Hyperthermia Increases Natural Killer Cell Cytotoxicity against SW-872 Liposarcoma Cell Line

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ABSTRACT

Background: Although there is convincing data in support of the effectiveness of hyperthermia in tumor therapy, the molecular mechanisms underlying the clinical effects of hyperthermia are still poorly understood. **Objective:** To investigate natural killer (NK) cell cytotoxicity against heat-treated SW-872 and HeLa tumor cell lines. **Methods:** NKG2D ligands and HLA class I transcription were examined using quantitative real-time PCR in treated tumor cell lines at 0, 2, 4, 6 and 12 h following thermal treatment at 39°C and 42°C for 1 h. The expression of MICA/B, ULBP1 and ULBP2 were also determined by flow cytometry. NK92-MI cytotoxic activity against heat-treated target cell lines was assessed by LDH release as well as annexin-V and 7-AAD assays. **Results:** Our results showed that heat treatment at 39°C improved the cytolytic activity of NK cells against SW-872 cells without increasing NKG2D ligand concentration or decreasing HLA class I levels. **Conclusion:** The observed increase in the cytotoxicity of NK cells against SW-872 cells after hyperthermia does not coincide with changes in MICA/B, ULBP1 and ULBP2 ligands of NKG2, however, the expression of other ligands in target cells may have made the cells susceptible to the cytotoxic effect of NK cells.

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INTRODUCTION

Hyperthermia was recently proposed as a promising treatment in adjuvant therapy for cancer. In addition to its direct cytotoxic effect, hyperthermia induces innate and specific antitumor immune responses, enhancing the effects of conventional treatment modalities such as radiotherapy and chemotherapy (1). Although high temperatures have a direct cytotoxic effect on tumors, temperatures in the fever range usually induce immune-mediated antitumor responses (2). The beneficial effect of fever in tumor control was previously demonstrated by Coley, who induced artificial fever with a bacterial toxin (3). Whole-body hyperthermia in cancer treatment is generally done at fever-range temperatures, whereas higher temperatures, due to their adverse effects on normal cells, are used preferentially for local treatment (2). Thermotherapy in the fever range may induce thermal tolerance in tumor cells; therefore this treatment modality in tumor management is usually used in combination with radiotherapy or chemotherapy (4).

Important factors that influence tumor cell responsiveness are the origin of the cells, the thermal dose and exposure time (5). In addition to enhancing tumor immunogenicity, hyperthermia can also lead to changes in the number of immune cells and their activity (6). Both stimulatory and inhibitory effects of hyperthermia have been reported for various immune cell functions.

The critical role of natural killer (NK) cells in immunosurveillance and the elimination of neoplastic cells are unquestioned (7). Unlike cytolytic T lymphocytes, NK cells do not require prior stimulation by antigens (8). Their cell function is regulated by a balance between signals that are generated from activating and inhibitory receptors. Natural killer cells have been reported to induce apoptosis in heat-treated target cells (9). In addition, NK cells have also been demonstrated to be highly responsive to heat (10); however, the results of some studies of the effect of hyperthermia on NK cell activity are controversial (11-14). The molecular mechanisms that underlie the regulation of NK cells by heat are still poorly understood. Here, we investigated whether hyperthermia increased the expression of NKG2D ligands in human tumor cell lines and consequently increased their susceptibility to NK cell-mediated cytotoxicity.

MATERIALS AND METHODS

Cell Lines. Human liposarcoma cell line SW-872 (DSMZ, Braunschweig, Germany) and human cervical adenocarcinoma cell line HeLa (ATCC, Manassas, VA, USA) were cultured in RPMI 1640 medium (Gibco, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (Biosera, Brighton, UK), 100 U/mL penicillin and 100 µg/mL streptomycin. The human NK cell line NK92-MI (ATCC) was propagated in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate and 1 mM nonessential amino acids. Cultures were maintained at 37°C, 5% CO_2 and 90% humidity.

***In Vitro* Hyperthermia.** *In vitro* hyperthermia was induced by incubating the target tumor cell lines SW-872 and HeLa at 39°C or 42°C for 1 h in a CO_2 incubator with a humid atmosphere. Heat-treated cells were then transferred to 37°C and analyzed at 0, 2, 6 and 12 h.

