAGGGTGGTGGCTGTGGCCCTGA-3'	327
ULBP3	5'-CTGCAGGTCAGGATGTCTTGTGAG-3'	5'-TGAGGGTGGTGGCTATGGCTTTGG-3'	321
β-actin	5'-AGCCATGTACGTAGCCAT-3'	5'-TTTGATGTCACGCACGATTT-3'	232

MIC, MHC class I polypeptide-related sequence; ULBP, UL16 binding protein.

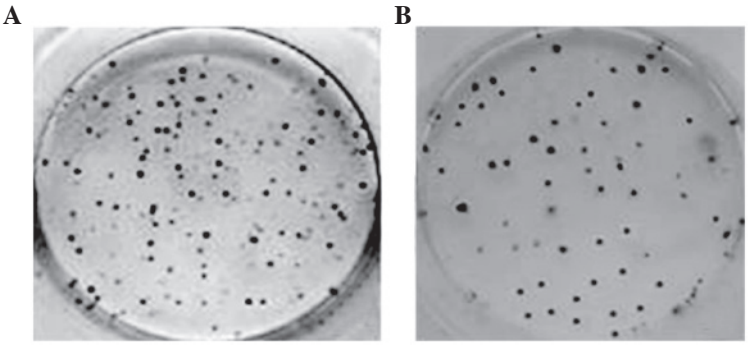

Figure 1. U251 cell clone formaton experiments. (A) 14 and (B) 7 days.

Cytotoxic activity of NK cells. The primary mouse monoclonal AMO-1 mAb antibody, specific for MICA and anti-MICA/B-PE (clone 6D4; 1:500; cat. no. NB100-78034PE), was purchased from Novus Biologicals (Littleton, CO, USA). The anti-ULBP 1-3 monoclonal antibodies used, human ULBP-1 MAB (clone 170818; 1:500; cat. no. MAB1380), human ULBP-2/5/6 MAb (clone 165903; 1:500; cat. no. MAB1298) and human ULBP-3 MAb (clone 166510; 1:500; cat. no. MAB1517) were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Each monoclonal antibody was incubated with K562 and U251 cells for 15 min at room temperature, then the solution was added to the NK cells. The cytotoxic activity of the NK cells was measured using an LDH detection kit. All antibodies were purchased from R&D Systems, Inc.

Statistical analysis. All data are presented as the mean ± standard deviation. An unpaired t-test was used to analyze differences between two groups and one-way analysis of variance was used to estimate differences among groups (the least significant difference method was used to perform multiple comparisons between groups). $P<0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS version 12.0 software (SPSS, Inc., Chicago, IL, USA).

Results

Colony formation. U251 cells formed colonies in culture and the colony forming efficiency was estimated by counting the number of colonies of each group in 6-well culture dishes. At day 7 and 14, colony forming efficiencies were 26.07±2.70 and 38.44±2.68%, respectively (Fig. 1).

Karyotype analysis. The karyotypes of 15 cells were determined under a microscope. All chromosomes were hyperdiploid, and the chromosome number ranged from 52 to 61 (Fig. 2); 33.3% contained 56 chromosomes, 20.0% contained 60 chromosomes, 13.3% contained 56 or 61 chromosomes, and 6.7% contained 52, 58 or 55 chromosomes. Chromosome with the two arms were present in 83.3% of cells, indicating that the U251 cells were malignant.

Purity of NK cells. Flow cytometry analysis indicated that the purity of cluster of differentiation $(CD)^-$, $CD16^+$ and $CD56^+$ cells (NK cells) was 89.56±2.37% following separation.

Cytotoxic activity of NK cells on K562 and U251 cells. The LDH method was used to determine the cytotoxic effect of NK cells on K562 and U251 cells. The results showed that there was significant difference between the cytotoxicity of NK cells on K562 and U251 cells at each effector (E):target (T) ratio by pairwise comparison ($P>0.05$; Figs. 3 and 4); the cytotoxic effect differed significantly between the different E:T ratios ($P<0.05$). Thus, NK cells demonstrated significant cytotoxic activity on the two cell types and the cytotoxic activity increased as the E:T ratio increased.

