Natural Killer Cell–Mediated Lysis of Hepatoma Cells via Specific Induction of NKG2D Ligands by the Histone Deacetylase Inhibitor Sodium Valproate

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Abstract

Natural killer (NK) cells as components of the innate immunity substantially contribute to antitumor immune responses. However, the tumor-associated ligands engaging activating NK cell receptors are largely unknown. An exception are the MHC class I chain-related molecules MICA and MICB and the UL16-binding proteins (ULBP) which bind to the activating immunoreceptor NKG2D expressed on cytotoxic lymphocytes. A therapeutic induction of NKG2D ligands that primes cancer cells for NK cell lysis has not yet been achieved. By microarray studies, we found evidence that treatment of human hepatocellular carcinoma cells with the histone deacetylase inhibitor (HDAC-I) sodium valproate (VPA) mediates recognition of cancer cells by cytotoxic lymphocytes via NKG2D. VPA increased transcription of MICA and MICB in hepatocellular carcinoma cells, leading to increased cell surface, soluble and total MIC protein expression. No significant changes in the expression of the NKG2D ligands ULBP1-3 were observed. The induction of MIC molecules increased lysis of hepatocellular carcinoma cells by NK cells which was abolished by addition of a blocking NKG2D antibody. Importantly, in primary human hepatocytes, VPA treatment did not induce MIC protein expression. Taken together, our data show that the HDAC-I VPA mediates specific priming of malignant cells for innate immune effector mechanisms. These results suggest the clinical evaluation of HDAC-I in solid tumors such as hepatocellular carcinoma, especially in combination with immunotherapy approaches employing adoptive NK cell transfer. (Cancer Res 2005; 65(14): 6321-9)

Introduction

Primary liver cancer or hepatocellular carcinoma is the fifth most common cancer worldwide with a continuously increasing incidence (1). The overall prognosis of hepatocellular carcinoma is poor because about 60% to 70% of the patients are diagnosed in advanced disease stages (2). In addition, still no standard treatment for patients with unresectable hepatocellular carcinoma exists, which, at least in part, is due to the particular resistance of these tumors to cytostatic agents leading to disappointing results using conventional chemotherapy (3, 4). Therefore, alternative hepatocellular carcinoma treatment strategies focus on the development of immunomodulatory approaches (5–7) and on novel substances lately discovered to mediate antineoplastic activity, among them histone deacetylase inhibitors (HDAC-I; ref. 8).

Recently, it was shown that remodeling of cellular chromatin, induced by an increased acetylation of DNA-associated histone proteins, leads to cell cycle arrest, redifferentiation, and apoptosis in a broad range of malignant cells. Strikingly, these effects are restricted to tumor cells without affecting nonmalignant cells (9). Histone protein hyperacetylation can be achieved by HDAC-I, which are drugs that inhibit the cellular enzyme histone deacetylase (HDAC). The underlying mechanisms of the tumor-specific activity of HDAC-I, however, remain unclear (10). Different HDAC-I were shown to display profound antineoplastic effects in hepatocellular carcinoma *in vitro* (11, 12) and in a hepatoma xenograft mouse model (13). In search for an *in vivo* applicable compound with a favorable pharmacologic profile, the well-known antiepileptic drug sodium valproate (VPA) has been found to mediate HDAC inhibition (14) and to display antineoplastic activity (15, 16). Up to now, VPA was given to a huge number of patients with well tolerable side effects. Recently, we showed that VPA treatment caused a marked antiproliferative response and induction of apoptosis in human hepatoma cells without displaying toxicity in nonmalignant primary human hepatocytes (PHH; ref. 17). This indicates that VPA might be a valuable new and tumor-selective drug for treatment of hepatocellular carcinoma.

The influence of substances with HDAC-I activity on antitumor responses of the innate immune system has not yet been clarified. Evidence for immunostimulatory effects of VPA is lent from investigations with patients suffering from convulsions being treated with VPA. In comparison with PBMC of healthy donors, PBMC of VPA-treated patients exhibited a significantly higher production of proinflammatory cytokines in response to mitogenic stimuli (18) as well as increased CD4/CD8 T-cell ratios and elevated IgM levels (19). However, the functional consequences of VPA treatment for cellular immunity remain unclear.

