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Original Article

Altered ganglioside GD3 in HeLa cells might influence the cytotoxic abilities of NK cells

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Abstract

Objective: Previously, we found that altered sialidases in HeLa cells in a natural killer-HeLa (NK-HeLa) coculture system contributed to the decreased cytotoxic ability of NK cells. However, changes that occur in the glycosylation of the HeLa cells in the NK-HeLa coculture system remain unknown.
Materials and Methods: An NK-HeLa coculture system was used to examine the changes that occur in the gangliosides of HeLa cells.
Results: GD3 expression in HeLa cells was significantly increased in the NK-HeLa coculture system. Exogenous ganglioside GD3 decreased the cytotoxic ability of the NK cells, which could be restored by the addition of the anti-GD3 antibody. Coadministration of GD3 and sialidase further decreased the cytotoxic ability of the NK cells, which could be partially restored by the addition of a sialidase inhibitor (DANA). GD3 expression in HeLa cells also decreased following DANA treatment.
Conclusions: This study suggests that interactions between ganglioside GD3 and sialidases in HeLa cells influence the cytotoxic ability of NK cells.

Keywords: cervical cancer; cytotoxicity; gangliosides; natural killer cells; sialidases

Introduction

Cervical cancer is the second most common malignancy that presents in women worldwide [1], and some studies have reported that immune cell function is influenced by cancer [2–4]. Natural killer (NK) cells play a crucial role in resisting viral infection in cervical tissues and are a key component in tumor immunosurveillance [5]. However, a lot of data indicates that NK cell cytotoxicity is downregulated during cancer development, including cervical carcinoma [6–9].

GD3 ganglioside, a glycosphingolipid that contains one or more sialic acid (SA) residues, is markedly overexpressed in almost all melanoma tissues and plays a role in the growth of malignant cells [10]. It has been proposed that GD3 ganglioside could possibly inhibit the immune response in humans [11, 12] and that the addition of the GD3 monoclonal antibody (R24) to NK cells could promote antibody-dependent cellular cytotoxicity (ADCC), thereby resulting in tumor shrinkage in cancer patients [13,14].

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Besides GD3 itself, terminal SA has also been reported to be involved in cellular interactions, communication, and possibly immune responses [15,16]. SA is almost always present on the glycoproteins and glycolipids that are widely distributed throughout nature [17]. Aberrant sialylation is closely associated with the malignant phenotype of cancer cells, including metastatic potential and invasiveness [18].

Sialidases (neuraminidase) influence cellular activity by removing terminal SA residues from glycoproteins and glycolipids. Four genetically distinct sialidases have been identified in mammalian cells, each with a predominant cellular localization [19,20]. Neu1 (the lysosomal form) plays a catabolic role in desialylating glycoproteins and is involved in the cellular signaling of immune responses. Neu2 (the cytosolic form) can desialylate glycoproteins and gangliosides and appears to play a role in myoblast differentiation. Neu3 (the plasma membranous form) plays a regulatory role in cellular activation, differentiation, and transformation. Neu4 (the lysosomal or mitochondrial form) has been identified and may be involved in cell apoptosis. It is well known that the expression levels of sialidases are related to cancer progression [21,22]. In addition, the enzymatic removal of all SA residues from NK cells by sialidase (neuraminidase) impairs the immune functions of NK cells [23,24]. In a previous study, we found that expression of sialidases, especially Neu2 and Neu3 in HeLa cells, is increased in an NK-HeLa coculture system [25]. However, the gangliosides that change in HeLa cells in this NK-HeLa coculture system are uncertain.

Materials and methods

Cell lines

The human cervical cancer cell lines—CC7T, CaSki, and HeLa—and the NK-sensitive cell line—K562—were obtained from the Culture Collection and Research Center, Food Industry and Development Institute (Hsin-Chu, Taiwan). CC7T cells and HeLa cells were grown in DMEM (Gibco, GibcoBRL, Grand Island, NY, USA) and supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS; 10,000 U/mL; Gibco). CaSki cells and K562 cells were grown in RPMI1640 medium (Gibco) and supplemented with 10% FBS and 1% PS. All cell types were cultured in 10-cm dishes at 37°C in a humidified atmosphere with 5% CO_2.

