Antibody-dependent Cell Cytotoxicity to Breast Cancer Targets Despite Inhibitory KIR Signaling

MARK N. STEIN, JAMES SHIN, OKSANA GUDZOWATY, ANDREW M. BERNSTEIN and JOHNSON M. LIU

Mount Sinai School of Medicine, New York, NY 10029, U.S.A.

Abstract. Background: Natural killer (NK) cells express killer immunoglobulin-like (KIR) inhibitory receptors, which recognize certain HLA class I molecules (KIR ligands), and stimulatory receptors such as FcγRIII. The purpose of this study was to test the possible influence of inhibitory KIR signaling on antibody-dependent cell cytotoxicity (ADCC) mediated by allogeneic NK cells against breast cancer targets. Materials and Methods: The cytotoxic activity of volunteer donor NK cells against the cell lines SKBR-3, T47D and MCF-7, which have high, low and no HER2 gene amplification, respectively, were studied. Both cell lines and donors were assigned to the C1 or C2 superfamily, defined by the structure of the HLA-Cw molecule. Results: It was found that ADCC mediated by allogeneic NK cells occurred despite combinations of NK cells and breast cancer targets predicted to trigger inhibitory KIR signaling. Conclusion: We suggest that adoptive immunotherapy with allogeneic NK cells and trastuzumab may be an effective combination against breast cancer targets.

NK cells express inhibitory receptors (killer immunoglobulin-like receptors or KIRs) that recognize certain HLA class I molecules (KIR ligands) and down-regulate NK cell function. If NK cells’ KIR receptors fail to recognize KIR ligands on tumor cells, the inhibitory signal is absent and NK cell activation proceeds. The clinical importance of absent NK inhibitory signals was most clearly demonstrated in haploidentical transplantation for leukemia. In this situation, mismatch between donor and recipient KIR ligands correlated with a remarkably low incidence of tumor relapse (1). Similar observations have been made regarding unrelated T-cell-depleted KIR-mismatched stem cell transplantation (2). These studies provide compelling clinical evidence that allogeneic NK cells can mediate an antitumor effect.

Current models of NK cell activation suggest that NK function is regulated not only by KIRs, but also by activating receptors, among which is FcγRIII (CD16). The FcγRIII receptor on NK cells binds the Fc moities of immunoglobulins, initiating cytotoxicity against antibody-coated target cells. NK cell-mediated antibody-dependent cell cytotoxicity (ADCC) has been found to be impaired in patients with advanced malignancy (3). Conversely, antitumor monoclonal antibodies work, at least in part, by engaging the FcγRIII receptors of NK cells. One such example is the antibody trastuzumab, which targets the human epidermal growth factor receptor, HER2, overexpressed in approximately 25-30% of breast cancers (4). Mice lacking FcγRIII receptors were markedly impaired in trastuzumab-mediated ADCC against breast cancer (5).

In order to determine whether inhibitory KIR signaling influences ADCC mediated by allogeneic NK cells, the cytotoxic activity of NK cells from volunteer donors against the breast cancer cell lines SKBR-3, T47D and MCF-7, which have high, low and no HER2 gene amplification, respectively (6, 7), were studied. These cell lines express KIR ligands that fall into one of two different superfamilies, as defined by a dimorphism at amino acids 77 and 80 on the HLA-C molecule (C1 group and C2 group, respectively). Donors likewise express KIR ligands from either the C1 or C2 groups. We investigated whether trastuzumab-mediated cytotoxicity by allogeneic NK cells against the different breast cancer cell lines is affected by C1- or C2-group compatibility.

Materials and Methods

Volunteer donors were screened for their HLA-C type using sequence specific primers to discriminate between the HLA-C
groups C1 and C2. Donor DNA was isolated using a QIAprep Spin Mini Prep kit (Qiagen, Valencia, CA, USA). The primers and PCR conditions used have previously been described (8). Two healthy donors were identified as being homozygous for either group 1 or group 2 HLA-C alleles. HLA typing of the donors and cell lines was performed using the Pel-Freeze SSP UniTray (Pel-Freeze Clinical Systems, Brown Deer, WI, USA). KIR typing of donor NK cells was performed using the Pel-Freeze KIR Genotyping SSP Kit (Pel-Freeze Clinical Systems).

