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Original Article

Increased concentration of sialidases by HeLa cells might influence the cytotoxic ability of NK cells

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Abstract

Objective: Cancer cells reportedly have the ability to escape from the immune system, mainly from natural killer (NK) cells. Although the real mechanisms are complicated, some inhibitors that are secreted from the cancer cells might play an important role. This study's aim was to investigate the potential mediator released by cancer cells (HeLa) that contributes to the decreased cytotoxicity of NK cells.
Methods and Materials: An NK-HeLa coculture system was used to test the hypothesis that the presence of the potential mediator from cancer cells contributes to the decreased cytotoxicity of NK cells.
Results: After coculturing with HeLa cancer cells, the cytotoxicity of NK cells was decreased. When the coculture medium and culture medium containing commercialized sialidase were used to culture NK cells, the cytotoxicity of the NK cells was also inhibited. However, cytotoxicity was partially restored by a sialidase inhibitor (DANA). Western blot analysis of the HeLa cells after coculturing with NK cells demonstrated increased Neu2 and Neu3 expression in HeLa cells.
Conclusions: The finding that Neu2 and Neu3 expression in cancer cells might be involved in the impaired function of NK cells, which could be restored by a sialidase inhibitor, provides a new concept that could be applied to the management of cancer.

Keywords: cervical cancer cell line; cytotoxicity; natural killer cell; neuraminidase; neuraminidase inhibitor; sialidase

Introduction

Natural killer (NK) cells were initially identified as a lymphoid population that represents 10–20% of peripheral blood mononuclear cells (PBMC), and they are important players in the first line of defense against diseases because of their ability to lyse major histocompatibility complex class I (MHC-I)-negative tumors and virus-infected cells and orchestrate the innate immunity of the organism. The rapid cytotoxic actions and broad target range suggest that NK cells may be promising candidates for use in cancer cell therapy, with the potential to target a wide range of malignancies [1]. However, cancer cells often escape from NK cells, which results in therapeutic failure. Although the tumor escape mechanisms developed by cancers are complicated and uncertain, some of them could be explained by inhibitors that are secreted from cancer cells [2]. The restoration of immune

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functions is of great importance to tumor eradication, most particularly in regard to the residual disease [3]. Therefore, restoration of the cytotoxicity of the NK cells by blockage or neutralization of these cancer cell-secreting inhibitors might provide be valuable for use in cancer treatment.

In this report, we used a coculture system that mixed NK cells and cancer cells (a cervical cancer HeLa cell line) to investigate the possible mechanisms of tumor-induced immunosuppression.

Materials and methods

Cell lines

A human cervical cancer cell line, HeLa, was obtained from the Culture Collection and Research Center, Food Industry and Development Institute, Taiwan. HeLa cells were grown in DMEM (GibcoBRL, Grand Island, NY, USA) that was supplemented with 10% fetal bovine serum (FBS). The NK-sensitive cell line, K562, was cultured in RPMI 1640 (GIBCO BRL) that was supplemented with 10% FBS. All cell types were cultured in 10-cm dishes at 37°C in a humidified atmosphere with 5% CO_2. Cell number and viability were determined by staining with trypan blue, and the cell count was determined using a hemocytometer (MARIENFELD/MATSUNAMI, Germany).

NK cells

PBMCs were separated from leukapheresed adult peripheral blood by Ficoll-Hypaque density gradient centrifugation (Amersham Biosciences, GE Healthcare Life Sciences. Piscataway, NJ, USA) [4]. PBMCs were resuspended at a concentration of 10^6 cells/mL and cultured for 18 days. Then, the NK cells were isolated from the PBMCs by auto-MACS (NK Isolation Kit; Miltenyi Biotec, Germany) and cultured for 1 day. The PBMCs and NK cells were maintained in RPMI 1640 medium that was supplemented with 10% FBS and 1000 U/mL recombinant human interleukin (IL)-2 (PeproTech Asia, Israel) at 37°C in a humidified atmosphere with 5% CO_2.