Natural killer (NK) cells as components of the innate immunity substantially contribute to the elimination of virus-infected cells (20, 21) as well as to antitumor immune responses (22). However, the tumor-associated ligands engaging activating NK cell receptors are largely unknown. Exceptions are several highly diversified MHC class I–related molecules which have been shown to bind to the homodimeric C-type lectin-like NKG2D receptor expressed on cytotoxic lymphocytes (23). The human NKG2D ligands (NKG2DL) include the stress-inducible surface glycoproteins MICA and MICB, expressed on many epithelial tumors and upon infection (24–28) as

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well as the UL16 binding proteins (ULBP), a multigene family with at least six functional members (28–30). NKG2DL expression is known to potently stimulate antitumor responses in mice, which are critically dependent on NKG2DL expression levels on the tumor cell surface (31, 32). Thus, NKG2D is thought to play an important role in tumor immune surveillance that was recently discussed as "a renaissance for the tumor immunosurveillance hypothesis" (33).

To date, little is known as to how antitumor modalities influence the expression of NKG2DL on tumor cells affecting their recognition and destruction by cytotoxic lymphocytes. In this study, we show that treatment of hepatoma cells with VPA induces expression of NKG2DL, leading to a significantly increased NK cell–mediated lysis of tumor cells due to enhanced NKG2D engagement. In contrast, VPA does not induce NKG2DL in nonmalignant PHH. Thus, the differential influence of VPA on malignant and nonmalignant liver-derived cells suggests VPA or substances with similar activities as a potential new hepatocellular carcinoma treatment option. They might be especially useful for long-term chemoprevention, control of residual minimal disease by increasing susceptibility of hepatoma cells to host immune surveillance or even enable adoptive NK cell transfer as a new treatment option.

Materials and Methods

Reagents and monoclonal antibodies. Sodium valproate (VPA, Ergenyl) was obtained from Sanofi-Synthelabo GmbH (Berlin, Germany). Anti-mouse IgG2a-horseradish peroxidase was from Southern Biotechnology (Birmingham, AL). The goat anti-mouse PE conjugate, mouse IgG2a and mouse-IgM were from Jackson ImmunoResearch (West Grove, PA). The blocking anti-NKG2D monoclonal antibody (mAb) was from R&D Systems (Wiesbaden, Germany) and mouse IgG1 (MOPC 31 c) was from Sigma (Munich, Germany). The mAb AMO1 (anti-MICA), BMO1 (anti-MICB), BAMO1 (anti-MICA/B), AUMO1 (anti-ULBP1), BUMO2 (anti-ULBP2; all of IgG1 isotype), the mAb BMO2 (anti-MICB) and BAMO3 (anti-MICA/B; IgG2a), the mAb CUMO2 (anti-ULBP3; IgM) and mAb W6/32 (anti-MHC class I; IgG2a), and recombinant sMICA*04 and sMICB*02 were produced as previously described (34).

Cells. Hep3B human hepatoma cells were maintained in DMEM supplemented with 10% FCS, HepG2 human hepatoma cells were cultured in MEM and DMEM (3:1) with 10% FCS. The NK cell line NK-92 was purchased from DSMZ (Braunschweig, Germany) and was maintained in alpha MEM with 12.5% horse serum, 12.5% FCS, and interleukin 2 (10 ng/mL). Primary NK cells were isolated from human peripheral blood mononuclear cells (PBMC). Buffy coats from healthy volunteers were separated by Ficoll Hypaque density centrifugation, and PBMC were incubated in RPMI 1640 with 10% FCS supplemented with 200 IU/mL recombinant human interleukin 2 (ImmunoTools GmbH, Friesoythe, Germany) for 48 hours. NK cells were isolated by negative selection using MACS columns (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Subsequent flow cytometric analysis confirmed that >90% of the purified cells expressed CD56 but not CD3. Media and supplements were purchased from Life Technologies (Karlsruhe, Germany). For treatment, cells were plated in 6-well plates at 100,000 cells per well and incubated for the indicated time periods. Primary human hepatocytes were kindly provided by T.S. Weiss (University Clinic Regensburg, Germany) and cultured as described previously (35).

