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KIR Receptor-Ligand Incompatibility Predicts Killing of Osteosarcoma Cell Lines by Allogeneic NK Cells

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Abstract

Background—The effectiveness of KIR incompatible, alloreactive NK cells has been primarily documented in hematological malignancies following stem cell transplant. This effect has not been thoroughly evaluated for pediatric solid tumors. In this study, we evaluated KIR receptor-ligand incompatibility of NK cells against osteosarcoma cell lines.

Procedure—Following the KIR receptor-ligand mismatch model, MHC I cell surface expression and KIR ligand mRNA content of 3 osteosarcoma cell lines was determined by flow cytometry and quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR), respectively. NK cells were isolated from healthy volunteer donor peripheral blood mononuclear cells (PBMCs) and KIR surface expression determined by flow cytometry. An Annexin-V based flow cytometric killing assay was used to determine % of dying osteosarcoma target cells by donor NK effector cells.

Results—One of 7 healthy volunteer donors tested lacked phenotypic expression of one KIR. However, variable expression of KIR ligands was observed in 3 osteosarcoma cell lines. The highest rates of dying cells were seen in osteosarcoma cells with the lowest KIR ligand expression. Following down-regulation of KIR ligand expression, an increased susceptibility to NK cell mediated killing was observed in a previously NK-resistant osteosarcoma cell line.

Conclusions—Variable MHC I and KIR ligand expression was observed in osteosarcoma cell lines and this resulted in variable susceptibility to NK cell mediated killing predicted by the degree of KIR receptor-ligand incompatibility. Collectively, these data provide rationale for the study of KIR incompatible stem cell transplant for osteosarcoma, although further studies with fresh osteosarcoma samples are necessary.

Keywords

NK cells; KIR; immunotherapy; osteosarcoma

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Introduction

The prognosis of metastatic, refractory, and recurrent osteosarcoma is dismal and new therapies are needed[1]. One exciting novel treatment involves the use of Killer Immunoglobulin-Like Receptor (KIR) incompatible haploidentical stem cell transplantation. In this therapy, mature donor natural killer (NK) cells eliminate residual host tumor cells, support engraftment, and may prevent graft-vs.-host disease (GVHD). This approach has demonstrated clinical benefit in adult and pediatric leukemia[3-5] as well as in vitro response for adult solid tumors[6-8]. Only two previous studies have examined the significance of KIR incompatibility in the treatment of pediatric solid tumors[9,10] and showed improved outcomes in autologous and allogeneic transplant in patients that were KIR receptor-ligand mismatched. These reports illustrate the clinical rationale for further research in this area.

KIRs are members of the immunoglobulin superfamily of receptors and are primarily expressed on human NK cells. They play a critical role in distinguishing self from non-self, through interactions with MHC class I antigens. Table I lists the three best characterized inhibitory KIRs and their cognate MHC class I ligands. It has been demonstrated that the binding affinity of inhibitory receptors is much stronger than that of corresponding activating receptors and contributes to self-tolerance[11]. Because the prevalence of the inhibitory KIRs is high, tumor expression of MHC class I ligands may play a significant role in determining NK cell alloreactivity versus inhibition[12]. Previous studies have shown that osteosarcoma cells, down-regulate class I, in vivo, likely to escape T-cell recognition and cell lysis[13]. These tumors are KIR incompatible, as they would lack ligand expression for inhibitory KIRs, and would be susceptible to NK-mediated killing. Therefore, we hypothesized that osteosarcoma cells would be effectively killed by KIR-incompatible allogeneic NK cells.

Methods

Cell lines

The following osteosarcoma cell lines were purchased from ATCC and used in all experiments: HOS (CRL-1543), SaOS (HTB-85), and U2OS (HTB-96). The cell lines were maintained in culture as detailed in the supplemental methods section. The erythroleukemia cell line, K562, was used as a positive control for flow cytometric killing assays because these cells are known to be sensitive to NK-mediated killing due to low MHC I expression.

MHC I and KIR ligand analysis

Cells from osteosarcoma lines were harvested from culture and MHC I expression was determined by flow cytometry using the pan-MHC I antibody, W6/32 (eBioscience, San Diego). RNA was then isolated from osteosarcoma cell pellets containing approximately 1 million cells using the RNeasy RNeasy -4 PCR Kit (Ambion, Austin, Tx) and then converted to cDNA by reverse transcription. Primer sequences (Invitrogen, Carlsbad, CA) for HLA class I KIR ligands and GAPDH control that were used for quantitative reverse transcription polymerase chain reaction (qRT-PCR), are listed in Supplemental Table I. Additional details for qRT-PCR are provided in the supplemental methods section.

