Upregulation of NKG2D ligands and enhanced natural killer cell cytotoxicity by hydralazine and valproate

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Abstract. Natural killer cells play a role in the immune antitumor response by recognizing and eliminating tumor cells through the engagement of NKG2D receptors with their ligands on target cells. This work aimed to investigate whether epigenetic drugs are able to increase MICA and MICB expression as well as NK cell cytotoxicity. Prostate, colon, breast and cervical cancer cell lines were analyzed for the expression of MICA and MICB at the mRNA and protein levels by RT-PCR, Western blot, flow cytometry and ELISA. The activating mark H3K4m2 at the MICA and MICB promoters was investigated by ChIP assays. Cytotoxicity of NK cells against the target epithelial cancer cells was investigated with the CD107 cytotoxicity assay. The results show that hydralazine and valproic acid not only increase the expression of MICA and MICB ligands of target cells, but also reduce their shedding to the supernatant. This upregulation occurs at the transcriptional level as revealed by increase of the H3K4 activating mark at the promoter of MICA and MICB genes. These effects are paralleled by increased cytotoxicity of NK cells, which was attenuated at different degrees by using blocking antibodies against the NKG2D receptor and ligands. In conclusion, our results demonstrate the ability of hydralazine and valproate to increase the NK activity against epithelial cancer cell lines and suggest that these drugs could reduce the levels of soluble MICA and MICB helping in avoiding tumor-induced suppression of NK cytotoxicity against the tumor.

Introduction

DNA hypermethylation and histone deacetylation are critical for determining a closed chromatin structure responsible for or related with aberrant gene transcription in malignancies (1). DNA methylation and histone deacetylase inhibitors exert antitumor effects by inhibiting cell proliferation, metastases, angiogenesis, and by inducing cell differentiation and/or apoptosis, as well as by increasing chemotherapy cytotoxicity (2-6). Nevertheless, chromatin remodeling agents also show promise for immune therapy of cancer. It is known that several immune escape mechanisms allow tumors to go undetected; for instances, epigenetic downregulation of genes involved in antigen processing and presentation has been described which is reversed by DNA methylation and histone deacetylation inhibitors allowing tumor recognition by the immune system (7-9).

DNA methylation and other epigenetic mechanisms are also important for the establishment and maintenance of diverse repertoires of clonally restricted KIR and Ly49 receptors in Natural killer (NK) (10) cells which are components of the innate immunity that substantially contributes to the elimination of virus-infected cells (11,12) as well as to antitumor immune responses (13). Epigenetic control of their expression produces specific combinations of multiple receptors on each NK cell, and a population-wide repertoire that allows correct responses to changes in the MHC ligand environment (10).

NKG2D is a type II transmembrane anchored C-type lectin-like molecule expressed as a disulfide-linked homodimer on the surface of NK cells and various T cell subsets including CD8+ αβ T cells, γδ T cells and NKT cells. In humans, NKG2D binds to MHC class I-related chain A (MICA), MHC class I related chain B (MICB), and a structurally distinct family of UL16-binding protein (ULBP) proteins (14,15). The role of NK cells in immunosurveillance against tumors has been widely demonstrated in syngeneic murine models where their depletion results in increased aggressive growth and metastatic ability of tumors (16,17). Likewise, NK cells deficiency or some of their receptors or effector molecules causes a high incidence of tumors in spontaneous or induced tumor models (18). NKG2D ligands are not expressed in normal cells, but are upregulated in cancer cells, which elicit a potent antitumor immune response in vitro and in vivo, when the tumor is not rejected or eliminated; the immune system sculpts the phenotype in tumor cells, and eliminate the cells more immunogenic favoring the growth of less immunogenic cells (19). Here we demonstrate that the
DNA demethylating inhibitor hydralazine in combination with valproic acid are able to enhance the natural killer cell-mediated cytotoxicity against epithelial cancer cells via increased expression of NKG2D ligands.