Interactions between NK and HeLa cells

A constant number of NK cells were directly cultured with the HeLa cells (at a 1:1 ratio) for 1.5 hours or 3.5 hours at 37°C in the NK-HeLa coculture system. Meanwhile, the cells were also treated with or without DANA (sodium 5-Acetamido-2,6-anhydro-2,3-dehydro-3,5-dideoxy-D-glycero-D-galactononanoate; DANA, CALBIOCHEM, La Jolla, CA, USA).

Cytotoxicity assay

K562 cells were incubated with 100 μCi of ^{51}Cr for 60 minutes at 37°C and then extensively washed with medium to remove any free ^{51}Cr. These radiolabeled target cells were seeded onto round-bottom 96-well microplates, then the NK cells or NK-HeLa cells were cultured with radiolabeled K562 cells (E:T ratio of 1:1) and incubated for 3 hours at 37°C. Supernatants containing the ^{51}Cr that was released from the lysed targets were collected and the radioactivity was counted using a Packard gamma counter (PerkinElmer Inc., United States). All experiments were repeated at least three times. Percent-specific cytotoxicity was calculated according to the formula: (sample ^{51}Cr released − spontaneously released)/(maximum amount released − amount spontaneously released) × 100 [5].

Sialidase and DANA treatment

NK cells were suspended at a concentration of 10^6 cells/mL in the RPMI 1640 medium and incubated in the presence or absence of 0.2 U/mL sialidase (N-6514; Sigma, St. Louis, MO, USA) and different doses of 1mM or 2mM DANA for 2 hours at 37°C followed by extensive washing with RPMI 1640 medium and 0.25% FBS.

Detection of sialidase activity and expression of HeLa cells in the coculture system

HeLa cells were seeded in 96well plate with 4×104 cells/well and fixed with cold methanol (100μL/well) for 5 minutes. Then remove cold methanol and incubated with the substrate 100μL per well contained 1 mM of X-Neu5Ac and 0.1 M sodium acetate (pH 4.8) and Fast Red Violet LB (0.1 mg/mL) for 1 hour at 37°C. The cells were then washed twice with PBS and detected using a fluorescence reader (Molecular Devices Corporation, SpectraMax M2, Molecular Devices, Sunyvale, CA, USA) with excitation wavelengths ranging from 520–550 nm and an emission wavelength of 580 nm [6,7]. In addition, the sialidase activity of the cocultured medium was detected using an Amplex Red Neuraminidase (Sialidase) Assay Kit (Invitrogen, San Diego, CA, USA), according to the manufacturer's recommendations.

Immunoblotting

Cells were homogenized and solubilized in SoluLyse-M Protein Extraction Reagent (Genlantis, San Diego, USA). The protein content was determined using the Bradford assay (Bio-Rad Laboratories, Inc., California, USA). The cell lysates were separated using SDS-PAGE and analyzed by immunoblotting with the respective antibodies using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, California, USA) [8]. The blots were probed using monoclonal anti-Neu2 antibody (Abnova, Taipei City, Taiwan) and anti-Neu-3 mAb (MBL Japan, Nagoya, Japan), respectively. Capture and analysis of the images of the protein bands was performed by using an imaging system equipped with a CCD camera (AlphaImager 2200; Alpha Innotech Corporation) and quantified by software AlphaEaseTM (Alpha Innotech Corporation, San Diego, CA, USA).

Statistical analysis

Statistical analysis was performed using the SPSS 10.0 software package (SPSS Science, Chicago, IL, USA). The statistics are presented as the means ± standard deviation (SD), and the n value of each group represents the individual case number that was performed and, in each case, every parameter was analyzed three times. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by an least significant difference (LSD) multiple range test to analyze differences in the group means in comparison with the control means. Statistical significance was defined as $p < 0.05$, as indicated by an asterisk (*), and $p < 0.01$, as indicated by a double asterisk (**).

Results

Cytotoxicity of the NK cells decreased after coculturing with HeLa cells

IL-2 was added during the differentiation and proliferation stages of the NK cells. During the culturing period, the cytotoxic effects of the IL-2-activated NK cells on the K562 cells gradually increased. To determine whether the cancer cells altered the cytotoxicity of the NK cells, the non-cocultured NK cells or NK cells were cocultured with HeLa cells for 1.5 or 3.5 hours to determine the 3-hour cytotoxic effects on the K562 cells. The results show that the cytotoxic abilities of the NK cells were significantly decreased (75 ± 8% in the 1.5-hour group and 70 ± 8% in the 3.5-hour group, respectively; Fig. 1A).

