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The Decreased Susceptibility of Bcr/Abl Targets to NK Cell-Mediated Lysis in Response to Imatinib Mesylate Involves Modulation of NKG2D Ligands, GM1 Expression, and Synapse Formation

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Chronic myeloid leukemia is a clonal multilineage myeloproliferative disease of stem cell origin characterized by the presence of the Bcr/Abl oncoprotein, a constitutively active tyrosine kinase. In previous studies, we have provided evidence that Bcr/Abl overexpression in leukemic cells increased their susceptibility to NK-mediated lysis by different mechanisms. In the present study, using UT-7/9 cells, a high level Bcr/Abl transfectant of UT-7 cells, we show that the treatment of Bcr/Abl target by imatinib mesylate (IM), a specific Abl tyrosine kinase inhibitor, hampers the formation of the NK/target immunological synapse. The main effect of IM involves an induction of surface GM1 ganglioside on Bcr/Abl transfectants that prevents the redistribution of MHC-related Ag molecules in lipid rafts upon interaction with NK cells. IM also affects cell surface glycosylation of targets, as assessed by binding of specific lectins resulting in the subsequent modulation of their binding to lectin type NK receptor, particularly NKG2D. In addition, we demonstrate that the tyrosine kinase activity repression results in a decrease of MHC-related Ags-A/B by binding of specific lectins resulting in the subsequent modulation of their binding to lectin type NK receptor, particularly NKG2D. In addition, we demonstrate that the tyrosine kinase activity repression results in a decrease of MHC-related Ags-A/B and UL-16-binding protein expression on Bcr/Abl transfectants UT-7/9. We show that NKG2D controls the NK-mediated lysis of UT-7 cells, and IM treatment inhibits this activating pathway. Taken together, our results show that the high expression of Bcr/Abl in leukemic cells controls the expression of NKG2D receptor ligands and membrane GM1 via a tyrosine kinase-dependent mechanism and that the modulation of these molecules by IM interferes with NK cell recognition and cytolysis of the transfectants. The Journal of Immunology, 2006, 176: 864–872.

Natural killer cells are cytotoxic effectors that play a major role in the first line of defense against pathogens and transformed cells because they can recognize their targets without prior immunization (1). The cytolytic activity of NK cells is the result of an integrative response emerging from activating and inhibitory NK receptors. Beyond well-characterized killer cell Ig-type receptors, which can recognize MHC class I alleles (2), NK cells possess lectin-type receptors whose carbohydrate ligands are still currently unknown (3). Some receptors are expressed as heterodimers composed of a common subunit (CD94) covalently bound to a distinct chain of the C-type lectin NKG2 family. Although NKG2A and its spliced variant, NKG2B, bind to the nonclassical HLA-E allele presenting HLA-A, -B, -C, and -G leader peptides and induce a negative signal into NK cell, the CD94/NKG2C receptor triggers a positive cytolysis signal when bound to HLA-E/peptide complexes (4, 5). Activating NKG2D receptor is another distantly related family member that does not interact with CD94 and that recognizes molecules induced by a cellular stress, namely, MHC-related Ags (MIC) and UL-16-binding proteins (ULBPs) (6–8). Several reports demonstrated that increased expression of these ligands correlated with the NK cell-mediated cytotoxicity (8, 9). It is established that activation of NK cells via NKG2D can overcome inhibitory signaling from self recognition and is triggered by signaling via a Syk-independent pathway activated by DAP10. Accumulating evidence indicates that the cytotoxic activity of NK cells is induced when activating receptors, known as natural cytotoxicity receptors (NCR), whose ligands are not yet well identified, are engaged. Nevertheless, the existence of carbohydrate determinants for the binding of such ligands has been suggested (10–12). One putative NCR ligand identified to date is the viral hemagglutinin that binds and activates NKp46 and NKp44 in a sialic acid-dependent manner (13, 14).