Expression levels of NKG2D ligands. The expression levels of NKG2D ligands, including MICA, MICB, ULBP1, ULBP2 and ULBP3, on the surface of U251 cells were marginally

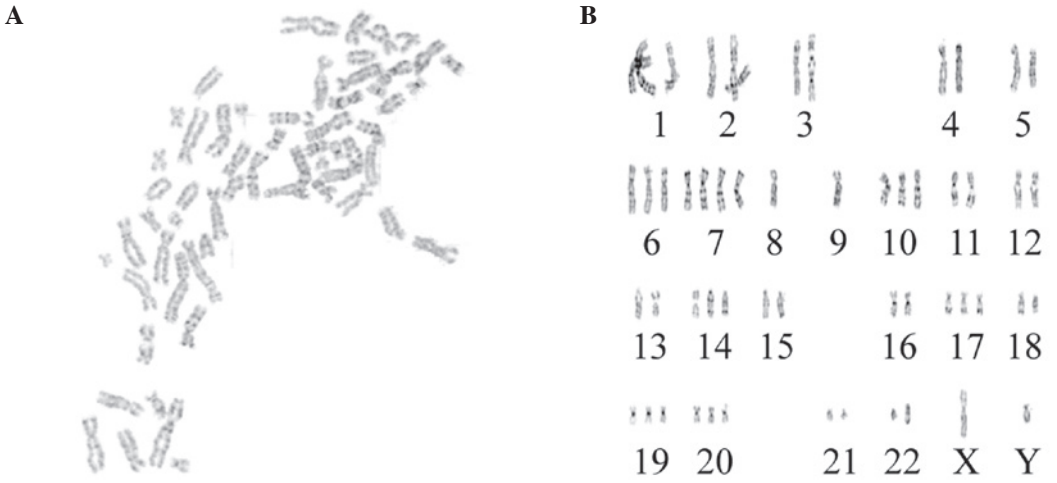

Figure 2. U251 cell chromosome karyotype analysis. Chromosome (A) number and (B) structure observation.

reduced compared with K562 cells. The highest expression level observed was ULBP3 (45.2%) and the lowest was ULBP1 (21.9%; Fig. 5). The expression levels of these ligands did not differ significantly between the two cell types (P>0.05; Table II).

RT-PCR. For RT-PCR analysis, five fragments of consistent length were amplified from the U251 and K562 cells, indicating that these products are mRNAs that encode the NKG2D ligands in U251 and K562 cells (Fig. 6).

Changes to the cytotoxic activity of NK cells. As demonstrated in Table III, when the E:T ratio was 20:1, AMO-1, BMO-1, M295, M310 and M551 bound to the relevant molecules on the K562 and U251 cell surfaces. Compared with the cytotoxic activity prior to blocking MICA/B and ULBP1-3, a significant difference was detected between the activity of NK cells on K562 and U251 cells (P<0.05). These results indicate that NKG2D ligands may be involved in the cytotoxic activity of NK cells on K562 and U251 cells.

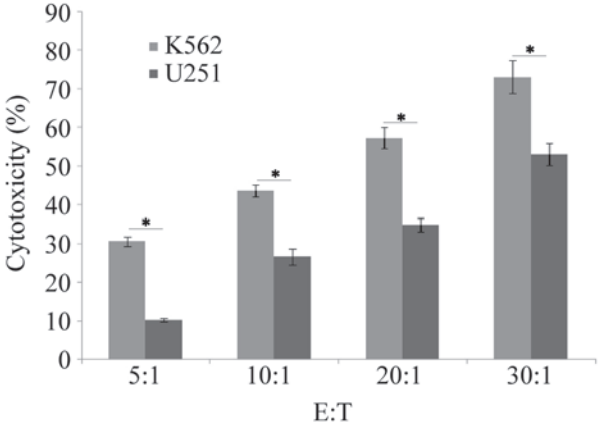

Figure 3. Cytotoxic activity of natural killer cells on K562 and U251 cells at different E:T ratios. *P<0.05. E, effector; T, target.

Discussion

NK cells are an important lymphocyte subpopulation and a primary factor involved in innate immunity. The activation and function of NK cells is determined by cell-surface activating and inhibitory receptors. The 'missing self' hypothesis was proposed in 1990 (9), which stated that NK cells selectively recognize and kill cells lacking, or with lower expression levels of, major histocompatibility complex I (MHC I) molecules via a specific mechanism, although NK cells do not kill healthy cells.