Increased sialidase activity of the cocultured medium

The sialidase activity of the cocultured medium was evaluated at 1.5 and 3.5 hours. As shown in Fig. 1B, the sialidase activity increased by 20% and 40% after 1.5 and 3.5 hours of incubation, respectively, in a time-dependent manner.

Effect of sialidase and DANA on NK cytotoxicity

The cytotoxic abilities of the sialidase-treated and non-treated NK cells were assessed to determine whether sialidase reduces the cytotoxic ability of NK cells. The results reveal that 0.2 U/mL sialidase does significantly impair the cytotoxic ability of NK cells (Fig. 2). The neuraminidase inhibitor, DANA (1 mM or 2 mM), did block the inhibitory effects of sialidase in a dose-dependent manner, although the effects were only partial (Fig. 2).

Cytotoxicity of NK cells was partially restored by DANA in the NK-HeLa coculture system

Based on the abovementioned findings, we determined (1) decreased cytotoxicity of the NK cells in the NK-HeLa coculture system, (2) increased sialidase activity in the cocultured medium, and (3) adding sialidase to the NK culture system mimicked the cocultured medium in terms of the

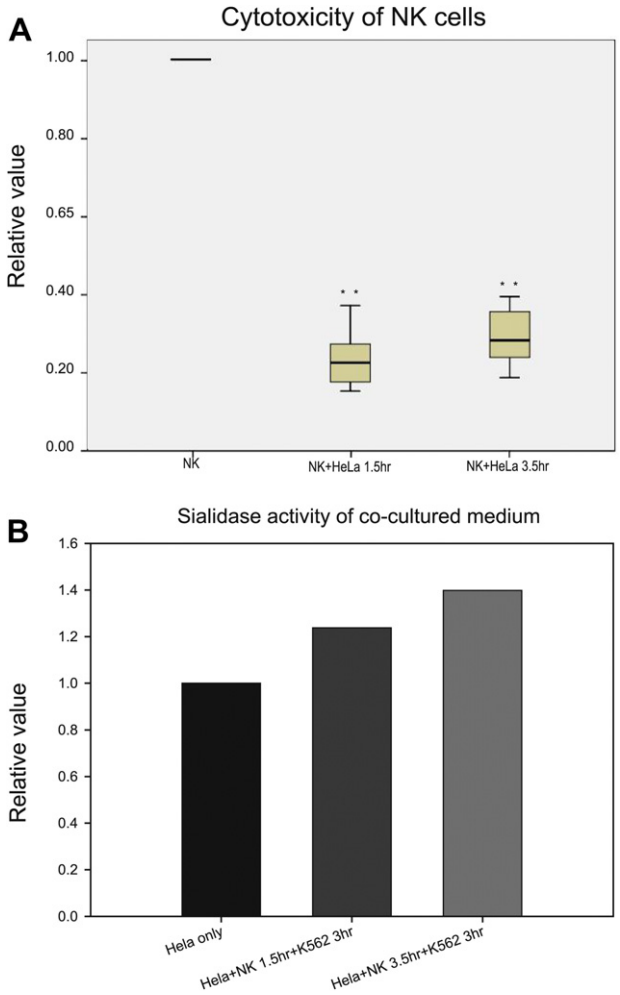

Fig. 1. (A) Cytotoxicity of cocultured NK cells. NK cells were cocultured with HeLa cells for 1.5 or 3.5 hours, and then K562 cells were added and incubated for an additional 3 hours. The cytotoxicity of the NK cells was measured relative to the NK+HeLa cells in the 1.5- or 3.5-hour group. (B) Sialidase activity of the cocultured medium. HeLa cells were cocultured with NK cells for 1.5 or 3.5 hours, and then K562 cells were added and incubated for an additional 3 hours. After coculturing, the medium was collected and tested for sialidase activity. Data on the cocultured medium is shown relative to the data of the HeLa cell medium. $^{**}p < 0.01$.