Analysis of NKG2D Ligands and HLA Class I Expression in Tumor Cell Lines. The expression of NKG2D ligands and HLA class I was assessed in untreated or heat-treated tumor cell lines at the mRNA and protein levels by quantitative real time polymerase chain reaction (qRT-PCR) and flow cytometry, respectively. Total RNA was extracted from 10^6 cells with RNX-Plus (CinnaGen, Tehran, Iran). RNA samples were treated with RNase-free DNase I (Fermentas, Vilnius, Lithuania) and then transcribed to cDNA with the RevetAid H Minus kit ((Fermentas, Vilnius, Lithuania). The relative expression of MICA/B, ULBP1, ULBP2 and HLA class I was determined by qRT-PCR in triplicate using SYBR Green (Applied Biosystems, Foster City, CA, USA) and normalized to GAPDH with the following forward and reverse primers (Bioneer, Daejeon, South Korea):
5′GGACCAGAAAGRAGGCTTGCATTCCC3′ and 5′AGAGGAAGAGCTCCCCAT CGTAGT3′ for MICA/B, 5′GATCCAACA AAACCACCCTCTCTG3′ and 5′ACAA CTCTCCTCATCTGCCAGCT3′ for ULBP1, 5′GCCTCCTCATCATCCTCCCCTG C3′ and 5′AGACCGTGCTCACAGGAGCC3′ for ULBP2, 5′GCGGAGATCAC ACTGACCT G3′ and 5′CAGAAGGCACCACC ACAGC3′ for HLA class I and 5′GGCTGGGGCTCATTTGCAGG3′ and 5′AGTTGGTGGTGCAGGA GGCA3′ for GAPDH. Real-time PCR conditions were 10 min at 95°C, 40 cycles of 15 s at 95°C, 1 min at 60°C. After amplification, a melting curve analysis was performed by collecting fluorescence data while increasing the temperature from 60°C to 95°C over 1 min. Relative changes in target gene expression were analyzed by Pfaffl method (15). To determine the surface expression of NKG2D ligands on the tumor cell lines, 0.5×10^6 cells were stained with PE-conjugated anti-human MICA/B monoclonal antibody (BD Biosciences, CA, USA), PE-conjugated anti-human ULBP1 monoclonal antibody and PE-conjugated anti-human ULBP2 monoclonal antibody (R&D Systems, Wiesbaden, Germany). The average percentage of cells that expressed each of the molecules studied was determined in three independent experiments and reported as the mean ± SD.

Evaluation of NK Cell Cytotoxicity by LDH Release Assay. Natural killer cell cytotoxic activity against heat-treated or untreated target cell lines was measured with the lactate dehydrogenase (LDH) cytotoxicity detection kit (Roche Applied Science, Bavaria, Germany). The target cell lines were cultured at 10^4 per well for 8 h in 96-well flat-bottomed culture plates. Then the plates were subjected to heat treatment, and 10^5 NK92-MI cells in 100 µL of complete medium containing 5% fetal bovine serum was added at certain time points. This assay was performed three times, each time in triplicate. The plates were incubated at 37°C for 12 h. Then the plates were centrifuged for 10 min at 250 g and LDH release was determined in the culture supernatant according to the manufacturer's instructions. The percentage of cytotoxicity was calculated with the following formula:

$$\text{Cytotoxicity \%} = \frac{(\text{Effector: Target cell mix} - \text{Effector cell control}) - \text{Low control}}{\text{High Control} - \text{Low Control}} \times 100$$

where effector: target represents LDH activity released from coculture of the effector and target cells, effector cell control represents spontaneous LDH release from effector cells, low control represents spontaneous LDH release from target cells, and high control represents maximum LDH activity released from target cells after lysis with 1% Triton X-100.