NK cell-activating receptors are divided into three classes: KIRs, C type lectin receptors and non-MHC class I-specific activating NK cell receptors. KIRs are predominantly expressed on the surface of NK cells and certain T cells. The gene encoding KIRs is located on the long arm of human chromosome 19 (19q) (10). C type-lectin receptors are a type of MHC-I receptor in the C-type lectin superfamily. The receptor is composed of a dimer of CD94 and NKG2 type II transmembrane proteins, and the gene encoding these receptors is located on the short arm of chromosome 12 (12p) (10,11). Expression of NKG2D does not require CD94 or combination with CD94, and it can combine with MICA or B to activate the cytotoxic activity of NK cells, mediated by the polypeptide DNAX-activating protein 10 (DAP10) (12). Unlike MICA and MICB, the NKG2D-DAP10 complex also recognizes and combines with retinoic acid early-inducible protein 1 and human cytomegalovirus glycoprotein ULBP, which acts as an anchor junction with glycosyl phosphatidyl inositol in target cells (13). Three NK cell stimulating receptors that are non-MHC dependent belong to the Ig superfamily (NKP46, NKP44 and NKP30) and are known to be natural cytotoxicity receptors (14). The ability of NK cells to kill target cells mediated by these three receptors is associated with cell surface receptor density. The current study primarily demonstrates the cytotoxic activity of NK cells on U251 glioma cells and the primary underlying mechanisms.

In the present study, the sensitive K562 cell line served as a positive control and the LDH cytotoxic release assay was used to detect the cytotoxic activity of allogeneic NK cells on U251 cells at different E:T ratios *in vitro*. The current study demonstrated that NK cells exhibited significant cytotoxic activity against the two cell types and that the cytotoxic effect

Table II. Expression levels of MICA/B and ULBP1-3 on U251 and K562 cell surfaces.

	Expression level (%)				
Cell line	MICA	MICB	ULBP1	ULBP2	ULBP3
K562	57.3±2.47	46.8±2.41	43.3±1.98	65.3±3.06	53.9±2.66
U251	34.2±1.35	40.4±1.27	22.7±1.12	39.4±1.43	45.4±1.75

No significant difference was detected between expression rates of each molecule on the surfaces of the two cell types (P>0.05). Values are presented as the mean ± standard deviation. MIC, MHC class I polypeptide-related sequence; ULBP, UL16 binding protein.

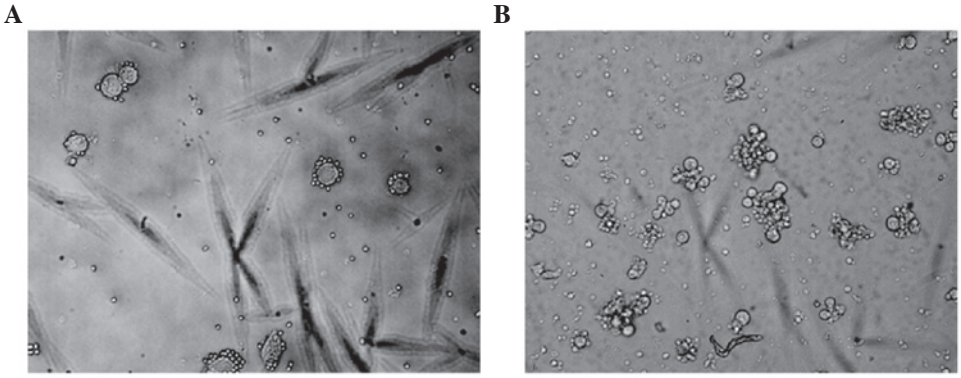

Figure 4. Combination of NK cells with U251 cells and cytotoxic U251 cells. (A) NK and U251 cells co-cultured for 1 h (U251 cells combined with NK cells). (B) NK and U251 cells co-cultured for 12 h (certain U251 cells were dissolved). Magnification, x100. NK, natural killer.