inhibitory effects on the cytotoxicity of the NK cells, which could be partially restored by adding DANA. It is rational to suppose that adding DADA to the NK-HeLa coculture system would restore the cytotoxicity of NK cells. Therefore, we added 1 or 2 mM DANA to the NK-HeLa coculture system and, as predicted, we found that DANA did restore the cytotoxic ability of the NK cells in the NK-HeLa coculture system, ranging from 10–25% in a dose- and time-dependent manner, although the effect was not significant in the 1.5-hour group, regardless of the concentration of DANA (Fig. 3A–B).

Sialidase activity of the HeLa cells after coculturing with NK cells

The sialidase activity of the HeLa cells in the coculture system was determined using a fluorometric assay that used

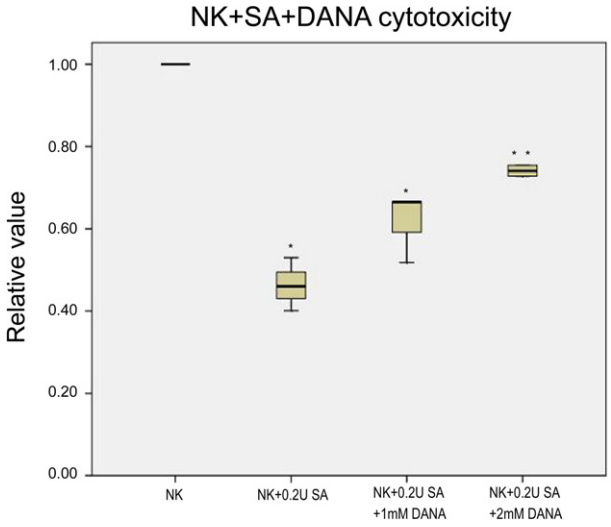

Fig. 2. Cytotoxicity of the NK cells treated with sialidase (SA) or sialidase inhibitor (DANA). NK cells were treated with 0.2 U/mL SA or 1 mM or 2 mM DANA at 37°C for 2 hours. NK cells were then cocultured with ^{51}Cr-labeled K562 tumor cells at E:T cell ratios of 1:1 for 3 hours. The cytotoxicity of the treated NK cells is shown relative to cytotoxicity of nontreated NK cells. $*p < 0.05$; $**p < 0.01$.

4-MUα-Neu5Ac. Compared to non-cultured HeLa cells (control), sialidase activity of the HeLa cells which were cultured with NK cells for 1.5hour or 3.5hour increased 1.4 ± 0.4 and 1.6 ± 0.4-fold, respectively, suggesting that the sialidase secreted from HeLa cells might decrease the cytotoxic effects of NK cells (Fig. 4A).

Increased Neu2 and Neu3 expression of the HeLa cells in the coculture system

Using Western blot analysis, we further evaluated the protein expression levels of the HeLa cells after coculturing with NK cells. In agreement with the enzyme activity of the sialidase expression of Neu2 and Neu3 in the HeLa cells after coculture with NK cells, the expression levels of Neu2 protein were increased by 22% and 33% in the 1.5-hour and 3.5-hour groups, respectively, in the HeLa cells after coculture with NK cells in comparison with the controls (Fig. 4B). The expression level of Neu3 protein in the HeLa cells was also increased 24% and 42% in the 1.5-hour and 3.5-hour groups, respectively (Fig. 4C).

Discussion

Changes in the immune system can be a consequence of an underlying malignancy or they can be induced by antineoplastic treatment [9]. Both affect the risk of infectious complications and relapse. Over the past decade, there have been a series of major developments in the laboratory assessment of immune dysfunction, particularly as it relates to the complex interactions between distinct components of the immune system [9]. Among these assessments, the use a coculture system might be a good tool for evaluating the interactions between NK and cancer cells. We could use this