It was recently emphasized that NK cell-mediated cytolysis requires close contact between target and effector, leading to the formation of an immunological synapse that facilitates the triggering, concentrates the tyrosine kinase receptors, and favors their signaling (15). The formation of the synapse also involves polarization of the target toward the NK cell and is accompanied by cytoskeleton remodeling and raft mobilization in the target that are required for optimal activation of resting NK cell (16).

Chronic myeloid leukemia (CML) is a clonal myeloproliferative disorder characterized by the presence of the Bcr-Abl oncogene, which results from reciprocal 9;22 chromosome translocation. The
oncoprotein Bcr/Abl has elevated tyrosine kinase activity, which leads to the activation of multiple cellular signaling pathways (17, 18). A selective inhibitor of the Bcr/Abl tyrosine kinase activity, STI-571, now imatinib mesylate (IM), has been used both in vitro and in clinical trials. Besides spectacular clinical responses, multiple resistances have emerged from IM therapy, and these results outline the need to better understand Bcr/Abl repression through this component (19, 20). In this context, stem cell transplantation remains the only treatment to cure CML in a large proportion of cases. Although these grafts result in the rapid generation of NK cells, the role of these effectors in graft-vs-leukemia is not fully understood (7, 21). Hence, it is of great interest to better understand the biological implications of IM therapy and their biological consequences with respect to NK reactivity toward leukemic targets. In our previous work (22) we have demonstrated that the susceptibility of leukemic cells to in vitro CD34+ differentiated NK lysis correlated with the level of Bcr-Abl expression. Furthermore, we report in this study that in vitro activated mature NK cells from peripheral blood are able to recognize and lyse Bcr/Abl transfectants with high efficiency. More importantly, we demonstrate that IM-mediated inhibition of Bcr/Abl tyrosine kinase activity alters the formation of an efficient immunological synapse (NK/CML), thereby decreasing the NK-mediated lysis of Bcr-Abl transfectants. Furthermore, we provide evidence that this process interferes with the surface expression of GM1, the sialylation state, and the control of MIC expression.

Materials and Methods

Cell culture

NK cells were sorted from the peripheral blood of normal volunteers by negative immunoselection using RosetteSep mixture Ab (StemCell Technologies). After gentle mixing, diluted sample was subjected to Ficol-Paque density centrifugation (20 min at 2000 rpm). The NK fraction was carefully removed and washed twice with saline buffer. NK cells were expanded in RPMI 1640 medium containing 10% human serum (Sigma-Aldrich) supplemented with 500 IU/ml IL-2 and used as effectors between days 6 and 18 of in vitro culture.

Leukemic cells were cultured in RPMI 1640 medium containing 10% FCS. Medium was supplemented with 10 ng/ml GM-CSF to expand the parental UT-7 cell line, whereas the Bcr-Abl high-expressing UT-79 cell line was cultured with factor independent (23). CIR and its MICA transfectants (A. Toubert, Institut National de la Sante et de la Recherche Medicale, Unité 662, Paris, France) were cultured in RPMI 1640 medium containing 10% FCS and subcultured twice a week at 0.3 × 10^6 cells/ml. MICA-transfectants were cultured in the presence of 500 μg/ml genelicin (G418; Sigma-Aldrich). In some experiments, cells were treated with a low dose of IM (0.5 μM; 24–48 h). It was previously shown that IM (2 μM; 48 h) reduces (40–50%) the proliferation of UT-79 cells and has no effect on UT-7 cells (24).

Phenotypic analysis

FITC-, PE-, or PE-Cy5-conjugated mAbs specific for the following markers were used for the surface expression study of the NK cells and targets. CD56, CD16, CD3, and control mouse Ig were purchased from Immunotech. Unconjugated anti-NKG2D mAb (MAB139, IgG1) was purchased from R&D Systems. On targets, MICA/B (SR99, IgG1) primary Ab was used in an indirect immunofluorescence analysis with an FITC-conjugated goat anti-mouse secondary Ab (Immunotech). The expression of NKG2D ligands was also assessed using NKG2D-Fc (R&D Systems), followed by PE-conjugated goat anti-human Ig. GM1 was detected upon staining with the FITC-labeled B subunit of cholera toxin (CT-B; Sigma-Aldrich) at 8 μg/ml on ice for 30 min. Flow cytometric analyses were performed on a FACSort (BD Biosciences), and 10,000 events were collected and analyzed using CellQuest software (BD Biosciences).