NK cells

Peripheral blood mononuclear cells (PBMCs) were separated from leukapheresed adult peripheral blood by Ficoll-Hypaque density gradient centrifugation (Amersham Biosciences, GE Healthcare Life Sciences. Piscataway, NJ, USA) [26]. PBMCs were resuspended at a concentration of 10^6 cells/mL and cultured for 18 days. Then, NK cells were isolated from the PBMCs by auto-MACS (NK Isolation Kit; Miltenyi Biotec, Germany) and cultured for 1 day. PBMCs and NK cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 1% PS, 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

Using the same procedure as a previous study [25], the interactions between NK and HeLa cells were analyzed using a constant number of NK cells, which were directly cultured with HeLa cells (in a 1:1 ratio) in RPMI 1640 medium that had been supplemented with 10% FBS, 1% PS, and 1000 U/mL IL-2 for 3.5 hours at 37°C in an atmosphere that contained 5% CO_2. In the sialidase inhibition experiment, the cocultured cells were treated with or without the sialidase inhibitor DANA (sodium 5-Acetamido-2,6-anhydro-2,3-dehydro-3,5-dideoxy-D-glycero-D-galactononanoate; Calbiochem, MERCK, Darmstadt, Germany).

NK cell cytotoxicity assay

2×106 K562 cells were incubate with 100 μCi of 51Cr in 1mL RPMI1640 medium at 37°C for 60 minutes, and then centrifuged at 3000×g to remove the supernatant. The K562 cells were washed by RPMI1640 medium then centrifuged at 3000×g to remove supernatant. These radiolabeled target cells were seeded onto round-bottom 96-well microplates onto which the NK cells, NK/CC7T, NK/CaSki, or NK/HeLa cells were cultured (NK:target ratio of 2:1, 1:1, and 0.5:1, respectively) and incubated for 3 hours at 37°C in an atmosphere that contained 5% CO_2. Supernatants containing the ^{51}Cr that was released from the lysed target cells were collected, and the radioactivity was counted using a Packard gamma counter (PerkinElmer Inc., United States). All experiments were repeated at least three times. The percentage of specific lysis was calculated according to the following formula**:** (sample ^{51}Cr released− spontaneously released) × 100/(maximum amount released − amount spontaneously released) × 100 [27].

Flow cytometric analysis

Mouse monoclonal anti-human GD3 antibody (R24; Abcam, Abcam Ltd, Cambridge, UK) was labeled with fluorescein isothiocyanate (FITC) using the E-Z FITC labeling kit (Pierce, Rockford, IL USA), according to the manufacturer's directions. The FITC-conjugated monoclonal anti-human CD56 antibody, isotype control, and propidium iodide were purchased from Biolegend (Bio-legend, San Diego, CA, USA).

To detect GD3 expression in the HeLa cells, the cells were incubated with FITC-labeled anti-human GD3 antibody or the isotype control antibody for 30 minutes at 4°C. After washing with PBS, the cells were analyzed using an FACSort flow cytometer (BD Biosciences, California, USA). To determine the viability of the NK cells, the cells were incubated with FITC-labeled anti-human CD56 antibody for 30 minutes at 4°C, followed by a PBS wash, and then propidium iodide was added to exclude the dead cells. Data analysis was performed using BD CELLQuest software (BD Biosciences, California, USA).

GD3 ganglioside and sialidase treatment

NK cells were suspended at a concentration of 10^6 cells/mL in RPMI 1640 medium that was supplemented with 10% FBS and incubated in the presence of each of the following combinations: (1) GD3 ganglioside (10 or 20 mg/mL; Matreya, Inc., PA, USA"; monoclonal anti-GD3 antibody R24 (10 or 20 mg/mL; Abcam Ltd., Cambridge, UK; (2) 0.2 U/mL sialidase (N-6514; Sigma-Aldrich, USA) with or without DANA (1 or 2 mM, Calbiochem); (3) 10 or 20 μg/mL GD3 ganglioside with or without 0.2 U/mL sialidase for 3.5 hours at 37°C, followed by extensive washing with RPMI 1640 medium supplemented with 0.25% FBS and testing using a cytotoxicity assay.

The HeLa cells were seeded at a concentration of 1×10^5 cells/well on 48-well plates and grown in 200 μL DMEM supplemented with 10% FBS. The cells were treated with 0.2 U/mL sialidase, with or without 1 or 2 mM DANA, for 3.5 hours at 37°C, followed by extensive washing with DMEM medium supplemented with 0.25% FBS, then tested using a flow assay to detect GD3 expression. The cells were then washed twice with PBS and analyzed using a fluorescence reader (Molecular Devices, Sunnyvale, CA, USA) at excitation wavelengths ranging from 520–550 nm and an emission wavelength of 580 nm [28–30].