Human tissue samples. A pair of samples from primary hepatocellular carcinoma and from noncancerous liver tissue was obtained from five female and five male patients undergoing surgical resection due to primary hepatocellular carcinoma (mean age, 65.5 years; range, 53-76), among them five with hepatitis C infection, one with alcohol abuse, one with hepatitis B infection, and three patients without obvious risk factors for hepatocellular carcinoma development. Surgical resections were done according to standard procedures at the University Hospital of Berlin, Germany, between 1998 and 2003. Immediately after resection, the tissues were rinsed in sterile PBS solution (137 mmol/L NaCl, 2.68 mmol/L KCl, 8.06 mmol/L Na_2HPO_4, 1.47 mmol/L KH_2PO_4) and stored in liquid nitrogen until RNA isolation. The study was done according to the ethical guidelines of the 1975 Declaration of Helsinki and informed consent was obtained before the resection procedure.

Microarray expression analysis. Gene expression profiles of HepG2 cells cultured with or without 1 mmol/L VPA for 24 hours, and of human tissue samples, were determined using Affymetrix microarray analysis and the Human Genome HU133A oligonucleotide array chip (Affymetrix, Santa Clara, CA). The labeling of RNA and hybridization was done by the Microarray Facility at the Eberhard Karls University Tübingen. HU133A contains 22,000 full-length annotated genes together with additional probe sets designed to represent expressed sequence tag sequences. We standardized for sample loading and variations in staining by scaling the average of the fluorescence intensities of all genes on an array to constant target intensity of 150 for all arrays used. The signal intensity for each gene was calculated as the average intensity difference, represented by Σ [(PM − MM) / (number of probe pairs)], where PM and MM denote perfect match and mismatch probes. In the samples, the mRNA of a gene was considered expressed ("present") and for HepG2 cells changed in expression during incubation with VPA when the detection P and change P were <0.05. To determine the Ps, a signed rank analysis was carried out on the PM and MM differences comparing each probe pair. The resulting Ps were used to make the change calls. Genes with significantly varied expression in VPA-treated versus untreated cells were identified using Data Mining Tool (Affymetrix).

Immunofluorescence staining. Fluorescence microscopy was done as previously described (36). In brief, cells were grown on coverslips until 70% confluency before treatment with VPA. After 48 hours, cells were fixed with 4% paraformaldehyde at room temperature. Coverslips were blocked and subsequently incubated with anti-MICA/B (BAMO1) or isotype control, respectively, followed by incubation with a secondary PE-conjugated antibody (Dianova, Hamburg, Germany). Specimens were examined using an Olympus fluorescence microscope.

Flow cytometry. Hepatoma cells were detached from culture dishes using 0.25% Trypsin/EDTA, incubated with the NKG2DL-specific mAb or the respective isotype control followed by incubation with the goat anti-mouse-PE conjugate and finally analyzed on a FACScan (Becton Dickinson, Mountain View, CA). Specific fluorescence indices (SFI) of NKG2DL staining were calculated by dividing median fluorescences obtained with the specific mAb by median fluorescences of the corresponding isotype control.

ELISA. Detection of sMICA and sMICB was done using a previously described sandwich-ELISA (34). In brief, for detection of sMICA the mAb AMO1 and BAMO3 were used at 5 and 1 μg/mL, respectively, with recombinant sMICA*04 as a standard. For determination of the levels of sMICB mAb BAMO1 and BMO2 were used at 2 and 1 μg/mL, respectively, with recombinant MICB*02 as a standard. Both assays were processed using anti-mouse IgG2a-horseradish peroxidase (1:8,000) and developed using the TMB Peroxidase Substrate System (KPL, Gaithersburg, MD). The absorbance was measured at 450 nm.

Cellular cytotoxicity assay. NK cell cytotoxicity against hepatocellular carcinoma cells was analyzed using a standard lactate dehydrogenase release assay. Target cells were incubated with VPA or control medium for 48 hours, then cells were washed and NK cells (NK-92 or human NK cells isolated from PBMC) were added to the target cells and incubated for 4 hours at 37°C. In blocking experiments, the blocking anti-NKG2D mAb and mouse IgG1 as isotype control were added to the NK cells at 10 μg/mL 30 minutes before the coculture. An aliquot of 100-μL medium was used for detection of lactate dehydrogenase activity using the lactate dehydrogenase Cytotox assay (Sigma). Spontaneous release of target cells alone was <15% of the maximum release as determined with target cells lysed in 1% Triton X-100. The experimental release was corrected by subtraction of the spontaneous release of effector cells at corresponding dilutions. Percentage of lysis was calculated as follows: 100 × (corrected experimental release − spontaneous release) / (maximum release − spontaneous release). Experiments were done in triplicates.