NK cell isolation and KIR phenotyping

In a University of Wisconsin Institutional Review Board approved protocol, whole blood was drawn from healthy volunteer donors and PBMCs were collected. NK cells were isolated by negative selection using an NK Cell Isolation Kit and a Midi-MACS separation system (Miltenyi, Auburn, CA) according to the manufacturer's recommendations.

Following NK cell isolation, KIR surface expression was analyzed by flow cytometry using the following antibodies: PE-conjugated anti-KIR2DL1 (HP3E4, BD Pharmingen), anti-KIR2DL2/2DL3 (CHL, BD Pharmingen), and anti-KIR3DL1 (DX9, Miltenyi) and counter-stained with CD3-PE-Cy5 and CD56-FITC. A FACSCalibur flow cytometer and Cell Quest acquisition software (BD Biosciences, San Jose, CA) were used in all flow cytometry experiments to measure 1,000 events.

Flow cytometry killing assay

NK cells were incubated for 12 hours with RPMI and 1000 U/ml rH-IL-2 (Peprotech, Rocky Hill, NJ). Tumor cells were harvested with TrypLE Select (Invitrogen) and then washed prior to staining with PKH-26 (Sigma-Aldrich), that allowed identification of PKH-26 + tumor cells and PKH-26 – NK cells. Healthy donor NK effector (E) cells and osteosarcoma target (T) cells were then distributed into a 96-well V-bottom plate at various E:T ratios in RPMI media containing 1000 U/ml rH-IL-2, and incubated for 3 hours. Annexin V Binding Buffer (BD Bioscience) containing Annexin-V-FITC (BD Pharmingen) was added to all wells and incubated in the dark, and following this, the samples were fixed with 1% paraformaldehyde. The percentage of PKH-26 and Annexin V double positive cells was assessed by flow cytometry and % of dying cells was determined after subtraction of spontaneous annexin V binding.

Statistical analysis

Statistical comparisons between groups were performed using a 2-tailed Student *t* test for 2 samples assuming unequal variances. Calculation of Pearson correlation coefficients (*r*) and drawing of the best-fit lines were performed using Microsoft Excel software (Redmond, WA).

Results

High prevalence of inhibitory KIR cell-surface expression in a donor NK cell population

While expression of the inhibitory receptors, KIR2DL1, KIR2DL2/2DL3, and KIR3DL1 is not ubiquitous, previous analysis has shown genotypic expression in greater than 90% of study populations with leukemia or other malignancies[3,5,7,8,12]. However, disparities have been observed in which the donor KIR gene was present but the receptor was not expressed on the cell surface[15]. As shown in Table II, 6 of 7 healthy volunteers expressed all three inhibitory KIRs in their NK receptor repertoire. Only one donor lacked an inhibitory KIR (KIR2DL1). Of note, the percentage of CD56+CD3-cells that were positive for a phenotypically expressed individual KIR was variable and ranged from approximately 10-60% in this group. Most individuals expressed KIR2DL2/2DL3 on the highest percentage of NK cells compared to the other inhibitory KIRs. The functional significance of this finding is unknown.

Variable expression of MHC class I and KIR ligands by three osteosarcoma cell lines

Tsukahara et al. found loss or down-regulation of MHC I in the majority of osteosarcoma and other sarcoma samples[13]. Although KIR ligands were not specifically assessed, this report suggests that osteosarcoma cells might be susceptible to KIR-incompatible NK cells. Therefore, in our study, cell-surface MHC class I expression was measured using the pan-MHC I antibody, W6/32. Mean fluorescence intensity (MFI) was measured by subtracting fluorescence of isotype controls from fluorescence of MHC class I positive cells. As shown in Figure 1, the three osteosarcoma lines tested exhibited varying levels of class I antigens on their cell surface. HOS osteosarcoma cells expressed extremely low levels of cell-surface class I protein in a manner similar to K562 cells. Conversely, two other osteosarcoma cell

lines, SaOS and U2OS, expressed relatively high levels of class I antigen. Furthermore, most of the SaOS cells (88% positive) and nearly all of the U2OS cells (99% positive) were MHC class I+ while 99% of the HOS cells were class I-. Representative histograms illustrate these clear differences.