Statistical analysis

Statistical analysis was performed using the SPSS 17.0 software package (SPSS Science, Chicago, IL, USA). The statistical significances of two groups were determined using the unpaired *t* test, and more than two groups were analyzed using one-way ANOVA followed by the post hoc *t* test with the least significant difference (LSD) correction. Differences between two conditions were considered statistically significant at $p < 0.05$ (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

Results

Reduced NK cell cytotoxicity when cocultured with cervical cancer cell lines

Consistent with a previous study [25], NK cells were isolated from the PBMCs of healthy people and cocultured with various kinds of cervical cancer cell lines (CC7T, CaSki, and HeLa cells), followed by NK-mediated lysis using a chromium release assay that used K562 cells as the target cells. As shown in Fig. 1A significant decrease in the killing efficiency was observed in the cocultured NK cells in comparison with NK cells alone. NK cells that were cocultured with HeLa cells showed the lowest cytotoxic activity compared with NK cells

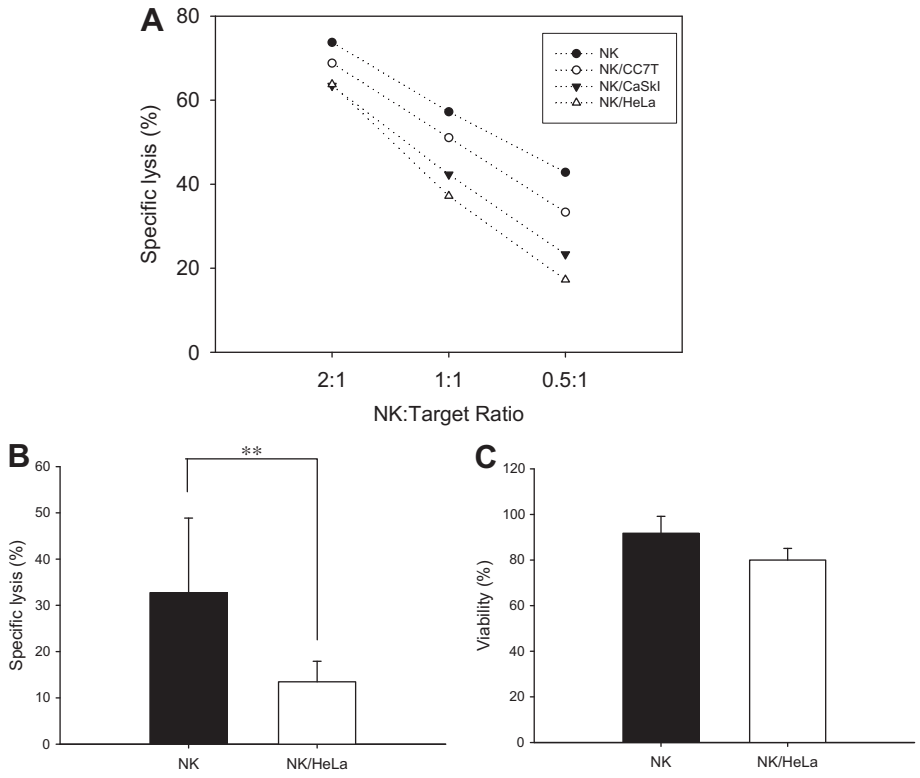

Fig. 1. Coculture with cervical cancer cell lines decreases the cytotoxicity of NK cells (A) NK cells were cocultured with or without CC7T (○), CaSki (▼), and HeLa (▽) tumor cell lines, respectively. After 3.5 hours of coculturing, the NK cells were assessed for their ability to kill K562 target cells using a chromium release assay at the indicated NK:target ratio. (B) NK cells were cocultured with (gray bar) or without HeLa cells (black bar) for 3.5 hours, then tested using a chromium release assay at an NK:target ratio of 1:1. Data are shown as the means ± SD ($n = 6$). (C) After the NK cells and HeLa cells were cocultured, the viability of the NK cells was assessed by flow cytometry using FITC-labeled anti-human CD56 antibody and propidium iodide staining. The viability of the cocultured NK cells (white bar) was compared with NK cells alone (black bar). Data are shown as the means ± SD ($n = 3$).

that were cocultured with the other cell lines; therefore, the NK cells that were cocultured with HeLa cells were used as the experimental model for the following analyses (Fig. 1B). The viability of the NK cells was comparable between NK cells that were cultured alone and cocultured NK cells (Fig. 1C).

