Binding Affinities of NKG2D and CD94 to Sialyl Lewis X-Expressing \(N\)-Glycans and Heparin

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Lectin-like receptors natural killer group 2D (NKG2D) and CD94 on natural killer (NK) cells bind to \(2,3\)-NeuAc-containing \(N\)-glycans and heparin/heparan sulfate (HS). Using recombinant glutathione \(S\)-transferase-fused extracellular lectin-like domains of NKG2D (rGST-NKG2Dlec) and CD94 (rGST-CD94lec), we evaluated their binding affinities \(\left(K_D\right)\) to high sialyl Lewis X (sLe\(X\))-expressing transferrin secreted by HepG2 cells (HepTf) and heparin-conjugated bovine serum albumin (Heparin-BSA), using quartz crystal microbalance (QCM) and enzyme immunoassay (EIA) microplate methods. \(K_D\) values obtained by linear reciprocal plots revealed good coincidence between the two methods. \(K_D\) values of rGST-NKG2Dlec obtained by QCM and EIA, respectively, were 1.19 and 1.11 \(\mu\)M for heparin-BSA \(>0.30\) and 0.20 \(\mu\)M for HepTf, while those of rGST-CD94lec were 1.31 and 1.45 \(\mu\)M for HepTf \(>0.37\) and 0.36 \(\mu\)M for heparin-BSA. These results suggested that these glycans can interact with NKG2D and CD94 to modulate NK cell-dependent cytotoxicity.

Key words natural killer group 2D; CD94; sialyl Lewis X; heparin; natural killer cell

Natural killer (NK) cells play important roles in innate immunity and in immune surveillance of malignant transformed cells and viral-infected cells. The cytotoxic activity of NK cells is regulated by a balance of opposing signals through activating and inhibiting cell-surface receptors of the immunoglobulin and C-type lectin superfamilies.

CD94 forms disulfide-linked heterodimers with NK group 2 (NKG2) A, B, C, or \(H\), which recognize the ligand leukocyte antigen (HLA)-E, which is constitutively expressed on human cells. Inhibitory receptors NKG2A and B have two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic domains, while activating receptors NKG2C, E, and H have a positively charged residues within their transmembrane regions and associate with the immunoreceptor tyrosine-based activating motif (ITAM)-containing 12 kDa adaptor molecule DNAX-activating protein (DAP12).

CD94 forms a salt-bridged hexamer with two homodimers of the YINM motif-containing adaptor molecule DAP-10 in humans and recognizes several major histocompatibility complex (MHC) class I related ligands, such as MHC class I related chain family proteins (MIC) A/B and the UL 16-binding proteins (ULBPs) 1–4 in humans.

The C-type lectin-like receptors CD94 and NKG2D lack most of the conserved Ca\(^{2+}\)-binding residues, and the glycans for NKG2D homodimer and CD94/NKG2S heterodimers have yet to be resolved. Mouse melanoma B16-F1 cells transfected with the fusosyltransferase (FUT) 3 gene and overexpressing sialyl Lewis X (sLe\(X\)), NeuAc\(2,3\)-Gal\(β1,4\)(Fuc\(α1,2\))GlcNAc-R, have been reported to be more susceptible to lysis by NK cells in \textit{vivo}, which is prevented by pretreatment with anti-CD94 and anti-sLe\(X\) antibodies. Similarly, we found that FUT 3 gene-transfected K562 (K562/FUT) cells selected for high expression of sLe\(X\) were more susceptible than K562 wild to lysis by NK-derived KHYG cells in \textit{vivo}, and that this susceptibility was suppressed by pretreatment of K562/FUT cells with anti-sLe\(X\) and KHYG cells with anti-NKG2D and anti-CD94 antibodies. In previous reports, we found that recombinant glutathione \(S\)-transferase (GST)-fused extracellular domains of NKG2D AA 73-216 (rGST-NKG2Dlec) and CD94 AA 68-179 (rGST-CD94lec) bound to plates coated with multivalent sLe\(X\)-expressing HepG2-derived transferrin (HepTf), \(α2,3\)-linked NeuAc-remodeled human \(α\)-acid glycoprotein (\(α2,3\)-NeuAc AGP), and heparin-bovine serum albumin (HepTf).

In the present report, we further characterized the binding affinities of rGST-NKG2Dlec and rGST-CD94lec to glycan ligands using quartz crystal microbalance (QCM) and enzyme immunoassay (EIA) microplate methods, finding that rGST-NKG2Dlec binds to HepTf with higher affinities than heparin, while rGST-CD94lec binds more preferably to heparin than to HepTf. These results suggested that these glycans can interact with NKG2D and CD94 to modulate NK cell-dependent cytotoxicity.