Statistical analysis. Evaluation of ELISA and cellular cytotoxicity results was done with Student's t test using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

Results

Sodium valproate induces NKG2DL mRNA in hepatocellular carcinoma cells. In an attempt to identify genes that are altered by VPA treatment of hepatoma cells, microarray analysis revealed that the mRNA of the NKG2DL MICA, MICB, and ULBP1 were constitutively present at low levels (compared with the transcripts of the housekeeping gene *GAPDH*) in the hepatoma cell line HepG2 (Table 1, control cells). Treatment with VPA (1 mmol/L) for 24 hours increased both MICA and MICB mRNA levels 1.7- and 2-fold, respectively, with a change $P < 0.002$ (Table 1; VPA-treated cells), whereas the signal for *ULBP1* or the housekeeping gene *GAPDH* did not change significantly, according to the algorithm described in Materials and Methods. *ULBP2* and *NKG2D* transcripts were not detected before or after VPA treatment; *ULBP3* probes were not included in the employed microarray setting. These results suggested that the HDAC-I VPA might be able to prime hepatoma cells for NK cell lysis by increasing NKG2DL expression.

Furthermore, microarray analysis of 10 human hepatocellular carcinoma samples revealed expression of *MICA* and *MICB* transcripts in 7 of 10 samples (Table 2). In comparison with the housekeeping gene *GAPDH*, the expression levels of *MICA* and *MICB* (signal mean, right column, Table 2), represented by the calculated mean fluorescence intensity signal, in the human hepatocellular carcinoma samples were found to be relatively low (3,704 versus 76 and 95, respectively; Table 2), which is in agreement with the transcript levels in HepG2 cells without VPA treatment. Taken together, *MICA* and *MICB* transcripts could be detected in the majority of the examined human-derived hepatocellular carcinoma samples with transcription without VPA treatment occurring at a low transcript level.

Sodium valproate increases NKG2DL protein levels in hepatocellular carcinoma cells. To determine whether VPA-induced changes in MIC mRNA were mirrored by altered protein expression, we did an immunofluorescense analysis with the human hepatoma cell lines HepG2 and Hep3B. Using an antibody that recognizes both MICA and MICB (BAMO1), weak binding was detected on naïve HepG2 and Hep3B cells (Fig. 1*A, left*). Treatment with 1 mmol/L VPA for 24 hours markedly increased binding of BAMO1 to HepG2 and Hep3B cells (Fig. 1*A, right*); notably, nuclei were not stained. MIC expression levels detectable by immunofluorescence analysis seemed higher in HepG2 compared with Hep3B both before and after VPA treatment.

To quantify the up-regulation of MICA and MICB proteins following VPA treatment, we used an ELISA for analysis of whole cellular MICA or MICB contents. Equal numbers of HepG2 or Hep3B cells were cultured in the presence or absence of 1 mmol/L VPA. MIC content was monitored during 48 hours. After the indicated time of incubation, lysis of treated and untreated cells was done using a previously described buffer (1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris-HCl [pH 7.5]; ref. 36). In HepG2 cell lysates, 1 ng/mL MICA protein was detected after 48 hours, whereas after VPA treatment, 1.3 ng/mL MICA protein were present in the cell lysates. This increase closely failed to reach statistical significance ($P = 0.07$, Student's t test; Fig. 1*B, left*). Regarding MICB, a higher constitutive amount of protein was observed in untreated HepG2 cells (5.2 ng/mL after 48 hours), and VPA-treated cells contained 12.3 ng/mL MICB protein. This increase of MICB proved to be statistically significant ($P = 0.01$, Student's t test, Fig. 1*C, left*). Using the hepatoma cell line Hep3B, we found a constitutive expression of 1.0 ng/mL MICA and 5.9 ng/mL MICB, respectively, in untreated cells after 48 hours. VPA treatment for 48 hours led to a statistically significant increase of MICA up to 1.4 ng/mL (Fig. 1*B, right*) and of MICB (Fig. 1*C, right*) up to 7.3 ng/mL ($P = 0.01$ for MICA and $P = 0.02$ for MICB, Student's t test). Taken together, these results show that the observed increase of MIC mRNA is mirrored by an increase of cellular protein with different reaction patterns in the investigated hepatoma cell lines.