Exogenous GD3 ganglioside and sialidase treatment reduces the cytotoxic effects of NK cells

GD3 ganglioside, which has been reported as a possible inhibitor of NK cell cytotoxicity, is expressed at tremendously high levels in almost all melanoma tissues and cell lines [9,10]. To examine whether NK cell cytotoxicity is inhibited by exogenous GD3, NK cells were incubated with different doses of GD3 and the GD3 antibody, by analysis using the chromium release assay. After treatment with 10 or 20 μg/mL GD3 ganglioside, NK cell cytotoxicity decreased in a dose-dependent manner. The decreased cytotoxicity of the NK cells was restored following treatment with GD3 plus the GD3 antibody (Fig. 2A). In addition to GD3 ganglioside, sialidase treatment also reduced NK cell activities. Consistent with a previous study [25], sialidase inhibitor (DANA) restored the inhibition of NK cell cytotoxicity (Fig. 2B). When the NK cells were cotreated with sialidase and GD3, sialidase enhanced the suppression of NK cell cytotoxicity that was caused by GD3 (Fig. 2C).

Upregulation of GD3 and sialidase in HeLa cells when cocultured with NK cells

Our data show that exogenous GD3 and sialidase down-regulate NK cell cytotoxicity; however, we would also like to know whether GD3 and sialidase were induced by coculturing the NK and HeLa cells. To determine this, cocultured HeLa cells were stained with the FITC-labeled anti-human GD3 antibody. Flow cytometric analysis showed that the GD3 expression of the cocultured HeLa cells was approximately 1.8-fold higher than non-cocultured HeLa cells (Fig. 3A). Our data suggest that the high GD3 expression level of the cocultured HeLa cells could be a factor in the interaction between HeLa and NK cells. As shown in Fig. 3B and 3C, sialidase activity was also upregulated in the cocultured HeLa cells, as well as in the cultured medium, after the HeLa and NK cells were cocultured.

Upregulation of GD3 in HeLa cells when treated with sialidase

Based on the abovementioned findings, ganglioside plus sialidase treatment can enhance the suppression of NK cell cytotoxicity, so we tested whether ganglioside and sialidase interact together by examining the effects of DANA. HeLa cells that were cocultured with NK cells and incubated with