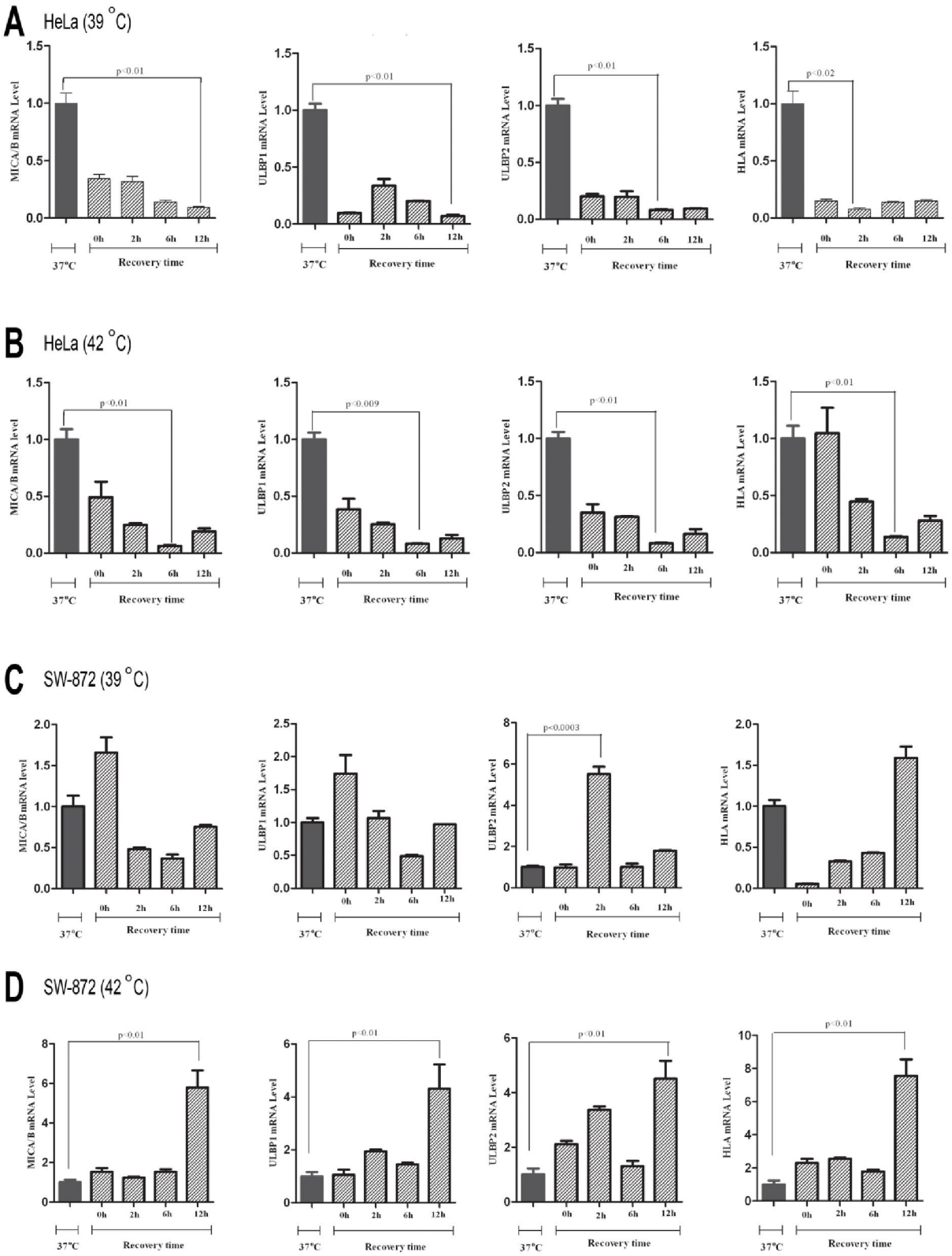

Figure 1. MICA/B, ULBP1, ULBP2, and HLA class I mRNA levels in HeLa cells and SW-872 cells after heat treatment at 39°C (A and C) and at 42°C (B and D) for 1 h and recovery at 37°C at different time points (0, 2, 6 and 12 h).

Evaluation of NK Cell Cytotoxicity by Annexin V and 7-Amino-Actinomycin. SW-872 cells at 10^5 per well were grown in 24-well culture plates, and after heat treatment at 39°C for 1 h the cells were mixed with 10^6 NK92-MI cells at different time points and incubated at 37°C for 12 h. Then cells were harvested using cell dissociation buffer (sodium chloride 8 g/L, potassium chloride 0.4 g/L, potassium phosphate 0.06 g/L, glucose 1 g/L, sodium phosphate, 0.048 g/L, sodium bicarbonate 0.35 g/L, 2 mM EDTA). Target cells were stained with PE-conjugated anti-human MICA/B monoclonal antibody and cytotoxicity was assessed by FITC annexin V apoptosis detection Kit with 7-amino-actinomycin (7-AAD) (Roche Applied Science, Bavaria, Germany). Samples were then analyzed by flow cytometry on a FACS Calibur apparatus (BD Biosciences, CA, USA). To exclude the effector cells, the gate was set on forward scatter versus PE-anti MICA/B and apoptosis or necrosis was calculated in target cells. This test was performed in triplicate.