Cytotoxicity assay

The susceptibility of leukemic cell lines to activated peripheral NK cell lysis was tested in a 4-h 51Cr release assay. E/T cell ratios ranged from 10:1 to 1:1. Determinations were performed in triplicate, and lysis percentages were determined as previously described (22). SDs were <10%. In some experiments, cell lines were treated with 0.5 μM IM for 48 h before the test. To block NKG2D engagement, anti-NKG2D mAb (20 μg/ml) was added at the onset of the cytotoxic assay.

Lectin staining

*Sambucus nigra* (SNA; Vector Laboratories) lectin is highly specific for sialic acid α2,6-linked to galactose, as found in N-linked oligosaccharides; sialic acid α2,6-linked to GalNAc, as found in O-linked oligosaccharides; and *Maackia amurensis II* (MAL II; Vector Laboratories) lectin recognizes α2,3-linked sialic acids. For cell sialylation analysis, target cells were probed with biotinylated MAL II (0.1 μg/ml in PBS) and SNA (0.1 μg/ml in PBS) lectins. After incubation with lectins, cells were washed three times with PBS, then streptavidin-PE conjugate (20 min at 4°C) was added. Cells were washed three times, then fixed with PBS/1% parafomaldehyde. Incubating cells with only secondary reagent determined basal fluorescence. In some experiments, cells were treated for 48 h with IM (0.5 μM) before staining. To control lectin-specific binding, leukemic cells were treated with 60 μl of *Vibrio cholerae* sialidase for 1 h at 37°C in the following buffer: 150 mM NaCl and 4 mM CaCl2, (pH 6). After sialidase treatment, cells were washed twice in serum-rich medium, then used for lipid raft-patching experiments, phenotypic analyses, and cytotoxicity assays. The viability of cells after sialidase treatment was determined by a 48-h MTT proliferation assay. Removal of α2,3- or α2,6-linked sialic acid residues was controlled using MAL II and SNA lectins.

Western blotting

To assess sialylation of glycoproteins, cells were lysed for 20 min at 4°C in the following buffer: 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 1% (v/v) Triton X-100 containing a protease inhibitor mixture (Roche). After centrifugation (20,000 rpm, 15 min, 4°C), cytosolic fractions were carefully removed, and total protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce). Equal amounts of protein lysates were run on 10% SDS-PAGE, then blotted onto nitrocel lulose. Membranes were blocked using 5% Tween 20 in TBS, then probed with 0.066 μg/ml SNA lectin or 0.033 μg/ml MAL II lectin. Blots were washed with TBS/1% Tween 20, then probed with HRP-conjugated streptavidin (0.033 μg/ml in TBS/1% Tween 20; Vector Laboratories). After extensive washings with TBS/1% Tween 20, blots were rinsed once with distilled water. Lectin staining was revealed by chemiluminescence using the ECL system (Amersham Biosciences). To ensure that lectin staining was specific, samples were treated for 90 min at 80°C with 2 M acetic acid (to liberate sialic acids) before electrophoresis.

MIC-A detection was performed using a specific goat antiserum (SCS5459; Santa Cruz Biotechnology) on protein lysates, leading to a 40– to 45-kDa band.

*Patching of lipid rafts with CT-B and MIC molecule redistribution*

For GM1 cross-linking, UT-79 cells were labeled with CT-B (Sigma-Aldrich; 8 μg/ml) on ice for 30 min before incubation with anti-CT-B rabbit anti-rabbit IgG (Molecular Probes) for 20 min at 37°C. Immediately after patching, cells were fixed in 2% paraformaldehyde for 15 min at room temperature. After washing, cells were incubated with primary Ab (anti-MIC) for 45 min at room temperature, washed, and incubated for 45 min with Alexa Fluor 546 goat anti-mouse IgG (Molecular Probes). The samples were conserved in mounting medium (Vectashield; Vector Laboratories) at 4°C and analyzed by laser scanning confocal microscopy (LSM5; Zeiss). Cells were considered to have patched receptors if the staining pattern showed large granular staining on the cell membrane. In some experiments, patching was induced after treatment with sialidase (60 μU for 1 h) or IM (0.5 μM for 48 h).