While the pan-class I antibody was useful for tumor cell surface expression of HLA I, it is not specific for the KIR ligands and does not directly identify KIR-ligand polymorphisms. Currently, monoclonal antibodies are not available for the protein quantification of each individual KIR ligand. Because of this methodological shortcoming, previous studies have quantified KIR ligand expression using RT-PCR without confirmation of cell-surface expression[5,7,8]. This information gap limits the functional analysis of KIR-KIR ligand interactions as it hinders identification of tumor cells transcribing KIR ligand genes but ultimately failing to express class I proteins at the cell-surface. Such cells might erroneously be considered KIR-compatible with NK cells expressing the corresponding inhibitory KIRs yet demonstrate exquisite susceptibility to lysis by the same NK cells.

To mitigate this problem, we combined evidence of KIR ligand transcription with cell-surface pan-class I protein expression to stratify KIR ligand positivity amongst the three osteosarcoma cell lines used in this study. Forward and reverse RT-PCR primers used to quantify KIR ligand transcripts (HLA-Bw4, HLA-C group 1, & HLA-C group 2) are listed in Supplemental Table I and the expression of KIR RNA by the three osteosarcoma cell lines is summarized in Table III. HOS cells express only HLA-Bw4 but not HLA-C groups 1 or 2. However, because of the absence of class I cell-surface expression (Figure 1), HOS cells are categorized as KIR ligand negative in the subsequent functional assays. As detailed earlier, U2OS and SaOS cells both have high surface expression of MHC I. U2OS cells lack HLA-C group 1 but express both HLA-Bw4 and HLA-C group 2. SaOS cells were found to express all three inhibitory KIR ligands. Therefore, following the KIR receptor-ligand model, we predicted that HOS cells would be most susceptible to NK-mediated lysis, SaOS cells would be the most NK-resistant and U2OS cells would display an intermediate sensitivity.

The rate of NK-mediated apoptosis in 3 osteosarcoma cell lines is predicted by the degree of KIR receptor-ligand incompatibility

The initial model of KIR incompatibility was proposed by Ruggeri et al.[3] and predicted NK cell alloreactivity based on the presence of donor KIR ligand in the absence of recipient KIR ligand (ligand-ligand model). This model assumes that the donor expresses the corresponding KIR for this ligand which is not universally true[5]. Therefore, we used a receptor-ligand model proposed by Leung et al.[5] that demonstrated the ability to predict relapse risk for leukemia patients receiving haploidentical (HLA-mismatched) transplant. In this model, patients with KIR receptor-ligand mismatch are predicted to have lower rates of relapse than those who are matched. Two healthy donors were selected as a source of effector cells for all experiments. Donor 1 NK cells lack expression of only KIR2DL1 while Donor 2 NK cells express the three inhibitory KIRs. The receptor-ligand model would predict that killing of the HOS cells (KIR ligand negative) would be higher than SaOS (3 KIR ligands) or U2OS cells (2 KIR ligands) for either donor. Furthermore, the level of observed apoptosis should correlate with the degree of receptor-ligand incompatibility.

The NK cytotoxicity assay results are summarized in Figure 2. As shown, osteosarcoma cells with surface expression of KIR ligands (SaOS and U2OS) displayed significantly less susceptibility to killing by either donor NK group when compared to the HOS cell line that lacks cell-surface KIR ligand expression ($p < 0.05$ for both cell lines compared to HOS cells). Figure 3 plots the relationship between the degree of receptor-ligand mismatch and the activity observed in the same killing assay. Target cell killing correlated directly with the

degree of receptor-ligand incompatibility between the two healthy donor allo-NK cells, as determined by KIR phenotypic expression, and the osteosarcoma cell lines, as determined by mRNA transcript expression of KIR ligands ($r=0.96$). Together, these findings support that cell-surface KIR ligand expression decreases susceptibility of these 3 osteosarcoma cell lines to allo-NK cell mediated killing in accordance with the receptor-ligand model.