Sodium valproate treatment increases surface expression of NKG2D ligands on hepatocellular carcinoma cells. To investigate whether changes of NKG2DL expression could be

Table 1. Microarray analysis of NKG2D signaling components in VPA-treated HepG2 cells

ID number*	Gene name	Control cells		VPA-treated cells		Fold-change†
		Signal‡	Detection§	Signal‡	Detection§	
205821_at	*NKG2D*	42 ± 15	A	87 ± 11	A	NC
205904_at	*MICA*	151 ± 4	P	248 ± 23	P	1.7
206247_at	*MICB*	155 ± 17	P	322 ± 6	P	2
221323_at	*ULBP1*	24 ± 6	P	65 ± 9	P	NC
221291_at	*ULBP2*	4 ± 1	A	8 ± 1	A	NC
M33197_3_at	*GAPDH*	3,035 ± 300	P	2,959 ± 194	P	NC

NOTE: Gene expression profile of HepG2 cells (control cells) and HepG2 cells cultured in the presence of 1 mM VPA (VPA-treated cells).
Abbreviations: NC, no change; A, absent; P, present.
*ID number of Human Genome HU133A oligonucleotide array chip.
†Calculated mean fluorescence intensity signal.
‡Classification of gene expression as absent or present, according to the calculation of the scores of 11 perfect match and of 11 mismatch probes for the same hybridized gene using the Microarray Suite software.
§Proportion of the signals from experiments with VPA treated cells to control cells for genes with changed expression (fold change >1 for increased and <1 for decreased gene expression).

observed on the cell surface where NKG2D signaling takes place, the surface expression of various NKG2DL was investigated by flow cytometry. NKG2DL expression was quantified by calculation of SFI. Overall, constitutive expression of NKG2DL was higher on HepG2 compared with Hep3B cells (Fig. 2*A*). Both MICA and MICB were constitutively expressed on HepG2 and Hep3B cells as determined by using mAb BAMO1, an antibody that recognizes both MICA and MICB (Fig. 2*A*; SFI levels 2.6 and 2.2, respectively). Both cell lines revealed only a weak constitutive expression of ULBP1, and low levels of ULBP2 expression were seen on HepG2 cells (Fig. 2*A*). ULBP3 could not be detected on either cell line.

Corresponding to the results of the microarray analysis, VPA treatment did not significantly alter the surface expression of ULBP proteins (Fig. 2*A*). In contrast, expression of MIC molecules was markedly increased after VPA treatment. After 24 hours of incubation with VPA, the expression of both MIC molecules was increased 2.2- and 1.6-fold on HepG2 and Hep3B cells, respectively. More specifically, in HepG2 cells, a 1.7-fold increase of MICA and a 2.2-fold increase of MICB surface expression were observed, whereas Hep3B cells revealed a 1.6-fold increase in MICA with no obvious changes (1.1-fold) in MICB surface expression. Importantly, MHC class I surface expression was not altered by VPA treatment showing that the observed changes were due to alterations in NKG2DL surface expression and not due to an unspecific effect of the compound (Fig. 2*A*). Thus, the increase of total MIC protein in VPA-treated hepatoma cells is paralleled by up-regulation of these proteins at the cell surface.

Sodium valproate treatment alters the release of soluble MIC into culture supernatants. MIC molecules can be shed and released from the cell surface leading to elevated levels of soluble MIC (sMIC) proteins in sera of patients with tumors of epithelial and hematopoietic origin (34, 37). To extend these studies to hepatocellular malignancies, we investigated whether hepatocellular carcinoma cell lines release sMIC in culture supernatants and whether this is altered by VPA. Whereas treatment with VPA for 48 hours did not change the detected levels of sMICA released by Hep3B cells, it significantly ($P = 0.007$, Student's t test) increased (1.6-fold) the detectable levels of sMICA in culture supernatants of HepG2 cells (0.25-0.4 ng/mL; Fig. 2*B*). Regarding sMICB, VPA treatment resulted in a statistically significant ($P = 0.009$ and $P = 0.028$ for HepG2 and Hep3B, respectively) increase in the amount of soluble protein present in supernatants of both Hep3B and HepG2 cells as determined by Student's t test (Fig. 2*C*). After 48 hours, for HepG2 cells, a 2.1-fold increase (0.7-1.5 ng/mL) and for Hep3B cells a 1.9-fold increase (1.8-3.5 ng/mL) of MICB was detected (Fig. 2*C*). These results show that human-derived hepatoma cells are able to release soluble MIC proteins, and this release is altered by VPA treatment.