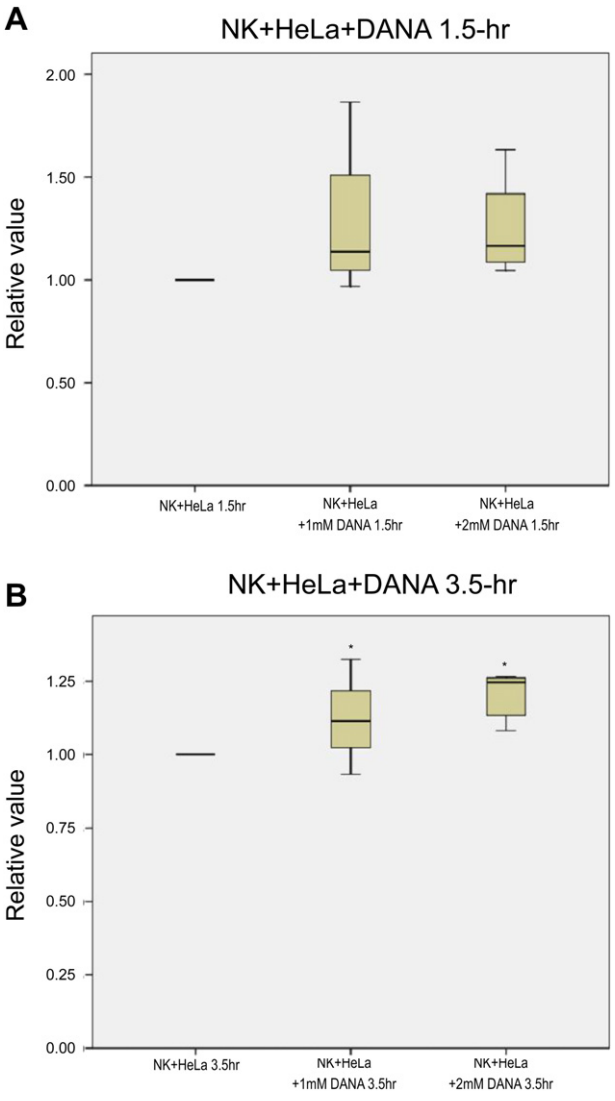

Fig. 3. NK cell cytotoxicity relative to NK+HeLa cells in the 1.5- or 3.5-hour groups. NK cells were cocultured with HeLa cells with or without DANA for (A) 1.5 hours or (B) 3.5 hours, then K562 cells were added and incubated for an additional 3 hours. The cytotoxicity of the DANA-treated groups is shown relative to the cytotoxicity of the group that was not treated with DANA. $*p < 0.05$.

system to evaluate the candidate mediators and investigate the potential candidate molecules that can restore the original conditions because the restoration of immune functions is of great importance to tumor eradication, and we should be able to overcome the various tumor escape mechanisms that have developed in cancer cells. In this study, we used the NK-HeLa coculture system to clearly demonstrate that the cytotoxicity of NK cells is impaired by HeLa cancer cells. The inhibitory effect of the NK-HeLa coculture system suggests that the interactions between NK and HeLa cancer cells do occur and that the HeLa cancer cells might have affected the NK cells, thereby contributing to the tumor escape mechanisms. We suppose that the HeLa cancer cells might have secreted some mediators, which are involved in the decreased cytotoxicity of the NK cells. Therefore, the culture medium of the NK cells, with and without HeLa cancer exposure, might provide us