Changes in KIR ligand expression by an individual osteosarcoma cell line result in altered susceptibility to NK cell mediated lysis

If KIR ligand expression by SaOS and U2OS cells provides protection from lysis by KIR-matched NK cells, then loss or down-regulation of KIR ligand expression should increase susceptibility. Previous studies have demonstrated loss or down-regulation of MHC I in a variety of tumors and tumor cell lines during growth or expansion[16-19]. Tsukahara et al. observed loss or down-regulation of HLA I in sarcoma tumor samples, including osteosarcoma[13]. Such changes might alter the susceptibility of osteosarcoma cells to NK-mediated killing. We observed down-regulation of KIR ligand expression in late-passage culture (>10) of SaOS cells. As shown in Figure 4, KIR ligand transcripts in late-passage SaOS cells decreased 16-fold for HLA-Bw4, 4-fold for HLA-C, group 1, and 16-fold for HLA-C, group 2, compared to transcript levels observed in early passage (<10). When subjected to the same cytotoxicity assay, late-passage SaOS cells experienced significantly higher rates ($p < 0.05$) of apoptosis by either donor NK cell group when compared to early-passage cells. Thus, while the change in KIR ligand transcripts demonstrates a limitation of using cell lines, loss of KIR ligand transcripts by late-passage SaOS cells correlated with the loss of protection from NK cell mediated killing suggesting less protection existed at these reduced levels.

Discussion

Though experience with allogeneic stem cell transplant for osteosarcoma is limited, suggestion of a graft-vs.-tumor effect was observed in a male with metastatic osteosarcoma who received an HLA-matched sibling transplant and achieved long-term survival[20]. Osteosarcoma may be a good candidate disease for KIR incompatible transplants as evidenced by recent observations of loss or down-regulation of MHC class I antigens in 52% of primary osteosarcoma tumors and in 88% of metastatic osteosarcoma specimens[13]. Our study shows variable MHC class I expression in 3 osteosarcoma cell lines and extends the current information to demonstrate variability of KIR-ligand transcripts. In this regard, the HOS cell line was found to have nearly absent MHC I (and thus KIR ligand) expression compared to SaOS and U2OS cells. The variability of MHC I and KIR ligand expression underlines the importance of developing a risk-stratification model for further pre-clinical studies with fresh tumor specimens and the subsequent clinical study of KIR incompatible transplantation in pediatric osteosarcoma.

Using the KIR receptor-ligand incompatibility model, we hypothesized that donor NK cells would kill HOS cells (low class I expression) more effectively than both U2OS and SaOS cells (high class I expression). We also expected that SaOS cells would exhibit the least susceptibility to NK mediated killing due to high-level expression of the 3 KIR ligands. As expected, significantly higher lysis of HOS cells was seen when compared to both U2OS and SaOS cells, using NK cells from two different donors. No statistical difference in killing of SaOS and U2OS cells existed suggesting that the difference between 0 and 1 receptor-ligand mismatches may not be functionally relevant. Additionally, early passage SaOS cells were less susceptible to allo-NK cell mediated killing compared to HOS cells as would be predicted using the KIR receptor-ligand incompatibility model because of the substantial differences in MHC class I expression between the two lines. Late passage SaOS cells

appeared to lose MHC I and KIR ligand expression, resulting in increased susceptibility to NK-mediated killing.

The potential for alterations in tumor MHC I and KIR ligand expression emphasizes the need to develop better tools for the delineation and monitoring of this critical parameter. Alterations in class I expression have been documented to occur *in vivo* and may have significant clinically relevant consequences in the response to KIR incompatible transplantation therapies. Additionally, in the setting of conventional therapies in patients with initially high HLA class I and KIR ligand expression, this expression could be monitored throughout treatment. If decreased expression is detected and patients are not responding well to therapy, these patients may benefit from KIR incompatible haploidentical stem cell transplantation as a treatment for relapsed disease.

Few studies have evaluated the effectiveness of NK cell alloreactivity and KIR incompatibility in solid tumors, mostly in the adult population[6-8]. One recent publication by Leung et al. evaluated KIR incompatibility and pediatric solid tumors in the conventional setting of autologous stem cell transplantation[9]. The basis of this prospective study was an earlier finding by this group that a percentage of the population will have self NKs that express an inhibitory KIR(s) without expression of the corresponding ligand(s)[5]. In that report, the authors found that patients with 2 KIR mismatches had prolonged survival while patients with 1 mismatch or complete KIR compatibility were more likely to develop treatment failures[9].