*Confocal microscopic analysis of UT-79/NK conjugates*

For NK/target cell interaction analysis, NK cells were incubated with FITC-CT-B for 1 h on ice to label lipid raft-associated GM1 gangliosides. Targets washed twice in PBS were allowed to settle onto slides for 30 min at 37°C before NK cells were added. Synapse formation was examined at various times after the preparation of the conjugates (15, 30, and 45 min) that were fixed with PBS/4% paraformaldehyde. After fixation, NK/targets conjugates were stained with Alexa Fluor 568-phalloidin (Molecular Probes). Cells were considered to have clustered receptors if the staining pattern was crescent shaped, and stained molecules polarized to one side of the cell. A total of 100–200 conjugates/slide was analyzed to evaluate percentages.
Results

Altered immunological synapse formation between IM-treated Bcr/Abl targets and NK cells

We previously reported that high level Bcr/Abl transfectant UT-7/9 cells derived from the UT-7 cell line were lysed more efficiently by activated NK cells, and that IM treatment decreased their susceptibility to NK-mediated lysis, whereas it had no such effect on parental UT-7 cells (22, 24). It is known that NK/target interactions require tight contact, resulting in the formation of an immunological synapse that concentrates the triggering receptors, favoring cell signaling. In this study, we compared the abilities of targets treated, or not, with IM to form NK/target cell synapses during cocultures with activated NK cells. FITC-labeled CT-B-labeled NK cells were incubated with target cells treated, or not, with IM, and conjugates were analyzed for lipid raft coalescence toward targets and cytoskeleton rearrangements after staining with a phalloidin-based probe specific for F-actin. In three independent experiments, optimal results were obtained in 30-min conjugates that led to the coalescence of lipid rafts in 35–40% of the NK cells toward nontreated targets and lower in 15-min conjugates (20–25%), whereas 45-min incubation led to target cell destruction (Fig. 1B). Toward nontreated cells, NK/target conjugates are characterized by flattened interface, coalescent lipid rafts, and polymerization of F-actin filaments at the cell-cell contact (Fig. 1A). As shown in Fig. 1A, when UT-7/9 cells were treated with IM, conjugates displayed an absence of raft coalescence and an absence of polymerized F-actin filaments, compared with UT-7/9/NK cells conjugates, which display a brighter signal at the cell-cell contact.

FIGURE 1. IM alters the formation of the immunological synapse in NK/UT-7/9 conjugates. A, Target cells were cultured in medium or in the presence of IM (0.5 μM; for 48 h). Confocal microscopy was performed of FITC-CT-B-labeled NK/target conjugates stained with phalloidin-AlexaFluor 568 to probe actin F filaments. In most NK/target conjugates, lipid rafts in NK cells coalesce and form large patches polarized at the NK/target interface. In IM-treated UT-7/9/NK conjugates, coalescence of lipid rafts is attenuated and GM1 staining remains uniform on the NK cell surface. There is a decreased polymerization of F-actin filaments in IM-treated UT-7/9/NK cell conjugates. IM exerts no effect on UT-7/NK conjugates. B, Quantitative analysis of target/NK conjugates. The percentages of NK cells forming synapses (lipid raft coalescence and polarization of phalloidin staining) in NK/target cultures incubated in the presence (IM) or the absence (Med) of imatinib are depicted. One representative experiment of three is depicted.
than in the rest of the cell. A synapse was identified in 99 of 189 (53%) UT-7/9/NK conjugates, whereas 23 of 145 (16%) conjugates formed a synapse toward IM-treated UT-7/9 cells. IM did not alter the synapse structure in UT-7/NK cell conjugates, because 76 of 190 vs 58 of 116 conjugates had synapses after IM treatment (Fig. 1B).