Materials and methods

Human prostate cancer cell line DU145, colon cancer cell line SW480, breast cancer cell line T47D and cervical cancer cell line CaSKi, obtained from the ATCC, were cultured at 37°C with 5% CO₂ in DMEM-F12 medium supplemented with 10% heat-inactivated fetal bovine serum. Hydralazine (H) and magnesium valproate (V) were obtained from Sigma and added at 10 µM and 1 mM for 5 days. Medium with freshly prepared drugs was replaced daily.

RNA extraction and real-time PCR. RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcription of 1 µg RNA was carried out using GeneAmp RNA PCR Core kit (Applied Biosystems) and stored at 20°C until use. NKG2D ligands gene expression was carried out using an iCycler (Bio-Rad) with specific primers for MICA sense 5'CTGGTGCTTCCAGGTACTTG-3', antisense 5'-GAAATCCGAGCTGTGTGGCAT-3'; and MICB sense 5'-GACCAAGACACACTATCGCG-3', antisense 5'-CATGTCACGGTGATGTTGCC-3'.

Chromatin immunoprecipitation (ChIP) assay. Cancer cell lines treated with H and V as above described, were fixed with 1% formaldehyde for 10 min at room temperature and neutralized by addition of glycine at a final concentration of 0.125 M for 5 min. Fixed cells were suspended in 1 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.0, 0.2 mM PMSF, 5 µg/ml aprotinin, 1 µg/ml leupeptin, and 5 µg/ml sonicated salmon sperm DNA. Following 30 min incubation on ice, samples were sonicated for 10x20 sec, with at least 1 min intervals on ice. Samples were then spun for 10 min at a maximum speed at 4°C, and the supernatants were collected. Protein-chromatin complexes were immunoprecipitated overnight with dimethyl-H3 (Lys4) antibody (Millipore) and rotated at 4°C. The chromatin was collected with protein A-agarose/salmon sperm DNA (Millipore). The beads were washed sequentially with 1 ml of each following buffer for 5 min: IP dilution buffer (0.1% SDS, 1% Triton X-100, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate, 10 mM Tris-HCl, pH 8.0, and 150 mM NaCl), washing buffer (0.1% SDS, 0.5% Na-deoxycholate, 10 mM Tris-HCl, pH 8.0, and 500 mM NaCl), and LiCl buffer (0.25 M LiCl, 0.5% Na-deoxycholate, 10 mM Tris-HCl, and 10 mM Tris-HCl, pH 8.0), followed by washing for 3 times with cold TE buffer (pH 8.0). Precipitates were then extracted by incubating with elution buffer (1% SDS, 0.1 M NaHCO₃) at 65°C overnight and with 1/25 volume of 5 M NaCl for 4-6 h at 65°C. DNA was purified with phenol/chloroform/isooamyl alcohol for PCR-amplification. The following PCR primers were used for amplifying the human promoter MICA sense 5'-CGACGTCGCCACCCTCTCA-3', antisense 5'-CCAGGTTCGCCCATTTCCATA-3' and promoter MICB sense 5'-CTACGTGCACCCTTCTCAGTGG-3', PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining.

Figure 1. Effect of HV on the expression of MICA and MICB genes. The gene expression levels relative to GAPDH were determined using real-time PCR. Each column and error bar represents the mean ± SD of the ratios of the NKG2DL. Results from three independent experiments. *p<0.05, ns=non-significant.

Immunofluorescence staining. Fluorescence microscopy was done as previously described (20). In brief, cells were grown on coverslips until 60% confluence before treatment with HV. After 5 days, cells were fixed with acetone at 4°C. Coverslips were blocked and subsequently incubated with mouse anti-MICA/B (6D4) (Santa Cruz Biotechnology, Inc.) or isotype control, respectively, followed by incubation with a secondary goat anti-mouse AF-594 (Invitrogen) and mounted with ProLong Gold DAPI (Invitrogen), coverslips were examined using a fluorescence microscope.