Up-regulation of NKG2DL expression following sodium valproate treatment enhances natural killer cell lysis of hepatocellular carcinoma cells. NKG2D stimulation by specific ligands such as MICA/B has been shown to enhance innate antitumor immunity including NK cell–mediated lysis of tumor cells (23). Therefore, we addressed the functional relevance of altered NKG2DL expression on hepatoma cells following VPA treatment. To this end, hepatoma cells were subjected to a lactate dehydrogenase release assay using the NK cell line NK-92. NK-92 expresses high levels of NKG2D and thus is well suited to test NKG2D-mediated cytotoxicity (data not shown). NK-92 cells lysed untreated HepG2 cells up to 15% at an E/T ratio of 30:1 (Fig. 3*A*). VPA treatment (1 mmol/L) for 48 hours markedly enhanced the hepatoma cell susceptibility to NK-92 mediated killing, resulting in a statistically significant (Student's t test, $P < 0.01$ between E/T ratio from 7.5:1 to 30:1) increase of HepG2 lysis (Fig. 3*A*; HepG2 + 1 mmol/L VPA). This increase in cytotoxicity was critically dependent on NKG2D/NKG2DL interaction, because it was completely abolished by addition of a blocking NKG2D mAb (Fig. 3*A*; HepG2 + 1 mmol/L VPA + anti-NKG2D), whereas addition of an isotype control mAb did not significantly decrease NK-92-mediated lysis of VPA-treated HepG2 cells (Fig. 3*A*; HepG2 + 1 mmol/L VPA + IgG).

These observations were extended by employing primary NK cells from healthy donors instead of the human-derived cell line NK-92 for hepatoma cell lysis. Priming of HepG2 cells with VPA (1 mmol/L, 48 hours) induced a significant (Student's t test, $P < 0.05$ for E/T ratio 7.5:1 to 15:1) increase in hepatoma cell lysis (Fig. 3*B*; HepG2 + 1 mmol/L VPA). As already observed using NK-92 cells, blocking the NKG2D/NKG2DL interaction markedly reduced the lysis of HepG2 cells by primary NK cells (Fig. 3*B*; HepG2 + 1 mmol/L VPA + anti-NKG2D), whereas no significant decrease of NK mediated lysis of VPA-treated HepG2 cells could be observed using an isotype control mAb (Fig. 3*B*; HepG2 + 1 mmol/L VPA + IgG). Taken together, these results show that the increased NK cell cytotoxicity against VPA-treated hepatoma cells is mediated through up-regulation of NKG2DL on the cell surface rendering hepatocellular carcinoma cells more susceptible for NK cell killing.

Considering this mechanism to be important for the specific elimination of tumor cells *in vivo* during treatment with HDAC-I, it was important to ascertain that VPA treatment does not induce NKG2DL expression in healthy, nonmalignant cells. Therefore, we incubated PHH from two different donors with VPA for 48 hours and determined the presence of MICA and MICB in whole cellular lysates by ELISA. As control, we used HepG2 cells at the same cellular density than PHH cells with or without addition of

Table 2. Microarray analysis of NKG2D-L in human hepatocellular carcinoma samples

ID number*	Gene name	Human hepatocellular carcinoma ($n = 10$)	
		Samples classified as mRNA present (%)†	Signal Mean ± SEM‡
205904_at	*MICA*	70	76 ± 10
206247_at	*MICB*	70	95 ± 11
221323_at	*ULBP1*	0	9 ± 2
221291_at	*ULBP2*	30	48 ± 6
M33197_3_at	*GAPDH*	100.0	3,704 ± 589

NOTE: Gene expression profile of human hepatocellular carcinoma tissue.
*ID number of Human Genome HU133A oligonucleotide array chip.
†% Sample gene expression classified as present, according to the calculation of the scores of 11 perfect match and of 11 mismatch probes for the same hybridized gene using the Microarray Suite software.
‡Calculated mean fluorescence intensity signal.