Statistical Analysis. One-way ANOVA and unpaired Student's *t-test* were used to compare each thermal state with the corresponding cell line under control conditions (at 37°C). All statistical analyses were done with SPSS 17.0 software and $p<0.05$ was considered significant.

RESULTS

Expression of NKG2D Ligands after Heat Treatment. To investigate the effect of hyperthermia on the expression of NKG2D ligands and HLA class I, total RNA was extracted from heat-treated HeLa and SW-872 cell lines and subjected to qRT-PCR. In HeLa cells, decreased levels of NKG2D ligands mRNA and HLA class I were observed at 0, 2, 6 and 12 h after heat treatment at 39°C and 42°C (Figure 1A and B). In SW-872 cells, transcription of NKG2D ligands and HLA class I was considerably increased 12 h after heat treatment at 42°C, but there were no substantial changes after hyperthermia at 39°C except for ULBP2, which was increased 2 h after heat treatment (Figure 1C and D).

The surface expression levels of MICA/B, ULBP1 and ULBP2 on heat-treated HeLa cells and SW-872 cells were assessed by flow cytometry (Figure 2A and B). In comparison to cultures at 37°C, we observed no significant changes in the surface expression of these molecules in HeLa (Figure 3A) or SW-872 cells (Figure 3B) after heat treatment.

NK Cell Cytotoxicity against Heat-Treated Tumor Cells. Natural killer cell cytotoxicity against heat-treated tumor cells at 39°C or 42°C was determined by LDH release assay in comparison to control target cells (37°C). The results showed that NK cell cytotoxicity against heat-treated SW-872 cells was clearly increased after 12 h, and returned to baseline levels after 24 h (Figure 4A). Despite the effect of hyperthermia on SW-872 cells, NK cell cytotoxicity against HeLa cells seemed to be unchanged after heat treatment (Figure 4B). To confirm the results of the LDH release assay, flow cytometry with annexin V and 7-AAD was performed. As shown in Figure 5, NK cell activity against SW-872 cells was increased 6 h after heat treatment at 39°C.