The rare and altered synapse formation in IM treated UT-7/9/NK cell conjugates (although synapses were normal toward untreated UT-7/9 cells) indicates that Bcr/Abl transformation may control mechanisms governing the NK/target cross-talk in response to IM treatment. This also suggests that besides reducing pTyr in Bcr/Abl targets, IM may modulate cell surface molecules important for NK target cross-talk.

**Inhibition of Bcr/Abl tyrosine kinase activity by IM induces a high surface expression of GM1 gangliosides**

Lipid rafts are cholesterol- and glycosphingolipid-rich membrane subdomains sequestering important ligands in NK/target cell interactions and surface glycosphingolipids. In this regard, GM1 gangliosides are an important component of the lipid rafts. To understand the absence of synapse formation between NK and IM-treated UT-7/9 cells, the modulation of membrane GM1 expression in response to tyrosine kinase inhibition was examined after staining with FITC-CT-B that specifically bound to cell surface GM1. Although a similar expression of GM1 was detected on
UT-7 and UT-7/9 cells (n = 6), IM treatment induced an increase in GM1 expression on UT-7/9 cells, but not on UT-7 cells (Fig. 2A, left panels). As an additional control, UT-7 and UT-7/9 cells were treated with *V. cholerae* neuraminidase (Neur), a sialidase that sheds sialic acids and allows better accessibility to GM1. Treatment with Neur of UT-7 and its Bcr/Abl transfectant induced increased binding of CT-B to membrane GM1 (Fig. 2A, right panels). Furthermore, the analysis of GM1 expression by confocal microscopy allowed us to obtain the precise effect of IM. To mimic surface receptor engagement and induce lipid raft aggregation or patching, CT-B-labeled GM1 were cross-linked into patches by aggregating Abs as previously described (25). GM1 patching resulted in the coalescence of large GM1 patches on the cell surface in 75–80% of UT-7 and UT-7/9 cells (Fig. 2B, Med). Interestingly, IM treatment of UT-7/9 resulted in an increase in the membrane expression of GM1, in agreement with flow cytometry data, but inhibited their patching upon cross-linking, leading to 60–80% of the cells displaying a dense and uniform membrane staining by CT-B after patching (Fig. 2B, right panels). IM had no effect on UT-7 cells, confirming that it is Bcr/Abl dependent. Treatment by Neur also prevented GM1 coalescence upon patching on 95–100% of UT-7 and UT-7/9 cells (Fig. 2B).

It is likely that Neur, by shedding sialic residues, may alter the sialylation of various membrane molecules important for NK-target cross-talk. We thus studied the NK-mediated lysis of Neur-treated targets. Neur treatment (1 h; 60 U/ml) did not affect cell viability, as assessed by MTT assay performed 24, 48, and 72 h after enzymatic treatment. Interestingly, such treatment of UT-7 and UT-7/9 cells led to a marked inhibition of NK-mediated lysis, resulting in low and similar lysis of both cell lines (Fig. 2C). In five independent experiments, a mean decrease of 45–50% in the lysis of Neur-treated cells was obtained. These results indicate that cell membrane sialylation is involved in target recognition and lysis and may play a role in the potentiation of Bcr/Abl target lysis by NK cells.