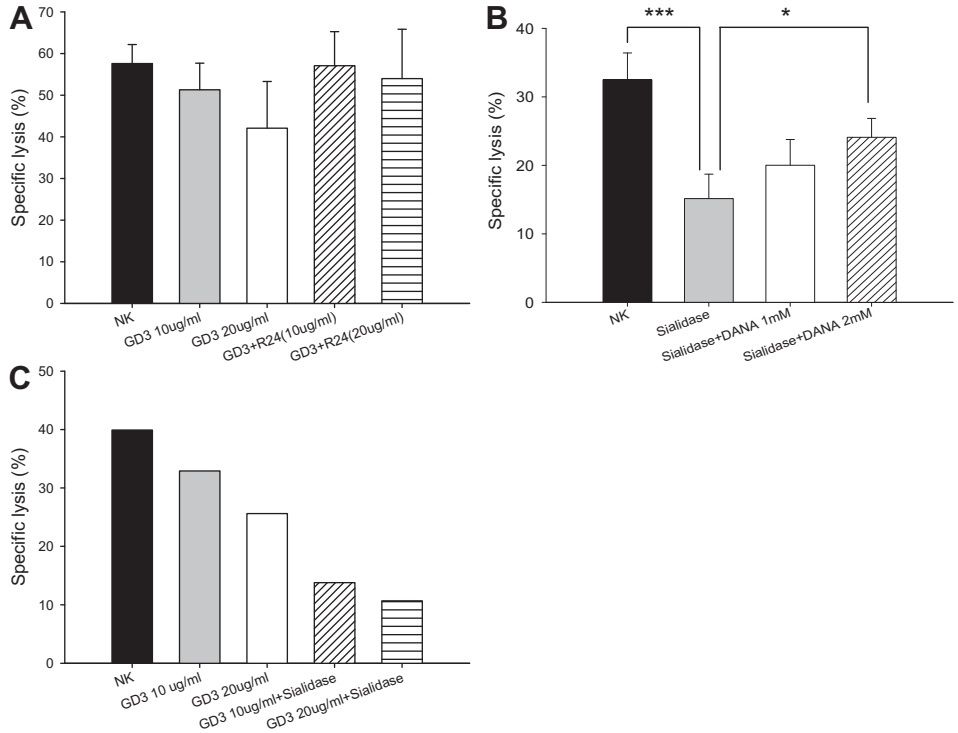

Fig. 2. Exogenous GD3 or sialidase treatment reduces NK cell cytotoxicity (A) NK cells were treated with GD3 ganglioside alone (gray and white bar) or GD3 plus GD3 antibody (R24) (slash and horizontal line bar) *in vitro*. (B) NK cells were treated with sialidase alone (gray bar) or sialidase plus the sialidase inhibitor DANA (white and slash bar) *in vitro*. After 3.5 hours of treatment, the cells were assessed for their ability to kill K562 target cells using a chromium release assay at an NK:target ratio 1:1. The killing efficiency of the treated NK cells was compared with that of the nontreated NK cells (black bar). Data are shown as means ± SD ($n = 3$). (C) NK cells were treated with GD3 ganglioside alone (gray and white bar) or GD3 plus sialidase (slash and horizontal line bar) *in vitro*. After 3.5 hours of treatment, the cells were assessed for their ability to kill K562 target cells using a chromium release assay at an NK:target ratio of 1:1.

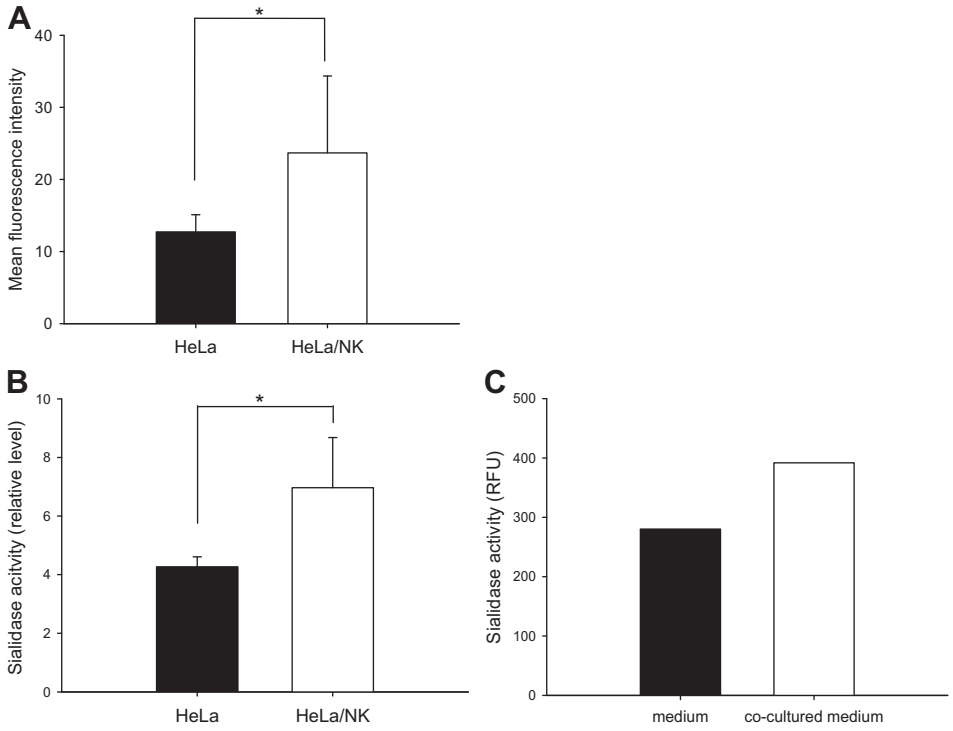

Fig. 3. GD3 and sialidase are upregulated in HeLa cells after coculturing with NK cells (A) After coculturing with (white bar) or without NK cells (black bar), GD3 expression of the HeLa cells was detected by flow cytometry using FITC-labeled anti-GD3 antibody. Data are shown as the means ± SD ($n = 5$). (B) After coculturing with (white bar) or without NK cells (black bar), the sialidase activity of the HeLa cells was detected as described in Materials and Methods section. The values shown are relative to the control (HeLa cells plus PBS) and as the means ± SD ($n = 4$). (C) After coculturing with (white bar) or without NK cells (black bar), the sialidase activity in the cultured medium was detected as described in Materials and Methods section.