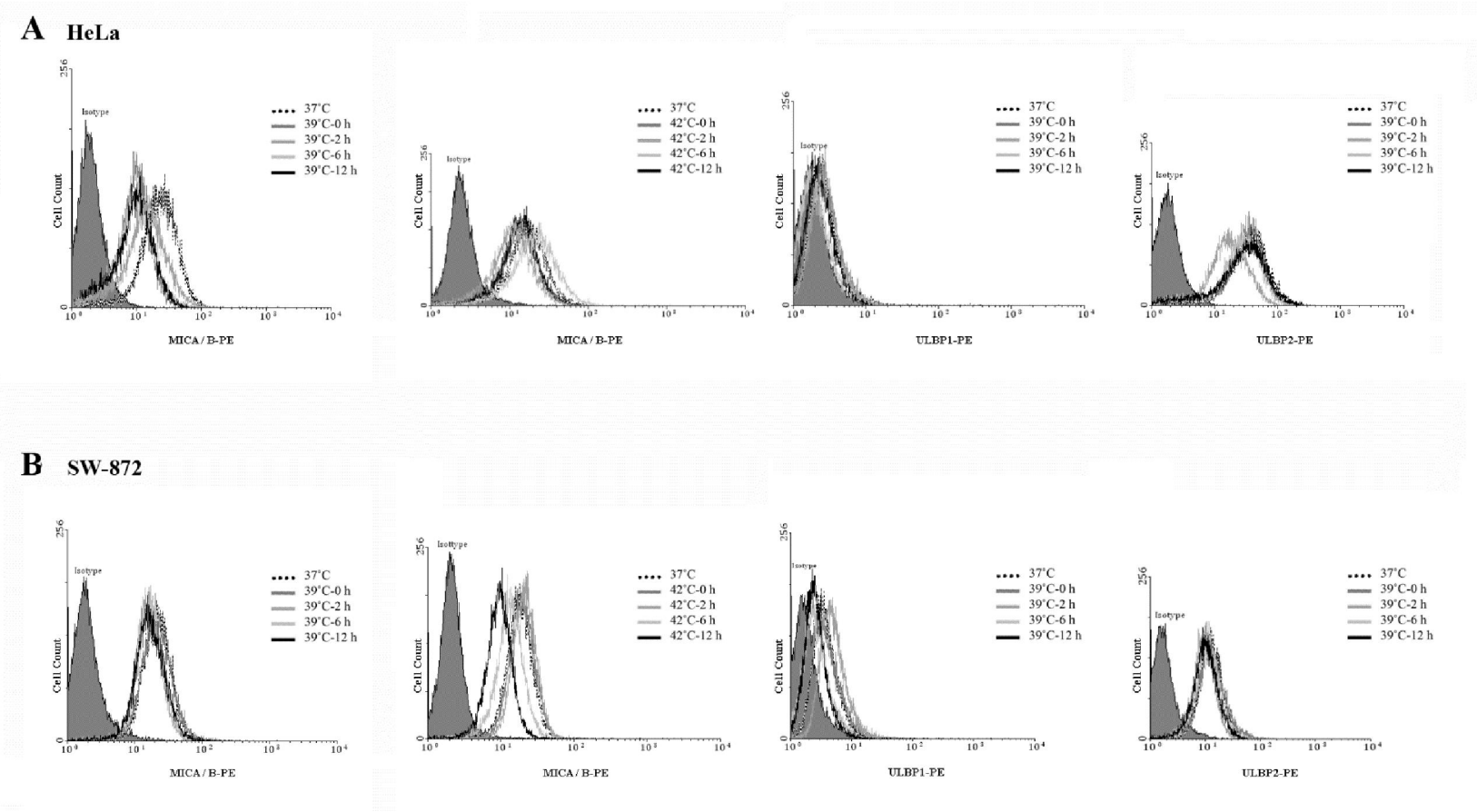

Figure 2. Profile of anti-MICA/B, ULBP1 and ULBP2 reactivity in heat-treated Hela (**A**) and SW-872 cells (**B**) analyzed by flow cytometry.