**Inhibition of Bcr/Abl tyrosine kinase activity by IM modulates target cell sialylation**

The above results indicate that target cell sialylation may have important consequences for cell recognition and lysis by activated NK cells. It is well known that aberrant sialylation is a characteristic of CML. Sialic acid residues are present on glycoproteins and thus may control the signaling of lectin-type NK receptors. In the course of these studies, target cell sialylation was assessed by staining with fluorescein lectins, namely SNA, which is highly specific for sialic acid α2,6-linked sialic acids, and MAL II lectins, which recognize (α2,3)-linked sialic acids. Increased Mal II lectin binding on UT-7/9 cells, compared with the parental cell line, was observed in 6 of 10 experiments, whereas SNA bound with similar efficiency to both cell lines. As shown in Fig. 3A, left panels, IM treatment of UT-7/9 transfectant significantly decreased cell surface sialylation, as measured by fluorescein SNA and MAL II lectin binding (n > 10), but had no significant effect on UT-7 cells. Lectin binding (SNA and MAL II), assessed by Western blotting, confirmed the modulation of sialylation of cytosolic proteins in UT-7/9 treated with IM. Increased sialylated glycoproteins (65 and 75 kDa, respectively) detected by both lectins were present in UT-7/9 cells, but not in UT-7 cells, after IM treatment (Fig. 3B). This was not related to induced expression of the corresponding proteins, as suggested by equivalent Ponceau Red staining. Together, these data suggest that IM prevents the surface expression of certain glycoproteins.

Treatment of UT-7 and UT-7/9 cells by *V. cholerae* sialidase, which sheds preferentially α(2,6)- and α(2,8)-linkages, resulted in greatly reduced binding of both MAL II and SNA lectin (Fig. 3A, right panels). It is intriguing that enhanced binding of the fusion molecule NGKD2D-Fc to UT-7/9 cells was detected, compared with the UT-7 cells, in response to Neur treatment. This indicates that sialic acids may hamper receptor ligation and triggering of NGKD2D receptor on a high-level Bcr/Abl transfectant (Fig. 3C). This effect of Neur may contribute to the abrogation of the differential recognition and lysis of Bcr/Abl targets compared with parental UT-7 cells upon enzymatic treatment (Fig. 2C).

**Down-regulation of MIC-A/B expression after inhibition of Bcr/Abl tyrosine kinase activity**

As the above results outline that MIC/NGKD2 interaction may be involved in the modulation of NK-mediated lysis of IM responder Bcr/Abl targets, flow cytometric analyses were performed to determine the membrane expression of MIC and ULBP proteins by Bcr/Abl transfectants, UT-7/9 and UT-7 cells. The results depicted in Fig. 4A, left panels, show a similar expression of MIC-A/B molecules on Bcr/Abl UT-7/9 transfectant and parental UT-7, as assessed by an anti-MIC-A/B mAb whose specificity was controlled on MIC-A transfectant derived from the MIC-A-negative C1R cell line (Fig. 4A, middle panels). In addition, staining with the NGKD2D-Fc fusion protein revealed similar NGKD2-D ligand expression on UT-7 and its Bcr/Abl transfectant. Interestingly, after treatment of targets with IM, the expression of MIC-A/B and NGKD2D ligands on UT-7/9 cells was clearly decreased, whereas it remained unchanged on UT-7 cells and C1R-MIC-A transfectant (Fig. 4A), indicating that, in UT-7/9 Bcr/Abl transfectant, MIC-A expression is under the control of tyrosine kinase activity. The expression of MIC-A was probed by Western blotting, revealing a band of 40–45 kDa in UT-7 and UT-7/9 cells that was decreased after IM treatment of UT-7/9 cells, but not of UT-7 cells (Fig. 4B).

Obviously, the modulation of GM1 expression by IM and its effect on lipid raft coalescence (Fig. 2) may alter the redistribution of NKR ligands, MIC and ULBP molecules, expressed by Bcr/Abl transfectants. We thus assessed the distribution of MIC molecules with respect to the lipid rafts by confocal microscopy when GM1 was cross-linked with anti-CT-B Abs to mimic engagement with the specific receptor. We show that in UT-7 and UT-7/9 cells, MIC molecules uniformly present on target cells were redistributed in the lipid rafts after patching by aggregating Abs (Fig. 4C). Interestingly, IM-treated UT-7/9 targets did not form patches after treatment with aggregating Abs and MIC molecules maintain a uniform membrane distribution with no sign of relocalization when cells were treated with neuraminidase (Fig. 4C).