Flow cytometry. Cell lines were detached from culture dishes using PBS/EDTA, incubated with mouse anti-human MICA/B (6D4), goat anti-human ULBP-1, rabbit anti-human ULBP-2, rabbit anti-human ULBP-3, antibodies purchased from Santa Cruz Biotechnology. FITC-labeled rat anti-mouse IgG, FITC-labeled goat anti-rabbit, FITC-labeled donkey anti-goat was purchased from ebioscience (San Diego, CA), finally analyzed on a FACSCalibur (Becton-Dickinson, Mountain View, CA). The data were analyzed with FlowJo 8.7 software (Tree Star, Inc.).
**ELISA assay.** The supernatants of cultured cells were harvested after 5 days with HV, the levels of soluble MICA were determined with an ELISA kit (DuoSet, RyD System) according to the manufacturer’s protocol.

**Isolation of NK cells.** Primary NK cells were obtained from peripheral blood lymphocytes and isolated by Ficoll-Hypaque density gradient centrifugation (Amersham Pharmacia Biotech) from heparinized venous blood obtained from normal healthy volunteer donors. NK cells were purified with a NK cell isolation kit (Miltenyi Biotec, Auburn, CA). Non-NK cells were magnetically labeled indirectly, using a mixture of biotin-conjugated antibodies and the NK Cell MicroBead Cocktail. Isolation of highly pure NK cells was achieved by depletion of magnetically labeled cells. The percentage of NK cells after isolation was evaluated using FITC-conjugated anti-CD3 mAbs and PE-conjugated anti-CD56 mAbs (BD PharMingen) in flow cytometry, and routinely exceeded 95%.

**CD107 cytotoxicity assay.** The CD107 assay with the cancer cell lines was set up as described (21). Briefly, cancer cells DU145, T47D, SW480 and CaSki, were plated at various cell numbers in a 96-well plate and incubated at 37°C and treated by 5 days with HV. On the day of the assay, the culture supernatant was removed from the wells and the effector NK cells were added to the wells at various E: T ratios (100:1, 50:1, 25:1). Control wells contained either only NK cells, only tumor target cells, anti MICA/B in target cells and anti NKG2D (1D11) in the well with NK cells. Ten microliters each of CD107-biotin was added to each well at the same time as addition of the NK cells. The plate was then centrifuged for 3 min at 1,000 rpm in order to facilitate immediate contact between the NK cells and the target cells at the bottom of the wells. The plate was then incubated at 37°C and after the first hour of incubation GolgiStop was added to each of the wells and the plate was returned to the incubator for an additional 4 h. At the end of the incubation period the cells were harvested into separate tubes and washed once with Pharmingen Stain Buffer (PSB, BD Pharmingen, CA) and incubated with Streptavidine-APC 30 min, and washed after which they were re-suspended in 1 ml PSB and stained with anti-human CD56 AF-488 for 30 min. The cells were then washed again and re-suspended in PSB for measuring Median Fluorescence Intensity (MFI) using a FACS Calibur flow cytometer (Becton-Dickinson).

**Results**

The four human epithelial cancer cell lines expressed basal levels of MICA and MICB mRNA. Upon HV treatment for five days at 10 µM and 1 mM respectively, a significant increase in MICA expression ranging from almost 3- to 6-fold was observed in all but 747D cells. MICB mRNA expression was also significantly increased in the four cell lines. Of note, the DU145 prostate cancer cell line was the most responsive to treatment (Fig. 1).

These results strongly suggest that these two ligands are epigenetically downregulated in epithelial cancer cells. Previous studies have shown the transcriptional activation of these molecules by a histone deacetylase inhibitor depends on the induction of Sp1 transcription factor and binding to the MICA and MICB promoters. To confirm the transcriptional activation by HV we evaluated by ChIP analysis the activating methylation mark H3K4m2. As expected, an increase in methylation was observed in the four cell lines in both MICA and MICB gene promoters except in 747D cells for MICA (Fig. 2) which strongly correlates with the RT-PCR results shown in Fig. 1.