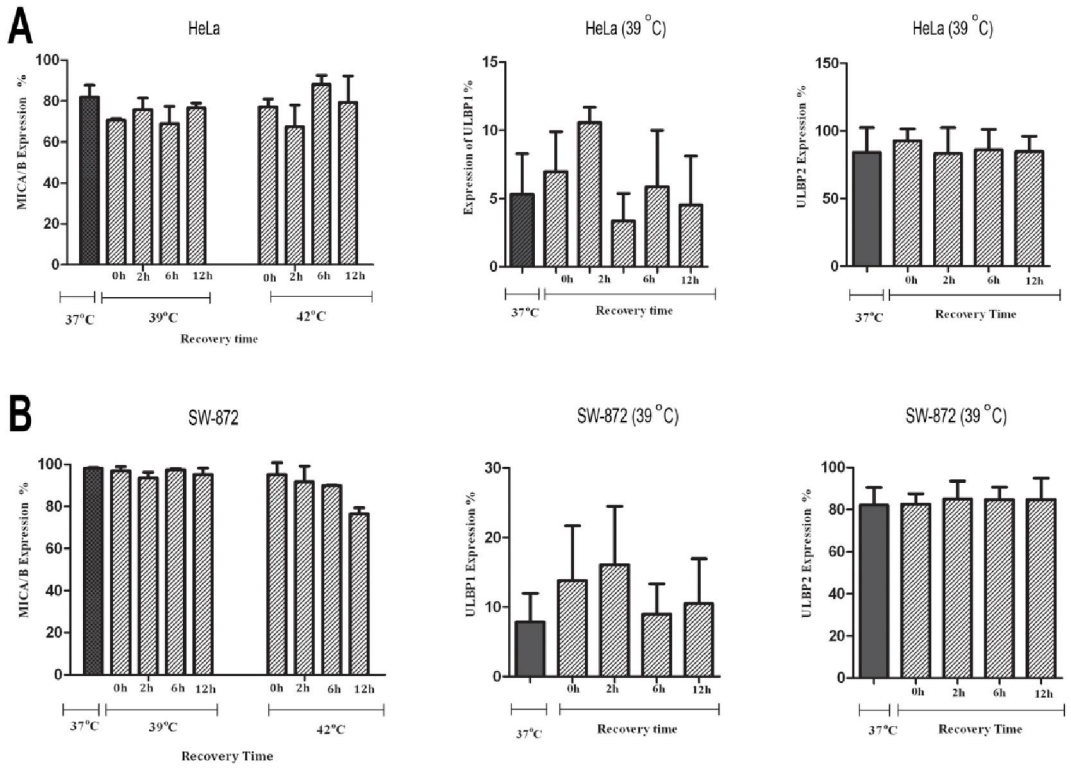

Figure 3. Percentage expression of MICA/B, ULBP1 and ULBP2 on the cell surface in HeLa cells (A) and SW-872 cells (B) after heat treatment at 39°C or 42°C for 1 h and recovery at 37°C at different time points (0, 2, 6 and 12 h).

DISCUSSION

In recent years, hyperthermia has been proposed as a feasible approach to cancer therapy. This method not only affects target cells directly, but can also kill tumor cells by activating the innate and the adaptive immune systems. Additionally, hyperthermia along with other therapeutic methods such as radiotherapy and chemotherapy can be effective in cancer treatment. In the current study, we examined the cellular and molecular effects of hyperthermia on SW-872 (liposarcoma) and HeLa (cervical adenocarcinoma) cell lines. Natural killer cell-mediated cytotoxicity against heat-treated tumor cells was also investigated.

Many studies have shown that hyperthermia increases the activity of the immune system. Some researchers believe that heat shock increases tumor antigenicity by enrichment of NKG2D ligands on tumor cells (16-19). A number of *in vivo* and *in vitro* experiments have assessed the effects of hyperthermia on NK cell activities; however, the results have been inconsistent (11-14). The cytotoxic activity of NK cells was reported to increase against KM12 cells at 42°C (18) and against Colo205 cells at 39°C (20), although other studies reported diminished NK cell cytotoxicity after hyperthermia (10,21,22).

The results of our study showed that cytotoxic activity of NK cells against HeLa cells was not changed after heat treatment at 39°C or 42°C (Figure 4B). Regardless of decreased MICA/B, ULBP1 and ULBP2 mRNA levels (Figures 1A and B), no

significant changes were observed in the corresponding proteins on the surface of this cell line (Figure 3A). It seems that NKG2D ligands on the surface of HeLa cells were preserved 12 h after hyperthermia, and may have decreased in parallel with their mRNA down-regulation. In agreement with our results, Kim et al. observed no augmentation in NK cell cytotoxicity up to 24 h after incubating HeLa cells at 42°C for 1 h. However, they found that the expression of NKG2D ligands was increased after 4 h (18).