MATERIALS AND METHODS

Preparation of rGST-NKG2Dlec and rGST-CD94lec rGST-NKG2Dlec and rGST-CD94lec were prepared as described in previous reports. Briefly, the extracellular domains of NKG2D (AA 73-216, NKG2Dlec) and CD94 (AA 68-179, CD94lec) were amplified from KHYG-derived cDNA using primers 5’-CACCATATGGAGTCTGATTCCCTAAAC-3’ (forward) and 5’-TTACAGTCCTTTGCA-3’ (reverse) for NKG2Dlec and 5’-CACCTACCGGT-GCAACTGTATCT-3’ (forward) and 5’-TTAAATGACGT-GTTGCTACAG-3’ (reverse) for CD94lec, respectively. The purified polymerase chain reaction (PCR) products were ligated into the pGEX4T-1 vector (GE Healthcare Bio-Science, Uppsala, Sweden) with Ligation High (Toyobo Co., Tokyo, Japan). The sequence was confirmed by DNA sequencing using a 3037xl DNA analyzer (Applied Biosystems, Foster City, CA, U.S.A.). The recombinant plasmids were transformed into Chaperone competent cells pTf16/BL21 (Takara, Otsu, Japan) and positive clones were confirmed by DNA sequencing.

After induction with 1 \(\text{mm}\) isopropyl-\(β\)-D-thiogalactopyrano-
noside (Promega Co., Madison, WI, U.S.A.) at 15 °C over night, the cells collected from 1000 ml culture medium by centrifugation at 3000 rpm for 10 min and suspended in 3 ml phosphate buffered saline (PBS) were sonicated for 10 s × 6 on ice. After centrifugation at 15000 rpm for 10 min and filtration with Ministart 0.2 mm-filter (Sartorius Stedim Biotech, Goettingen, Germany), soluble lysate was applied on a GStrapTM FF column (GE Healthcare) and recombinant proteins were eluted with 10 mM glutathione reduced form (GSH) according to the manufacturer’s instructions. The proteins dialyzed against PBS and concentrated by ultrafiltration, were separated on 0.1% sodium dodecyl sulfate (SDS) 10% polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie brilliant blue. Protein concentrations were determined using the Advanced protein assay reagent (BioFX Lab., Owings Mills, MD, U.S.A.) for 5 min at room temperature. The reaction was stopped by the addition of 100 μl of 1 M H2SO4, and absorbance was read at 450 nm with a Model DTX800 plate reader (Beckman Coulter, Fullerton, CA, U.S.A.). Non-specific binding to NorTF- and BSA-coated plates was subtracted from the binding to HepTF- and heparin-BSA-coated plates, respectively. Using linear reciprocal plots, [M]/ΔAbs versus [M], Kd values were calculated as described above.

**RESULTS**

**Determination of Binding Affinities of rGST-NKG2Dlec and rGST-CD94lec to HepTF and Heparin-BSA Using QCM** To confirm direct binding of NKG2D and CD94 to glycans, we constructed GST-fused extracellular domains of NKG2D (rGST-NKG2Dlec) and CD94 (rGST-CD94lec). To measure the binding affinities (Kd values) of rGST-NKG2Dlec and rGST-CD94lec to HepTF and heparin, we utilized a QCM system, in which the binding amounts on the QCM sensor can be directly measured by the decrease in frequency. We immobilized HepTF and heparin-BSA on the sensor followed by blocking with 0.1% BSA, and the frequency changes were monitored for 4 h after addition of rGST-NKG2Dlec and rGST-CD94lec to the solution at final concentrations of 0 to 1—3 μM (Fig. 1). In typical sensorgrams, the decreases in frequency (ΔF) were dependent on the amounts of rGST-NKG2Dlec and rGST-CD94lec added. As shown in Fig. 2, using linear reciprocal plots, [M]/ΔF versus [M], the Kd values of rGST-NKG2Dlec to HepTF and heparin-BSA were determined to be 0.30 (R²=0.9936) and 1.19 (R²=0.9980) μM, and those of rGST-CD94lec to HepTF and heparin-BSA were 1.31 (R²=0.9706) and 0.37 (R²=0.9935) μM, respectively.

**Determination of Binding Affinities of rGST-NKG2Dlec and rGST-CD94lec to HepTF and Heparin-BSA Using an EIA Microplate Assay** To further clarify the binding affinities, we prepared 96-well plates coated with HepTF and heparin-BSA and determined the binding of rGST-NKG2Dlec and rGST-CD94lec to these plates using EIA methods indicated that the Kd values of rGST-NKG2D were heparin>HepTF, while those of rGST-CD94lec were HepTF>heparin. These results suggested that soluble forms of HepTF and heparin can interact with NKG2D and CD94 to modulate NK
cell-dependent cytotoxicity through competition with the ligands on the target cells.