Recognition and NK cell cytotoxicity upon ligand binding against tumor cells require that NKG2D ligands are expressed at the cell surface; hence flow cytometry and immunofluorescence staining assays were performed using an antibody which recognizes both MICA and MICB. The results in Fig. 3A show that indeed HV not only transcriptionally activates their expression but led to a significant increase in the expression of these molecules in the four epithelial cancer cell lines. This was observed even in 747D cells which showed less transcriptional activation as compared to the other three cell lines. Fig. 3B supports these results as both cytoplasmic and membrane staining were
observed in the cell lines. To further explore this issue, it was important to investigate the effect of HV treatment in the shedding of these molecules. As shown in Fig. 4, the analysis with an ELISA assay of the supernatant of HV treated cells did not show statistical significance, however, there was a clear reduction in the amount of soluble MICA.

To determine whether increased MICA and MICB in target epithelial cancer cells could increase their susceptibility to NK cells, we measured the cytotoxicity of isolated NK cells from the peripheral blood of healthy individuals. Fig. 5 shows that as expected HV treatment for five days significantly increased the susceptibility of the four cell lines to cytolysis by NK cells. Further, Fig. 5 also shows that this increased lysis depended upon the interaction NKG2D-NKG2D-ligands. The addition of a blocking antibody against the NKG2D receptor in effector cells led to a very strong inhibition of killing. A lesser but still clear inhibition of cytotoxicity was also demonstrated when a blocking antibody against MICA/B was added to the assay. This latter result may suggest that HV could also induce UL16-binding protein (ULBP) 1, 2 and 3 which are other well-known ligands of the NKG2D receptor whose presence at the surface of target cells triggers NK cell activation and cytotoxicity upon engaging. Fig. 6 clearly shows HV treated cells had a significant increase of all three ULBP ligands at the membrane of the epithelial cancer cells, which may explain the only partial abrogation of killing by NK cells when MICA and MICB were blocked in the cytotoxicity assay.

Discussion

Hydralazine and valproate are being repositioned as epigenetic drugs for cancer treatment. The results of this study show that
in combination, they are able not only to increase the expression of NKG2D ligands at transcriptional level but also to enhance the membrane expression of these ligands in epithelial cancer cells which leads to enhanced NK cell cytotoxicity against these target cells.

Current evidence strongly suggests that activation of the immune system, mediated by engagement of NKG2D receptor with its ligands, plays an important role in immunosurveillance of cancer (22,23), and the expression of NKG2D-L has been described in multiple types of tumors (24,25). Because MICA and MICB are crucial for the activating receptor NKG2D and subsequent killing of tumor cells and it has been shown that these molecules can be downregulated by epigenetic mechanisms as a tumor evolving mechanism to minimize or avoid the response mediated by NKG2D ligands, here we prove that the DNA methylation and HDAC inhibitors hydralazine and valproate, respectively, are able to transcriptionally increase the expression of these ligands in four epithelial cancer cell lines. Though the specific epigenetic process involved in the regulation at the promoter of these genes is yet unknown, hydralazine and valproate, respectively, are able to transcriptionally increase the expression of these ligands in four epithelial cancer cell lines. These two effects as well as the increased expression of ULBP 1, 2 and 3 most likely were responsible for the significant increase in the cytotoxicity of NK cells upon the epithelial cancer cells as killing was avoided at different degree by blocking either the receptor or the ligands.

A number of studies have shown that valproate (29-31) and other epigenetic agents including HDAC inhibitors sodium butyrate (32) and trichostatin A (33), as well as the DNA methylation inhibitor 5-aza-2'-deoxycytidine (34) and arsenic trioxide (35) which also inhibits DNA methyltransferase (36) are also able to increase NKG2D ligands and NK cells cytotoxicity in a variety of hematological and non-hematological malignant models. Thus, our data are in line with previous studies which support the role of epigenetic agents for increasing NK activity against tumor cells. A previous study performed in the AML cell line HL60 and in primary AML blasts, showed an increased expression of ULBP1 by several agents. The highest expression was combining Vitamin D3, 5-aza-2'-deoxycytidine, trichostatin A, granulocyte colony-stimulating factor and γ interferon (30) nevertheless, the specific contribution for increased expression of either DNA methylation or HDAC inhibitors remains to be demonstrated. In a previous study it was shown that hydralazine and valproate had an additive effect upon the upregulation of HLA class-I antigen expression and antigen-specific CTL response in cervical cancer (37) which supports their use in combination.