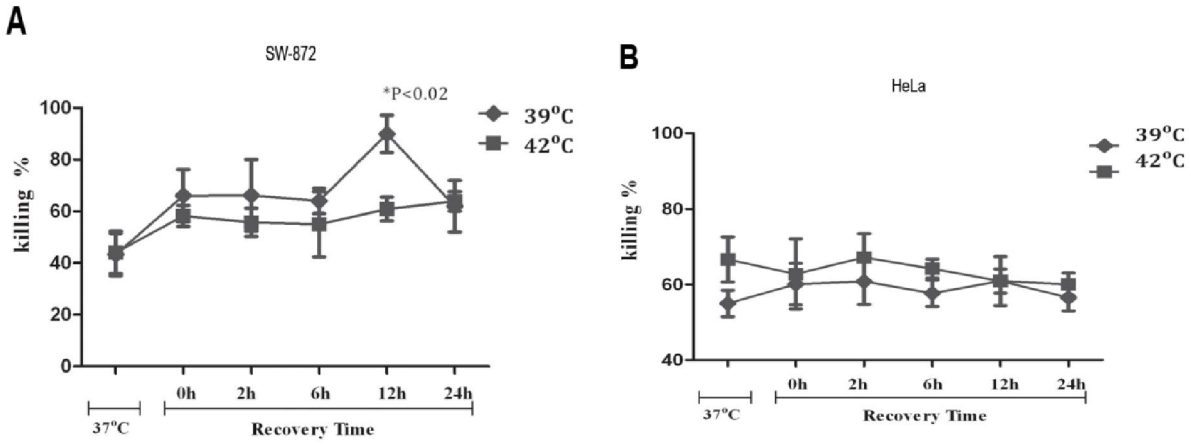

Figure 4. NK92-MI cytotoxicity against SW-872 (**A**) and HeLa (**B**) cells treated at 39°C or 42°C after 0, 2, 6, 12 and 24 h compared to untreated target cells by LDH release assay.

Because the effect of hyperthermia differs somewhat among various tumor cells, we compared the response to heat treatment in SW-872 and HeLa cells. Natural killer cell cytotoxicity against SW-872 cells was significantly increased 12 h after heat treatment at 39°C but not at 42°C ($p<0.02$) (Figure 4A). However, no changes were seen in MICA/B, ULBP1 or ULBP2 transcription (Figure 1C and D) or protein levels after hyperthermia at 39°C (Figure 3B). Moreover, transcription of HLA class I was increased in this condition (Figure 1C), which is in agreement with the results of Ito et al., who reported rat T-9 glioma cell growth suppression despite the increased MHC class I expression after hyperthermia at 43°C for 1 h (23). The down-regulation of MHC class I appears to have no critical effect on the induction of NK cell cytotoxicity after hyperthermia. The effect of hyperthermia appears to be reversible since NK cell cytotoxicity had decreased 24 h after heat treatment at 39°C (Figure 4A).

To confirm the results of NK cell cytotoxicity against heat-treated SW-872 cells according to the LDH release assay, we also used annexin V and 7-AAD to evaluate the rate of apoptosis and necrosis in target cells by flow cytometry. The results of this analysis showed maximum target cell death after 6 h, which returned to baseline levels after 12 h (Figure 5). Unlike the LDH release assay, which detects the late stages of cell death due to the rapid flip-flop of phosphatidylserine across the target cell plasma membrane, flow cytometry with annexin V detects the early stages of apoptosis.

Hyperthermia is followed by substantial alterations in the pattern of gene expression in tumor cells. Microarray studies have identified differences in the expression of 1,213 of 23,000 genes in heat-treated tissues compared to untreated tissues. Most immune-related genes have been shown to be down-regulated during the first few hours after hyperthermia. Immune responses are transiently inhibited shortly after hyperthermia,

and then enhanced following the upregulation of heat shock proteins (23,24). These molecules, especially HSP70, play an important role in NK cell activity. KLRD1 (CD94) has been proposed to be a HSP70 receptor on NK cells (25). Therefore the results of our findings based on NKG2D ligands and HLA class I mRNA down-regulation in the early hours after hyperthermia in the HeLa cell line is consistent with the results of gene expression studies involving large numbers of genes.

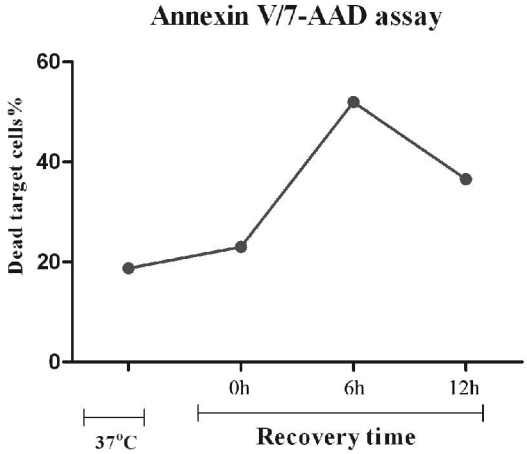

Figure 5. NK92-MI cytotoxicity against SW-872 cells treated at 39°C after 0, 6 and 12 h compared to untreated target cells by flow cytometry with annexin V and 7-AAD.

In conclusion, heat treatment at 39°C appears to enhance NK cell cytolytic activity against SW-872 cells without an associated increase in NKG2D ligand levels or HLA class I down-regulation. After hyperthermia, the expression of other ligands in target cells may make them susceptible to the cytotoxic effects of NK cells.

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