1 or 2 mM DANA were then tested using FACS (FACSort flow cytometer; BD Biosciences, United States) analysis in order to observe any changes in GD3 expression. The data show that DANA can significantly eliminate the high expression levels of GD3 in cocultured HeLa cells (Fig. 4A). We also treated HeLa cells with sialidase and DANA to study the effect of GD3 expression of HeLa cells using FACS analysis. As shown in Fig. 4B, the level of GD3 expression of the HeLa cells was increased following sialidase treatment, as well as that of the HeLa cells that were cocultured with NK cells, and this increase could be recovered by DANA. These results suggested that an interaction between GD3 and sialidase might be involved in mediating NK cell activity during the coculturing of HeLa and NK cells.

Discussion

It has been proposed that NK cell cytotoxicity is downregulated in patients with cervical carcinoma [7,8]. In this study, we found that NK cells from healthy people also exhibit

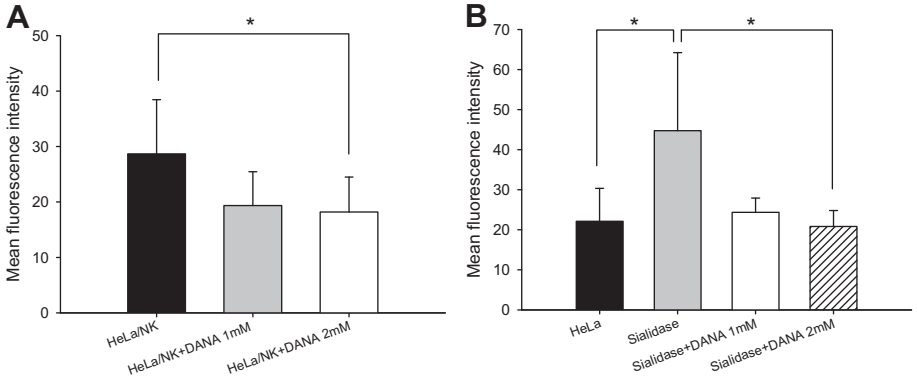

Fig. 4. GD3 expression is upregulated by sialidase and inhibited by DANA (A) HeLa cells were cocultured with NK cells alone (black bar) or NK cells plus DANA (gray and white bar). After coculturing, the level of GD3 expression in the HeLa cells was detected by flow cytometry using the FITC-labeled anti-GD3 antibody. Data are shown as means ± SD ($n = 5$). (B) HeLa cells were treated with sialidase alone (gray bar) or sialidase plus DANA (gray and slash bar) *in vitro*. After coculturing, the GD3 expression of the HeLa cells was detected by flow cytometry using the FITC-labeled anti-GD3 antibody. Data are shown as means ± SD ($n = 3$).

the same phenomenon after coculturing with HeLa cells [25]. These results suggest that cervical cancer could possibly escape the immune response by suppressing NK cell cytotoxicity. To investigate how cervical cancer interacts with NK cells and restrains their cytotoxicity, we used HeLa cells as the experimental model and cocultured HeLa cells with NK cells to test our hypothesis.

We suppose that sialidase and GD3 act synergistically to inhibit NK cell cytotoxicity. Although many studies have addressed activated NK cell resistance to virus-infected and tumor cells via a balance between inhibitory and activating receptors, such as NKp30, NKp44, NKp46, and NKG2D [31–33], GD3 and sialidase are less often reported in studies on the interactions between NK cells and cancer cells. We followed Nicoll's study by treating NK cells with sialidase and then testing the changes in the cytotoxicity of the NK cells [23]. However, our data show that NK cell cytotoxicity is directly inhibited by sialidase treatment without interactions between siglec-7 and GD3. These results differ from that of Nicoll, but are the same as those reported by Moebius [24]. In addition to sialidase treatment, NK cells incubated with GD3 showed decreased cytotoxic activities as well. When NK cells were cotreated with GD3 plus sialidase, suppression of NK cell cytotoxicity was evident. These results indicate that sialidase and GD3 could act synergistically to inhibit NK cell cytotoxicity.