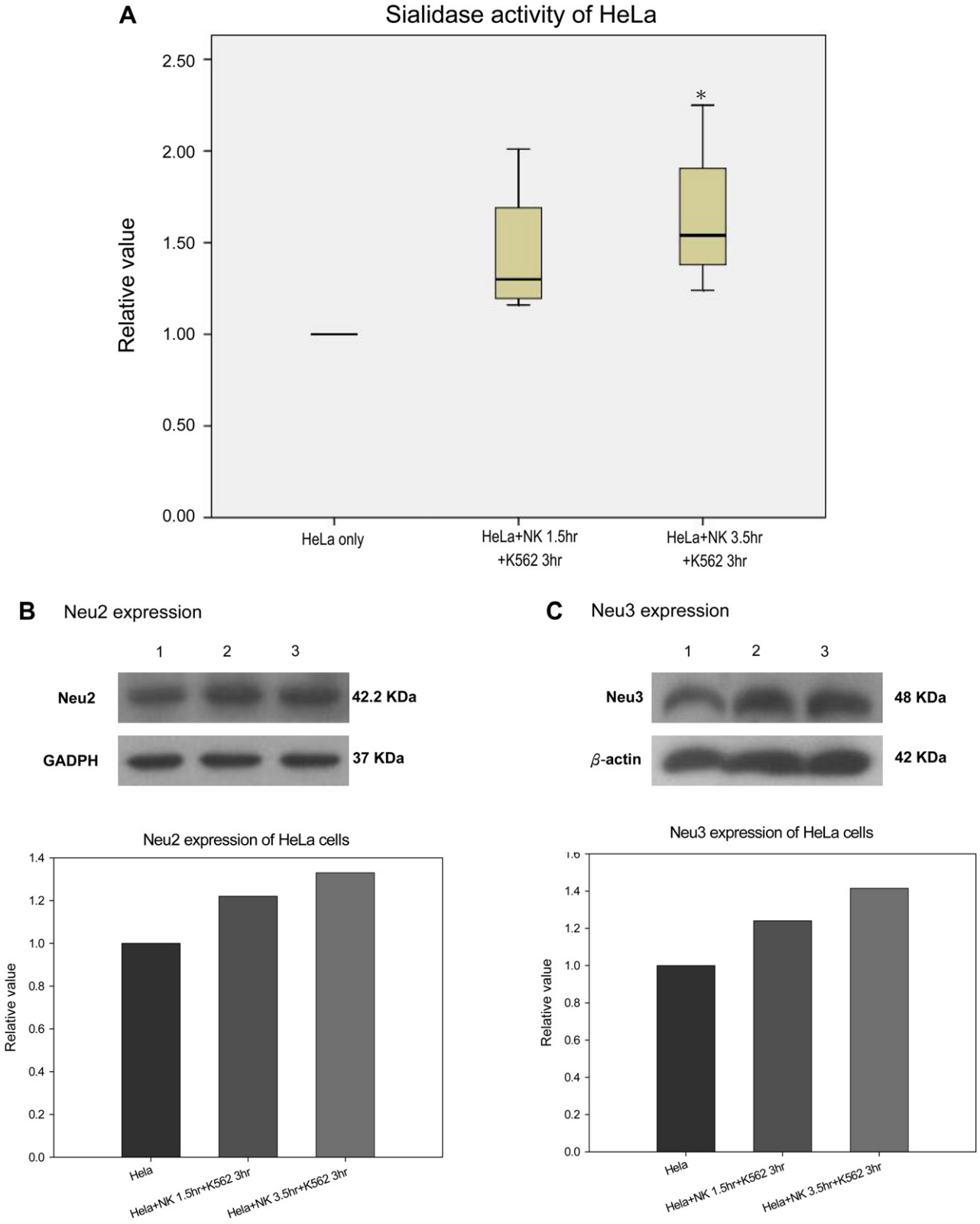

Fig. 4. (A) Sialidase activity of HeLa cells. HeLa cells were cocultured with NK cells for 1.5 or 3.5 hours, and then K562 cells were added and incubated for an additional 3 hours. After coculturing, the HeLa cells were fixed and tested for sialidase activity. The value of the cocultured HeLa cells is shown relative to HeLa cells. (B, C) Western blot analysis of the HeLa cells. 1) HeLa only; 2) HeLa cells cocultured with NK cells for 1.5 hours; 3) HeLa cells cocultured with NK cells for 3.5 hours. Protein expression levels were measured in the HeLa cells. 1) HeLa cells only; 2) HeLa cells cocultured with NK cells for 1.5 hours and 3) HeLa cells cocultured with NK cells for 1.5 hours or 3.5 hours. K562 cells were added and incubated for an additional 3 hours. $*p < 0.05$.