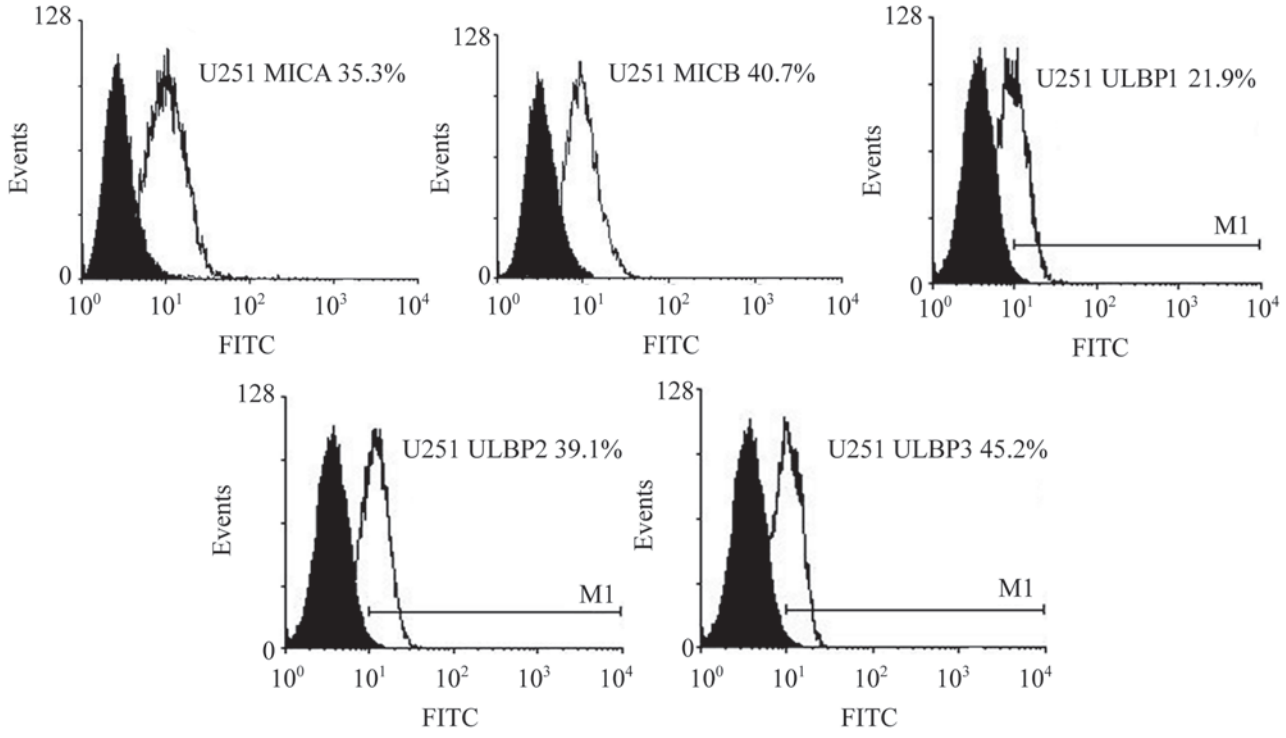

Figure 5. Expression levels of natural killer group 2, member D ligands on the U251 cell surface. MIC, MHC class I polypeptide-related sequence; ULBP, UL16 binding protein; FITC, fluorescein isothiocyanate.

increased as the E:T ratio increased. The mRNA expression of MICA/B and ULBP genes was detected in K562 and U251 cells by RT-PCR, and flow cytometry also indicated the presence of MICA/B and ULBP protein. These results indicate that signals for the activation of NK cells may be stimulated by binding of MICA/B and ULBP in U251 cells, and NKG2D

Table III. Cytotoxic activity of natural killer cells on K562 and U251 cells when the effector:target ratio is 20:1 blocking MICA/B and ULBP1-3.

	Cytotoxic activity (%)				
Cell line	AMO-1	BMO-1	M295	M310	M551
K562	49.82±1.74	50.26±2.41	52.42±1.58	50.74±2.16	51.57±1.66
U251	29.01±0.72	30.83±0.91	37.87±0.82	31.45±1.46	39.42±1.07

AMO-1 and BMO-1, and M295, M310 and M551 are monoclonal antibodies that bind to and block MICA/B and ULBL1-3, respectively. Values are presented as the mean ± standard deviation. MIC, MHC class I polypeptide-related sequence; ULBP, UL16 binding protein.

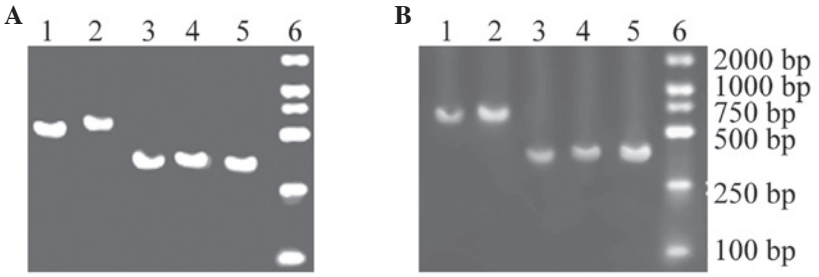

Figure 6. Expression of natural killer group 2, member D ligand genes in (A) K562 and (B) U251 cells using reverse transcription-polymerase chain reaction. Lane 1, MICA (635 bp); lane 2, MICB (690 bp); lane 3, ULBP1 (319 bp); lane 4, ULBP2 (327 bp); lane 5, ULBP3 (321 bp); lane 6, DNA marker. MIC, MHC class I polypeptide-related sequence; ULBP, UL16 binding protein.