DISCUSSION

In this report, we investigated the binding affinities of rGST-NKG2Dlec and rGST-CD94lec to glycans using QCM and EIA microplate methods. The \( K_d \) values obtained by the two methods were in good coincidence and indicated that rGST-NKG2Dlec binds more preferentially to HepTf (\( K_d: 0.3, 0.20 \mu M \), obtained by QCM and EIA, respectively) than to heparin-BSA (1.19, 1.11 \( \mu M \)), while rGST-CD94lec binds to heparin-BSA (0.37, 0.36 \( \mu M \)) with higher affinities than to HepTf (1.31, 1.45 \( \mu M \)). The \( K_d \) values of rGST-NKG2Dlec and rGST-CD94lec to sLeX-containing \( N \)-glycans and heparin are comparable to those of NKG2D homodimer to MICA (0.3—0.94 \( \mu M \)), MICB (0.79 \( \mu M \)), ULBP-1 (1.1 \( \mu M \)), and ULBP-3 (4 \( \mu M \))\(^\text{18}\) and to those of CD94/NKG2A heterodimer to HLA-E (1.23 \( \mu M \)) and CD94/NKG2C heterodimer to HLA-E (6.88 \( \mu M \))\(^\text{19}\).

In our previous report\(^\text{13}\), we demonstrated that susceptibility of K562/FUT cells to KHYG cells was suppressed by...
pretreatment of K562/FUT cells with anti-sLeX and KHYG cells with anti-NKG2D and anti-CD94 antibodies and that tyrosine phosphorylation of a 17-kDa protein in KHYG cells was enhanced by incubation on HepTf-coated plates and treatment with anti-NKG2D but not anti-CD94 antibody.

Y152 and Y199 in NKG2D are essential for binding to MICA/B and ULBP,20) and Q112, F114, N160, and L162 in CD94 are important for binding to HLA-E.21) In our previous mutagenesis analyses,14,15) the binding of rGST-NKG2Dlec (Y152A) and rGST-CD94lec (F114A and N160A) to sLeX, and the binding of rGST-NKG2Dlec (Y152A and Y199A) and rGST-CD94lec (F114A, N160A, and C166G) to heparin-BSA, were reduced, suggesting that glycan binding sites overlap partly with those of the protein ligands. Of the C-type lectin-like receptors on NK cells, several have been also reported to recognize sulfated glycans: mouse Ly-49A binds to fucoidan18,19,22); and osteoclast inhibitory lectin (OCIL, lectin-like transcript 1: LLT1) to fucoidan, λ-carrageenan,

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**Fig. 3. Binding of NKG2D and CD94 to HepTf and Heparin Using an EIA Microplate Assay**

Dose–response curves for rGST-NKG2Dlec (A, C) and rGST-CD94lec (B, D) binding to HepTf (A, B) and heparin-BSA (C, D) coated plates, respectively. rGST-NKG2Dlec and rGST-CD94lec (100 μM, 0 to 1 μM) were incubated on HepTf (A, B) and heparin-BSA (C, D) coated plates for 2 h, and binding was determined using POD-conjugated anti-GST antibody. The results are shown as mean±S.D. (n = 3).

**Fig. 4. Linear Reciprocal Plots for Binding of NKG2D and CD94 to HepTf and Heparin Using an EIA Microplate Assay**

Linear reciprocal plots for binding of rGST-NKG2Dlec (A, C) and rGST-CD94lec (B, D) to HepTf (A, B) and heparin-BSA (C, D) coated plates, respectively. [M]∆Abs vs. [M] was plotted. The results are shown as mean±S.D. (n = 3).
and dextran sulfate;\textsuperscript{23} and the binding of Ly-49A to H-2Dd competes with fucoidan.\textsuperscript{24} Further study is needed to clarify whether glycan ligands could compete with protein ligands to modulate NK cytotoxicity.

The sLeX antigen expressed on leukocytes, and its interaction with E-selectin on vascular endothelial cells, triggers the extravasation of leukocytes into inflammatory sites.\textsuperscript{25,26} Selectin-mediated adhesion of cancer cells to vascular endothelial cells is also involved in cancer metastasis, and the presence of cancer cells expressing sLeX and sLeA is correlated with poor prognosis.\textsuperscript{25,26} Retrospective analyses of clinical trials and prospective clinical studies have suggested that heparin may have suppressive effects on cancer survival and the formation of metastasis rather than the growth of primary tumors.\textsuperscript{27} The anti-inflammatory effects of heparin are mainly mediated by blocking P-selectin and L-selectin-initiated cell adhesion, in which the sulfate groups at C6 on GlcNS\textsubscript{6S} are essential.\textsuperscript{28} On the other hand, suppressed expression of heparin/heparan sulfate-degrading endosulfatase (HSulf) in cancer cells has been reported to induce high-sulfated heparan sulfate on the cell surface,\textsuperscript{29—31} and increased expression of heparanase in cancer cells enhances the cleavage of heparan sulfate in extracellular matrices to escape from NK cell surveillance.\textsuperscript{32,33}

In the present study, we confirmed the binding of NKG2D and CD94 to sLeX-containing N-glycans and heparin with high binding affinities. The present results, along with past evidence, indicate that sLeX, sLeA, and sulfate-containing glycans play complicated roles in cancer progression and metastasis.

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