Donor NK cells were isolated from peripheral blood lymphocytes using the Miltenyi NK Cell Isolation Kit II (Miltenyi Biotech, Auburn, CA, USA). Flow cytometric analysis of NK cell purity (utilizing the FACScalibur flow cytometer and CellQuest Pro software, BD Biosciences Pharmingen, San Diego, CA, USA) following NK cell enrichment demonstrated that the CD3−, CD56+ population of cells was routinely 60-80% of the effector cell population.

Breast cancer cell lines were obtained from ATCC (Manassas, VA, USA). SKBR-3 and T47D cells were grown in RPMI plus 10% fetal calf serum (FCS). MCF-7 was grown in DMEM plus 10% FCS. The HLA-C group of cell lines was determined using PCR SSP as above.

NK cell cytotoxicity against target cells was assessed by flow cytometric analysis (9). Target cells grown to confluence were trypsinized for 3 minutes, washed in culture medium without FCS, and counted. 106 target cells in 200 μL were labeled with 16 nM PKH-26 (Sigma-Aldrich, St. Louis, MO, USA) for 5 minutes at room temperature followed by incubation with 1 ml of FCS at room temperature to inhibit the labeling reaction. Target cells were washed twice with culture medium, and resuspended at a final density of 106 cells per ml. For cells treated with trastuzumab or rituximab (both from Genentech, South San Francisco, CA, USA), antibody was added at a final concentration of 12.5 μg/ml to the labeled target cells 30 minutes prior to the addition of the NK cells at 37°C. ADCC assays were performed in a reaction volume of 200 μl with varying E:T ratios for 4 hours at 37°C with 5% CO2 in 96-well plates after which time target cell viability was assessed. TO-PRO-3 iodide (TP-3; Molecular Probes, Eugene, OR, USA) was used to assess target cell viability. Flow cytometer parameters were adjusted so that unlabeled target cells were in the first log decade of the FL2 and FL4 channels (to respectively assess PKH-26 and TP-3 fluorescence) and dead target cells (permeabilized with IntraPrep Permeabilization Reagent; BD Biosciences, San Jose, CA, USA) were above the second log decade of the FL4 channel. To access baseline and experimental target cell viability, 5 μl of 1 μm TP-3 was added to individual samples immediately prior to flow cytometric analysis. The data was analyzed using CellQuest software. Cytotoxicity was calculated as the fraction of target cell events in cells taking up TP-3 divided by the total number of target cell events.

Results

HLA class I molecular typing of NK cell donors and target cell lines was determined by PCR analysis (Table I). Donor 1, SKBR-3 and T47D belonged to HLA-C group 1, whereas donor 2 and MCF-7 belonged to HLA-C group 2. KIR genotyping of donor 1 and donor 2 (Table II) demonstrated that donor 2 had the same KIR genes as donor 1 with the addition of KIR2DL5A (inhibitory), KIR2DS3 and KIR3DS1 (latter two activating). We would expect inhibitory KIR signaling to occur when the donor NK cells and breast cancer targets are matched at the major HLA-C group.

In assays using MCF-7 target cells (with no HER2 gene amplification), donor 1 NK cells (HLA-C disparate) and donor 2 NK cells (HLA-C compatible) exhibited some degree of cytotoxicity against the MCF-7 cells at E:T ratios from 30:1 to 3.75:1 (representative data from replicate experiments shown in Figure 1a). When MCF-7 cells were incubated with trastuzumab and NK cells at an E:T of 15:1, both donor 1 and donor 2 exhibited marginal increases in cytotoxicity (from 38% to 47% and from 21% to 32%, respectively). The relatively low ADCC activities seen were attributed to the low density of HER2 on MCF-7 cells.

In contrast, when SKBR-3 cells (with high levels of HER2 amplification) were incubated with trastuzumab (Figure 1b), donor 1 (HLA-C compatible) cytotoxicity increased by 90% (from 43% to 82%) at an E:T of 15:1, and donor 2 (HLA-C disparate) cytotoxicity increased by 86% (from 35% to 64%). The augmentation of cytotoxicity was specific to trastuzumab, as no increase in cytotoxicity was noted with trastuzumab, as no increase in cytotoxicity was noted with the humanized anti-CD20 antibody rituximab (data not shown). These experiments suggested that ADCC occurred without significant dampening by inhibitory KIR signaling.