receptors in NK cells, leading to the killing of U251 cells by NK cells. To support this hypothesis, monoclonal antibodies for MICA/B and ULBP were used to block NKG2D receptors in U251 cells, and changes to the cytotoxic activity of NK cells were observed. When the E:T ratio was 20:1 and the relevant NKG2D receptors on U251 cells were blocked by the administration of antibodies (AMO-1, BAMO-1, M295, M310 and M551) the cytotoxic effect of NK cells on K562 and U251 cells was significantly reduced ($P<0.05$). The findings of the current study demonstrated that the NKG2D-MICA/B interaction is important for the cytotoxic activity of NK cells on K562 and U251 cells, which also supports the hypothesis of the current study. However, the antibody blocking assay indicated that the cytotoxic activity was not completely inhibited, suggesting that other signaling pathways may contribute to the cytotoxic effect of NK cells on tumors, in addition to the activity mediated by NKG2D (15). As the expression rate of MICA/B and ULBP increased, the cytotoxic activity of NK cells also increased. Thus, expression levels of NKG2D ligands influences the effect of NK cells in the immune response.

Previous studies have demonstrated that if the expression of transforming growth factor-β (TGF-β) increases as the degree of malignant glioma increases, the expression levels of MICA, ULBP2 and 4 are reduced, whereas, the expression levels of MICB, ULBP1 and 3 are not affected. The expression levels of MICA and ULBP2 are upregulated following reduction of TGF-β and inhibitory metalloproteinases *in vitro*, and therefore the NKG2D-mediated cytotoxic effect can be restored. Thus, the major mechanism by which glioma escapes immune surveillance may be via the autocrine expression of TGF-β by neuroglioma cells, and the damage of MICA and ULBP2 by metalloproteinases (16,17). In a previous study, when LN-229 human glioma transfected with the gene encoding MICA were inoculated into nude mice, tumor growth was significantly delayed compared with wild-type LN-229 cells, and tumor size was significantly decreased (18). When rat glioma cells transfected with the genes encoding MICA/B and ULBP were inoculated into homologous mice following radiographic exposure, an immunoprotective effect was generated when SMA560 cells were re-vaccinated. This previous study suggests that upregulation of MICA/B and ULBP expression in tumor cells overcomes the inhibitory signal transduced by human leukocyte antigen-I molecules, and enhances the cytotoxic activity of NK cells. Wu *et al* (19) demonstrated that MHC I and the activating receptors of NK cells were not expressed in $CD133^+$ neuroglioma cells, but the expression of NKG2D receptors was upregulated following incubation with interferon (IFN)-γ. Neuroglioma cells were subsequently killed by NK cells. Thus, increased expression levels of the NKG2D receptor enhances the cytotoxic activity of NK cells and may provide a novel method for the development of immunotherapies.

Autologous NK cells have been applied as a treatment for neurogliosis in Japan (20). Autologous peripheral blood was obtained from patients with glioma then transfused to nine patients exhibiting glioma recurrence. Amplified NK cells possess 82.2±10.5% leukomonocytes (mean ± standard deviation) and the therapeutic effects of NK cells are enhanced by simultaneous venous injection with IFN-β. Clinical results demonstrate that autologous NK cells are safe and somewhat effective for the treatment of glioma (19-23). The present study indicates that allogeneic NK cells exhibit specific cytotoxic activity on glioma cells. Based on the theory of immune

escape, glioma cells evade the cytotoxic effect of NK cells resulting in the development of tumors. The results of the current study suggest that treatment with allogeneic NK cells will present as a superior method to treatment with autologous NK cells in glioma cases.

In conclusion, the current study indicates that allogeneic NK cells exhibit a therapeutic effect against glioma cells and that NK cell cytotoxic activity is associated with the expression of NKG2D ligands. Further research is required to address the mechanism by which the expression of NKG2D ligands may be increased to further enhance the anti-cancer activity of NK cells.

Acknowledgements

The present study was supported by Nanyang Municipal Science and Technology Research Projects (grant nos. 2011GG014 and 2012GG088).

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