Despite these encouraging results, some autologous transplantation recipients will not have this natural degree of autologous incompatibility and would be more likely to experience a treatment failure. To date, only one paper examining KIR receptor-ligand incompatibility in the setting of haploidentical stem cell transplantation for pediatric solid tumors has been published. In that report, Perez-Martinez et al. describe complete remission and partial response in 2 of 3 children with metastatic solid tumors[10]. The two patients who responded to treatment demonstrated KIR/KIR ligand mismatch while the non-responding patient was fully KIR/KIR ligand matched[10]. These findings suggest a graft-vs.-tumor effect toward pediatric solid tumors that may be predicted by KIR/KIR ligand mismatch.

Our study demonstrates that susceptibility of these osteosarcoma cell lines to NK-mediated apoptosis could be predicted based on the degree of KIR receptor-ligand mismatch. We also observed an increased susceptibility to NK-mediated osteosarcoma killing in a cell line that was found to down-regulate MHC class I and KIR ligand transcripts. While this finding strengthened our hypothesis, it does highlight a limitation in using tumor cell lines. Thus, it is necessary to confirm our findings through experiments that down-regulate expression of MHC I and KIR ligand expression (i.e. siRNA) and to perform related experiments in fresh osteosarcoma tumor samples. Following these future experiments, it would be pertinent to test this hypothesis in the clinical setting of haploidentical stem cell transplant for osteosarcoma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

HLA class I cell surface expression in osteosarcoma cell lines (A) HOS (MFI 10) cells were determined to have low HLA class I expression compared to K562 a known NK-susceptible leukemic cell line. SaOS and U2OS cells were found to have higher class I expression (MFI >200) when compared to HOS and K562 cells, although this was not significant ($p = \text{NS}$). (B) HOS cells were also found to have low frequency (<1%) of cells positive for HLA I. SaOS (88% positive) and U2OS (99% positive) cells had much higher frequency of cells expressing class I antigens when compared to HOS and K562 (<1%). (C) Sample histograms of class I expression in osteosarcoma cell lines. Shaded area represents the cell population with HLA I expression while the open area represents the isotype control. MFI of cells was in comparison to isotype control for W6/32 antibody.

Figure 2.

Osteosarcoma cell lines with greatest KIR receptor-ligand incompatibility are most sensitive to NK-mediated killing. Lysis rates were determined using the cell membrane marker, PKH-26, to identify target cells and annexin V was used to measure apoptosis. % dying cells (Annexin V+ cells) was calculated by subtracting spontaneous lysis of targets from the average of double-positive cells. The class I negative HOS cells had significantly greater killing from both donor's NK cells than SaOS and U2OS cells. * $p < 0.05$.

Figure 3. Correlation of KIR receptor-ligand mismatch and target cell apoptosis. As shown, the degree of receptor-ligand mismatch exhibited a strong correlation with the lysis of osteosarcoma cells by allogeneic donor NK cells ($R^2 = 0.93$).

Figure 4.

Down-regulation of inhibitory KIR ligands diminishes protection of late-passage SaOS cells from donor NK cell killing (A) Late-passage SaOS cells exhibit down-regulation of KIR ligand transcripts. (B) Late-passage SaOS cells (>10) demonstrate enhanced susceptibility to apoptosis by donor NK cells when compared to early-passage SaOS cells (<10). *p < 0.05.

Table I

Inhibitory KIRs and their cognate MHC class I ligands.

Receptor	Ligand
KIR2DL1 (CD158a)	HLA-C2 (Cw2, Cw4, Cw5, Cw6, Cw15, Cw1602, Cw17, Cw18)
KIR2DL2/2DL3 (CD158b)	HLA-C1 (Cw1, Cw3, Cw7, Cw8, Cw12, Cw13, Cw14, Cw16)
KIR3DL1 (CD158e)	HLA-Bw4

Table II

KIR receptor repertoire (% of positive cells) in peripheral blood NK cells harvested from seven healthy volunteers.

	KIR2DL1(%)	KIR2DL2/2DL3(%)	KIR3DL1(%)
DONOR 1	17	49	27
DONOR 2	27	32	21
DONOR 3	0	50	52
DONOR 4	20	38	11
DONOR 5	26	38	37
DONOR 6	24	32	11
DONOR 7	31	61	35

Table III

Presence of KIR ligand transcripts in 3 osteosarcoma cell lines.

	HLA-Bw4	HLA-C, group 1	HLA-C, group 2
HOS			
SaOS			
U2OS			

Open boxes represent absent KIR ligand transcripts while filled boxes show presence of transcripts.