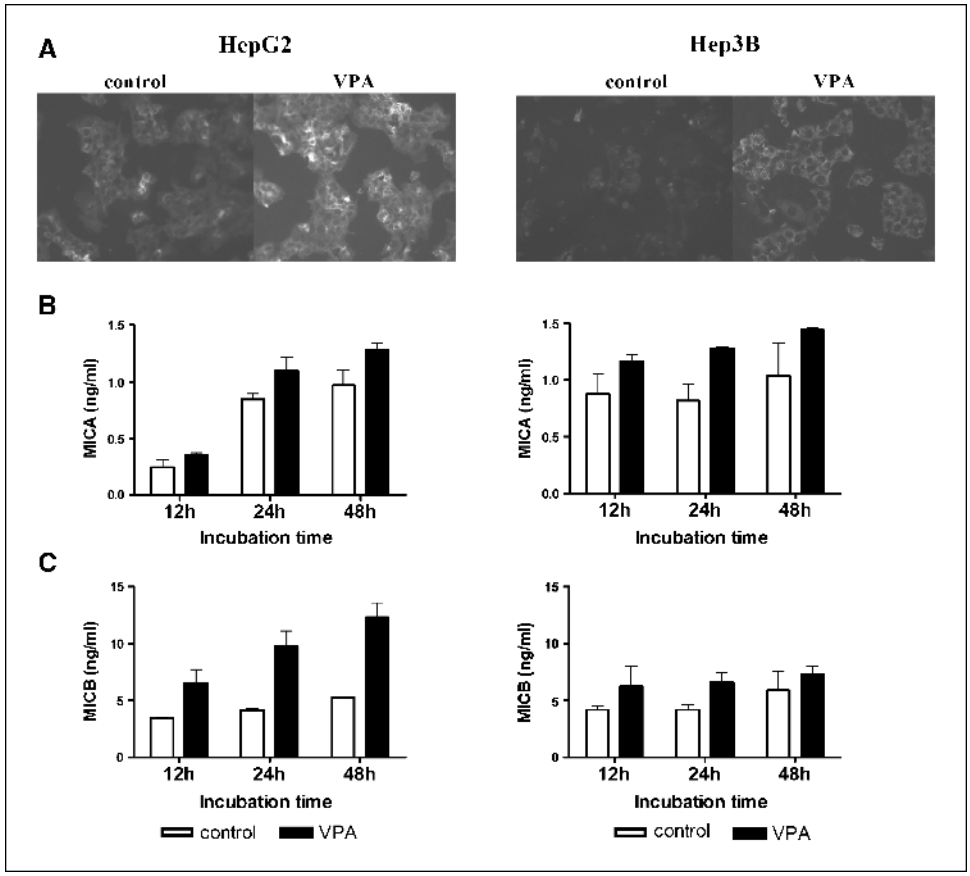

Figure 1. Increase of MICA/B protein in hepatoma cells following VPA treatment. *A,* immunofluorescence analysis using the MICA/B-specific mAb BAMO1 followed by goat anti-mouse-PE in untreated (*control*) and VPA-treated (1 mmol/L for 24 hours; *VPA*) hepatoma cells HepG2 (*left*) and Hep3B (*right*). *B* and *C,* HepG2 (*left*) and Hep3B (*right*) cells were cultured at 10^5 cells/cm^2 in medium containing 1 mmol/L VPA or control medium (*control*) for the indicated time. MICA (*B*) and MICB (*C*) protein levels were detected in whole cellular protein extracts by ELISA as described in Materials and Methods. Data of one representative experiment from a total of three experiments with similar results. *Columns,* means of triplicates; *bars,* SD.

VPA. Strikingly, whereas HepG2 cells displayed a strong up-regulation of both MIC proteins in the course of VPA treatment, no up-regulation of either MICA or MICB could be detected in PHH of both donors (Fig. 4). Moreover, in contrast to untreated HepG2 cells, PHH, as expected, did not reveal an obvious constitutive expression of MIC proteins. These results suggest that VPA induces differential NKG2DL expression in malignant in contrast to nonmalignant liver cells with functional relevance for specific NK cell–mediated lysis of tumor cells.

Discussion

Hepatocellular carcinoma cells display a marked resistance to conventional cytostatic agents resulting in disappointing clinical outcomes when currently available chemotherapeutic treatment strategies are employed. Alternative approaches, including promising new antineoplastic substances (4) or the stimulation of antitumor immunity, are needed to overcome treatment failure due to drug resistance.