with some useful information. We evaluated changes in the culture medium of the NK cells, with and without HeLa cancer cells, and found many candidate molecules, including cytokines and proteins, that were elevated in the coculture medium. Many of these, including IL-6 and matrix metalloproteinases (MMPs; especially MMP 2 and 9), have been previously reported [10,11]. Among the increased molecules that were identified in the coculture medium in this study, sialidase attracted us because sialidases remove sialic acids and greatly influence many biological processes by changing the conformations of glycoproteins, thereby affecting recognition and masking the biological sites of functional molecules [12]. Therefore, cellular functions and behaviors will be changed if an alteration to sialylation occurs [13,14]. It is rational to suppose that sialidases might be possible mediators by decreasing the cytotoxicity of NK cells when NK cells are exposed to HeLa cancer cells because killer lectin-like receptors on NK cells mediate cytotoxicity via the glycans on the target cells [15]. In this study, we used different strategies, including the use of a coculture medium, to replace the culture medium of the NK cells and administer commercialized sialidase into the culture medium of the NK cells. We found that the cytotoxicity of NK cells was indeed impaired, and that this inhibitory effect could be restored by the administration of a neuraminidase inhibitor (in this study, DANA), supporting the hypothesis that the sialidase in the NK-HeLa coculture system contributed to the decrease in the cytotoxicity of the NK cells. However, the origin of sialidase is still uncertain. The sialidase activities of the NK and HeLa cells in the coculture system were checked, and we found that the sialidase activity of the HeLa cells was increased, which suggests that the sialidase in the coculture medium originated from the HeLa cells. Western blot analysis further confirmed the increased expression of sialidases in the HeLa cells in the coculture system and that the types of sialidases were Neu2 and Neu3.

The relationship between the sialidases and cancer has been extensively reviewed by Miyagi [10]. Sialidases are glycosidases that catalyze the removal of α-glycosidically linked sialic acid residues from the terminal positions of the carbohydrate groups of glycoproteins and glycolipids, which is the initial step in the degradation of these glycoconjugates [10,12]. Sialidase activity may be implicated in cellular growth and differentiation, neoplastic transformation, and possibly cell-to-cell interactions [16−18]. There are four types of mammalian sialidases that have been identified and characterized so far: these are designated as Neu1, Neu2, Neu3, and Neu4. The first three are localized predominantly in the lysosomes, cytosol, and plasma membranes, respectively, and the fourth, Neu4, is a recently identified sialidase that has been suggested to exist in lysosomes, mitochondria, and certain intramembranous components [10]. Among these sialidases, Neu3 has been most often found to be elevated in various kinds of cancer cell lines, including renal cancer, colon cancer, and cervical cancer cell lines [10,19−23]. In this study, we also found that the exposure of HeLa cells to NK cells further increases the expression of Neu2 and Neu3, and that Neu2 and Neu3, at least partially, are secreted by HeLa cells after coculture with NK cells. Therefore, the use of DANA (a neuraminidase inhibitor) could successfully restore the inhibitory effects of NK cells in a medium that has been supplemented with sialidase. However, the administration of DANA could only partially restore the cytotoxicity of NK cells in the NK-HeLa coculture system, suggesting that other mediators might contribute to the decreased cytotoxicity of the NK cells. We also found that IL-6 was elevated in the coculture system (data not shown). IL-6 not only affects NK cell functions [24], but also enhances Neu3 promoter luciferase activity by 2.5-fold and significantly enhances endogenous sialidase activity in human RCC ACHN cells [10]. Therefore, the presence of at least IL-6 in the coculture medium might also contribute to the decreased cytotoxicity of NK cells.

We used PBMC NK cells as the effector cells in this study, and this is the main limitation that requires further attention because it was difficult to obtain an adequate number of NK cells from one adult donor in order to perform all of the experiments of this study. However, based on our findings, we can still conclude that HeLa cancer cells increase the secretion of sialidase (Neu2 and Neu3) while in coculture with NK cells, and that the secretion of sialidase contributes to the decreased cytotoxicity of NK cells. This inhibitory effect can be restored by a neuraminidase inhibitor, suggesting that the use of sialidase inhibitors might be valuable in the management of cervical cancer. This sort of therapy would be helpful for the restoration of immune functions in order to eradicate residual tumors, which might not be completely cured by the various kinds of established therapies already in use.

Acknowledgments

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