GD3 is a glycosphingolipid that contains α2,8-linked disialic acids, and sialidase is an enzyme that can remove terminal sialic acid residues from glycoproteins and glycolipids. GD3 is the substrate of sialidase, however we observed that both sialidase activity and GD3 expression were upregulated in HeLa cells after coculturing with NK cells. Moreover, sialidase treatment enhanced the level of GD3 expression in HeLa cells, and the higher GD3 expression could be recovered by the sialidase inhibitor, DANA. These data imply that after coculturing with NK cells, HeLa cells can secrete sialidase in order to increase GD3 expression, and then sialidase and GD3 act synergistically to inhibit NK cell cytotoxicity. This is possibly a self-protection mechanism used by cervical cancer to escape NK cell immunosurveillance.

The downregulated cytotoxicity of NK cells that was caused by HeLa cells can be partially recovered by DANA treatment. However, the inhibition of cytotoxicity in cocultured NK cells could not be recovered to the level of the non-cocultured NK cells by the use of DANA. These data indicate that there are other factors involved in the interaction between NK and HeLa cells. It has been reported that malignant cells could produce soluble factors, such as immunosuppressive cytokines and prostaglandins, in order to escape immune responses [34]; therefore, we evaluated the cytokines that were released by NK and HeLa cells during coculturing using "the Human Cytokine Protein Array 3.1 kit (AAH-CYT-3-4), an antibody-based array (RayBiotech, Inc, Norcross, Ga), which could detect 42 cytokines simultaneously, including epithelial neutrophil activating peptide (ENA)-78, granulocyte colony-stimulating factor (GCSF), granulocyte/macrophage colony-stimulating factor (GM-CSF), growth regulated oncogene (GRO), growth regulated oncogene-α (GRO-α), monokine-induced by interferon-γ (MIG), interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, interferon-gamma (IFN-gamma), monocyte chemoattractant protein-1 (MCP-1), MCP-2, MCP-3, macrophage colony-stimulating factor (MCSF), macrophage-derived chemokine (MDC), monokine-induced by interferon-γ (MIG), macrophage inflammatory protein-1δ (MIP-1δ), regulated upon activation, normal T-cell expressed and secreted (RANTES), stem cell factor (SCF), stromal cell derived factor-1 (SDF-1), thymus and activation-regulated chemokine (TARC), transforming growth factor-β1 (TGF-β1), tumor necrosis factor (TNF)-α, TNF-β, epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I), Angiogenin, Oncostatin M (OSM), Thrombopoietin, vascular endothelial growth factor (VEGF)-A, platelet-derived growth factor-β (PDGF-β), and leptin. We found that GRO, GRO-α, MCP-1, IL-6, -8, -10, -13, and IFN-γ were upregulated in the cocultured medium (data not shown). The cultured medium of NK cells and cocultured medium of NK and HeLa cells were detected by human cytokine antibody array. Compared to the cultured medium of NK cells (control), GRO, GRO-a, MCP-1, IL-6, -8, -10, -13, and IFN-gamma were increased in the cocultured medium of NK and HeLa cells. ELISA assays were used to confirm the result of cytokine array. The data showed that the cocultured medium of HeLa cells and NK cells from different peoples might result in different increase levels of IFN-gamma and IL-13. Furthermore, using the ELISA assay, we found that IFN-γ and IL-13 expression levels differed between the NK cells of individual people, but IL-6, IL-8 and IL-10 were comparable (data not shown). Based on these data, we suggest that IFN-γ and IL-13 are produced by NK cells and IL-6, IL-8 and IL-10 might be secreted by tumor cells. Among these cytokines, IL-6 (26-fold induction) and IL-10 (4.6-fold induction) might be the dominant cytokines involved in mediating NK cell cytotoxicity because they are believed to be present in the tumor microenvironment in order to promote tumor growth and suppress immune responses [35–38]. However, the manner in which these cytokines mediate NK cell cytotoxicity during coculturing requires further investigation.

Taken together, the interaction between cancer cells and immune cells might be more complicated than the parameters of our testing. However, based on our results, we have identified the important role played by both GD3 and sialidases in the interaction between cervical cancer cell lines and NK cells, specifically that both could inhibit the cytotoxic ability of NK cells. These findings are worthy of further investigation.

Acknowledgments

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