Hydralazine and valproate in combination have shown to upregulate the expression of hundreds of genes in preclinical and clinical studies (6,38-39). Current data on transcriptional changes induced in cancer cell lines by epigenetic agents indicate that not all upregulated genes have CpG islands
in their promoters and theoretically silenced by epigenetic mechanisms, but that gene upregulation in a proportion of them is secondary to pathway activation (39). On this basis, and without detailed analysis at the promoter of NKGD2 ligand-coding genes, the results here obtained could have resulted from pathway activation induced by these drugs. Nevertheless, the Sp1 transcription factor has an important regulatory role in the expression of MICA and MICB genes (32) and it is known that Sp1 can recruit repressor complexes, such as Sin3A HDAC1/HDAC2, to repress gene transcription (40,41) and that it directly interacts with DNA cytosine methyltransferase-1 (42) which suggests that Sp1 serves as a platform for recruitment of transcriptional repressors. This fact may explain that several epigenetic drugs share the ability of upregulating the expression of NKGD2 ligands by interfering with the inhibitory role of Sp1 in their regulation.

Regardless of the precise mechanism of action of hydralazine and valproate upon NKGD2 ligand expression and

Figure 5. Enhanced natural killer (NK) cell-mediated lysis following treatment with HV. The cells were incubated for five days with HV then examined for their sensitivity to killing by cultured NK cells from healthy individuals at different ratios (A). The 1:100 ratio is shown in bar graphs, it also examines the effect of preincubation of target cells with anti-MICA/B and incubation of NKG2D antibody with effector cells, *p<0.05 (B).
cytotoxic activity of NK cells, the findings of this study are of clinical relevance to further assess their immunotherapeutic potential in malignancies. So far, the combination of hydralazine and valproate has shown efficacy against myelodysplastic syndrome and a number of solid tumors (39,43–46) however, whether their clinical usefulness rely on their effect upon key processes of cancer such as proliferation, and apoptosis or on their effect upon the immune response to tumor remains to be demonstrated.

The major limitation of this and many studies on this issue are related to the model itself. Studies dealing with human NK cell cytotoxicity have been almost exclusively performed employing, as target cells, allogeneic tumor cell lines that express unrelated HLA class-I repertoires. Under these experimental conditions it is not possible to assess whether changes in HLA class-I expression in cancer cells may indeed affect NK cell-mediated killing (47). In addition, we have previously shown that hydralazine and valproate had an additive effect upon the upregulation of HLA class-I antigen expression and antigen-specific CTL response in cervical cancer (37). It is known that NK cells express different HLA-class I-specific inhibitory receptors (48,49) which detect allelic determinants on HLA class I molecules, therefore, the overall effect of this combination on the CTL response and NK cytotoxicity needs investigation.

Figure 6. Treatment with HV induces the expression of UL16-binding proteins (ULBPs)1-3. The graph represents three different tests. Mean fluorescence intensity (MFI). *p<0.05, **p<0.005, ns, non-significant.
In summary, our results clearly demonstrate the ability of hydralazine and valproate to increase the NK activity against epithelial cancer cell lines and suggest the potential of these drugs to reduce the plasmatic levels of soluble MICA and MICB helping in avoiding tumor-induced suppression of NK cytotoxicity against the tumor. Nevertheless, further research is needed to define the mechanism by which these drugs upregulate the NKG2D ligands and also to investigate in autologous models whether hydralazine and valproate could increase both NK and CTL cytotoxicity.

References