When T47D cells (low HER2 amplification) were used (Figure 1c), the results were similar to those seen with SKBR-3. In the presence of trastuzumab, NK cell cytotoxicity was significantly augmented across the full range of E:T ratios for both donor 1 (HLA-C compatible) and donor 2 (HLA-C disparate) NK cells. At an E:T of 25:1, trastuzumab augmented donor 1 cytotoxicity from 30% to 61% and donor 2 cytotoxicity from 20% to 54%.

Discussion

In the present study, the ability of isolated unstimulated human natural killer cells to kill human breast cancer cell lines in vitro was evaluated. Consistent with previous findings (10), purified NK cells mediated both cytotoxicity and ADCC against breast cancer cell lines. When breast cancer cell targets were incubated with trastuzumab over a

### Table I. HLA class I typing and HLA-C group of donor NK cells and cell lines.

<table>
<thead>
<tr>
<th>Donor 1</th>
<th>Donor 2</th>
<th>SKBR-3</th>
<th>T47D</th>
<th>MCF-7</th>
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Table II. KIR genotyping of donor 1 and donor 2.

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<th>KIR Genotype</th>
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</tbody>
</table>

X = gene present, empty box = gene absent.

Figure 1. Donor 1 (NK1) vs. donor 2 (NK2) +/- trastuzumab against breast cancer target cell lines. Cytotoxicity of donor 1 and donor 2 NK cells against breast cancer MCF-7 cells (a) with a low level of HER-2 expression, SKBR-3 cells (b) with high HER-2 expression and T47D cells (c) with intermediate HER-2 expression after a 4-hour incubation at 37°C, with and without trastuzumab at varying effector to target (E:T) ratios.
range of E:T ratios, ADCC was most dramatically increased in the SKBR-3 cell line, which has the highest HER2 gene copy number and the greatest cell surface expression of HER2. T47D and MCF-7, which have few or no extra copies of HER2, nevertheless showed augmentation of cytotoxic activity with trastuzumab. This finding is somewhat unexpected, as clinical studies have shown significantly better clinical efficacy of trastuzumab in patients with tumors with multiple copies of HER2 compared with patients whose tumors had a single copy of HER2 (11). We believe the high number of effector cells and the availability of drug to target in vitro may explain the sensitivity of cells expressing low levels of HER2 to NK cell ADCC.

In order to evaluate whether inhibitory KIR signaling influences ADCC mediated by allogeneic NK cells, two NK cell donors were selected based on HLA-C typing of the donor polymorphisms. Based on the "ligand-ligand" model of NK cell reactivity, donor 1 was predicted to encode KIR receptors specific for the HLA-C group C1, and donor 2 was predicted to encode KIR receptors specific for group C2. Breast cancer target cells were HLA-C typed to determine KIR ligand specificity. As predicted by this model, we would expect inhibitory KIR signaling to occur when the donor NK cells and breast cancer cells are C-group matched. We found that ADCC occurred despite combinations of NK cells and breast cancer targets predicted to trigger inhibitory KIR signaling. The variation (between donors 1 and 2) in cytotoxicity in the absence of trastuzumab may be due to complex differences in expression of both inhibitory and activating receptors [reviewed in (12)], including the killer lectin-like receptor (KLR) and natural cytotoxicity receptors (NCRs).

Our study suggests that transfer of allogeneic NK cells in combination with a cancer-targeting antibody such as trastuzumab may represent an effective approach to adoptive immunotherapy, which has historically focused on enhancing T-cell function (13-15). The therapeutic benefit of allogeneic lymphocytes administered following stem cell transplant has been demonstrated in hematological malignancies (16-18), renal cancer (19, 20) and breast cancer (21). However, this benefit comes at the expense of T-cell-mediated graft-versus-host-disease and toxicity from the conditioning regimen. Recently, Miller et al. (22) demonstrated the feasibility and potential efficacy of adoptive transfer of human haploidentical alloreactive NK cells in patients with cancer. We suggest that NK cells in combination with a monoclonal antibody may confer more rapid tumor killing, due to the additive benefit of the two modalities of treatment and the potent cytotoxic capability of NK cells. Phase I studies have demonstrated that purified allogeneic NK cells could be transfused without adverse effects (22-26). Our data suggest that immunotherapy with phenotypically defined donor NK cells primed with trastuzumab may be an effective strategy against breast cancer.

Acknowledgements

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References