**Inhibition of NGKD2D-induced lysis of Bcr/Abl transfectants UT-7/9 cells by activated peripheral NK cells after IM treatment**

We have previously shown that NK-mediated lysis of UT-7/9 cells was under the control of Bcr/Abl high level expression and that treatment with IM reduced the lysis, whereas it had no effect on UT-7. The present studies also implicate the activating receptor NGKD2 as an important pathway in the lysis of these leukemic targets by peripheral activated NK cells. In five independent experiments, addition of a specific anti-NGKD2 mAb resulted in greater inhibition of lysis of UT-7/9 cells, compared with that of UT-7, indicating a major role for this activating receptor in the killing of Bcr/Abl transfectant UT-7/9 cells. This fits well with the higher binding of the specific NGKD2D-Fc fusion protein. As a control, it was shown that C1R MIC-A transfectants were lysed with greater efficiency than parental C1R and that anti-NGKD2 mAb decreased lysis of the transfectants to the level of the parental cells, confirming the specificity of the blocking experiments (Fig.
After treatment with IM of UT-7/9 cells, addition of anti-NKG2D mAb in the cytotoxic assay did not significantly inhibit NK-mediated lysis \((n = 5; p < 0.0015)\), as depicted in Fig. 5B, indicating that IM inhibits the functional effect of NKG2D/MIC interaction.

**Discussion**

CML is the most susceptible hemopathy to allogeneic adoptive cellular therapy, as evidenced by the high rate of molecular remission and the efficiency of donor lymphocyte infusion to control relapse after bone marrow transplantation \((26, 27)\). Despite development of the specific tyrosine kinase inhibitor, IM, which has greatly improved the treatment of CML patients \((20)\), resistance after long-term administration has appeared, indicating that stem cell transplantation and adoptive cellular therapy remain the only curative treatments \((28)\). The antileukemic effect of allogeneic transplantation is mediated by cytotoxic immune effectors, namely, CTL and NK cells, but the respective roles of these two types of lymphoid effectors are as yet unclear \((29)\).

Over the past decade, major advances have been made in the field of NK biology, notably through identification of new receptors involved in NK recognition and cytolysis. It is now clear that NK cell function is regulated by a balance between inhibitory and activating signaling pathways \((30)\). Receptors triggering cytolysis by NK cells include NCR (NKp46, NKp30, and NKp44) from the Ig family. The ligands for these receptors on the targets are largely uncharacterized, but blocking experiments have shown that they are expressed by various tumor cells \((8)\). In addition, NK cells express lectin-type receptors that transduce positive or negative signaling depending on the presence of the motifs present in their intracytoplasmic regions \((31)\). Among them, the activating NK receptor NKG2D recognizes stress-induced molecules on infected and transformed cells. These lectin-type receptors recognize carbohydrates that were shown to have a regulatory role in NK cell-mediated killing \((32)\). In this regard, several reports have demonstrated that the increase in particular carbohydrate residues after transfection of tumor cells by glycosyltransferases or insertion of
abundantly glycosylated protein into cell membrane can protect targets from NK cytolysis (33–35).

In the present study, we investigated the mechanisms by which high level Bcr/Abl expression through its tyrosine kinase activity modulates susceptibility to allogeneic activated NK-mediated lysis of leukemic Bcr/Abl transfectants. Bcr/Abl triggers numerous signaling pathways, interfering with proliferation, migration, adhesion, and resistance to apoptosis. The cellular model UT-7, its Bcr/Abl transfectant UT-7/9, and use of the specific tyrosine kinase inhibitor allow dissection of the mechanisms controlling the susceptibility of high level Bcr/Abl transfectants to NK cells and its relation to tyrosine kinase activity. Therefore, a better understanding of how IM modulates the susceptibility of Bcr/Abl targets to NK cells may have important implications for the treatment of patients developing resistance to IM.

We previously showed that specific inhibition of tyrosine kinase activity by IM reduces NK2D ligand expression and prevents their redistribution after lipid raft aggregation. In IM-treated Bcr/Abl transfectants, increased GM1 induces membrane rigidity and prevents lipid raft coalescence, altering the synapse structure. It also prevents MIC molecule redistribution in the treated target, altering cell signaling. MIC-A/B molecules are expressed in specific membrane microdomains and may constitute a checkpoint in NK cell activation (39). The control treatment by Neur, a sialidase that sheds sialic acids and unmask GM1 at the cell surface, precludes lipid raft aggregation and MIC lateral mobility in a Bcr/Abl-independent manner. It also decreases lysis of C1R cells (data not shown), indicating that it exerts its effect through synapse. Thus, both high level expression of MIC-A/B and their redistribution in lipid rafts at the interface with NK cells are required for efficient engagement of the NKG2D receptor on NK cells. In addition, increased GM1 in response to IM...
FIGURE 5. NKG2D-induced lysis of Bcr/Abl target UT-7/9 cells is inhibited after specific tyrosine kinase inhibition by IM. A. Inhibition of the NK-mediated lysis of UT-7 and UT-7/9 cells in the presence of anti-NKG2D mAb. NK-mediated lysis toward targets was measured by a 
$^{51}$Cr release assay performed in the presence of anti-NKG2D mAb or isotypic control (clgG). One representative experiment of five is shown. Control specificity of the blocking mAb on C1R and C1R-MICA transfectant is also depicted (E:T cell ratio, 5:1). B. IM treatment of UT-7/9 cells abrogates the inhibitory effect of anti-NKG2D triggering on the lysis of Bcr/Abl targets. UT-7/9 cells were treated, or not, with IM (0.5 μM; 48 h) and used as targets in an NK-mediated cytolysis assay performed in the presence of anti-NKG2D mAb or isotypic controls (clgG; E:T ratio, 5:1).

may also be involved in resistance to apoptosis, because it may prevent the recruitment of signaling molecules inside lipid rafts. This corroborates previous reports demonstrating the importance of membrane fluidity in the signaling of growth factor receptor (40, 41).

The present study indicates a major role for NKG2D receptor in triggering the lysis of UT-7/9 and parental UT-7 cells and, thus, the importance of MIC and ULBP molecule expression to the susceptibility of these targets to NK-mediated lysis. The decreased membrane expression of MIC and ULBP as well as their reduced mRNA transcripts (data not shown) in UT-7/9, but not in UT-7, cells after IM treatment indicate that NKG2D ligand expression is under the control of Bcr/Abl tyrosine kinase activity. Furthermore, our data outline that IM-induced modulation of NKG2D ligand expression on UT-7/9 cells (and K562 cells; data not shown) is accompanied by their decreased susceptibility to NK-mediated lysis via an NKG2D-dependent mechanism. In addition, we have recently shown that the up-regulation of MIC molecule expression by Bcr/Abl immature dendritic cells activates NK cells, further outlining the role of NKG2D in susceptibility of CML to NK cells (42).

It is noteworthy that, in contrast to effectors of the adaptive immune response, recognition by activating NCR does not seem to involve classical protein entity recognition and may be related to cellular ligands involving carbohydrates, lipids, or both (43). This is in agreement with the observed relationship between modulation of the glycosylation status of proteins in IM-treated Bcr/Abl transfectant and their altered interaction with their receptor on NK effectors. Thus, modulation of carbohydrate-dependent target-effector interactions by high level Bcr/Abl expression may also be of crucial importance to additional tumor cell recognition and cytolysis events by activated NK cells.

Taken together, our present findings show for the first time the impact of high level Bcr/Abl expression, through its elevated tyrosine kinase activity, on glycosylation of leukemic transfectants. However, more intriguing is the effect of IM treatment on glycosylation and its unexpected impact on surface GM1 expression. One possible hypothesis is that IM may act through decreased activation of the transcription factor NF-κB that controls the expression of several genes, among which are several glycosyltransferases. Treatment with IM has recently been reported to exert diverse effects on innate and adaptive immune responses (44, 46). Those results and ours presented in this article strongly suggest that such effects of IM should be taken into consideration for long-term treatment with IM or the use of this compound in combination with other treatment modalities.

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Disclosures

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References