In the last few years, HDAC-I have been shown to exhibit profound antitumor effects in a broad range of tumor entities (9, 10), among them hepatocellular carcinoma models (11–13). However, a substance with a favorable pharmacologic and toxicologic profile *in vivo* suitable for treatment of hepatocellular carcinoma patients has not yet been described (8). Recently, we showed that two different substances with HDAC-I activity, including the well-known drug VPA, induce profound antineoplastic effects and apoptosis in hepatoma-derived cell lines without toxicity for primary human hepatocytes (17). The anticancer potential of these and other HDAC-I is thought to result from their ability to affect several cellular processes that are dysregulated in malignant but not in normal cells. Several studies have shown that HDAC-I modulate the expression of 2% to 10% of all cellular genes in transformed cells by increasing the acetylation pattern of histone proteins (38). However, besides described changes in the expression of genes involved in apoptosis signal transduction or cell cycle modulation, the mechanisms that contribute to the cancer-selective toxicity of HDAC-I or their antitumor effects *in vivo* remain unclear.

Because we previously have found a differential response of hepatoma cell lines and PHH to VPA suggesting the potential use of this HDAC-I for treatment of hepatocellular carcinoma, we further investigated the mechanisms responsible for the therapeutic benefit by microarray analysis comparing naive to VPA-treated human hepatoma cell lines.[4] Surprisingly, these investigations revealed a possible link between VPA and the innate immune system as we found alterations in the expression of various genes involved in the interaction with the cellular immune system, among them increased mRNA levels of ligands for the activating immunoreceptor NKG2D. To determine whether expression of NKG2DL in hepatoma cells also occurred *in vivo*, we did a microarray analysis of primary hepatocellular carcinoma samples which revealed that most tumors expressed MIC transcript levels comparable with those observed in untreated hepatoma cell lines

[4] In preparation.

indicating that NKG2DL expression in hepatocellular carcinoma is also of relevance *in vivo*. In humans, NKG2D, which is expressed on cytotoxic lymphocytes, associates with the DAP10 adaptor protein allowing transduction of activating signals (39). Among the NKG2D ligands (NKG2DL) are the stress-inducible surface glycoproteins MICA and MICB, which are expressed on many epithelial tumors and upon infection (24–28), and the UL16 binding proteins (ULBP), a multigene family with at least six functional members (28–30). Expression of NKG2DL on the cell surface renders cells susceptible to NK cell cytotoxicity despite the expression of MHC class I. This has been shown in mice, where a family of proteins structurally related to ULBP, the retinoic acid early inducible (RAE-1) molecules function as ligands for NKG2D (40, 41). RAE-1-transduced cell lines were eliminated *in vivo* due to NK and CD8 T-cell activity and induced tumor immunity against the parental cell line supporting an important role for NKG2D in tumor immune surveillance (31, 32).

The observed induction of MIC mRNA is of great interest, because several reports suggested that VPA might influence components of the immune system. A comparison between PBMC derived from healthy donors and from patients suffering from convulsions undergoing VPA therapy revealed that PBMC from VPA-treated individuals exhibited a higher production of IL-1α, IL-1β, and IL-6 in response to mitogen (18). Further evidence for the involvement of VPA in cellular immune reactions was provided by studies demonstrating increased CD4/CD8 T-cell ratios and elevated IgM levels in VPA-treated patients (19). However, as to now, the consequences of VPA treatment for cellular immunity remain unclear. Regarding other substances with HDAC-I activity, transcriptional activation of costimulatory molecules and type I and II IFNs leading to augmented immune recognition and activation of immune effector cells has been shown (9). Furthermore, addition of HDAC-I-like sodium butyrate to mixed-leukocyte reactions sensitized leukemia cells for cytolysis that has been attributed to significantly increased expression of the costimulatory molecule CD86. For these reasons, HDAC-I are currently being evaluated in clinical studies as a novel treatment of acute myelogenous leukemia (42).

In our study, analysis of NKG2DL mRNA expression in hepatoma cells revealed a marked induction of both MICA and MICB upon VPA treatment, whereas no ULBP2 mRNA and only low constitutive levels of ULBP1, which were not affected by VPA treatment, were detected. Whereas mRNA of NKG2DL has been detected in various normal cells, corresponding NKG2DL cell surface expression has not been detected indicating that NKG2DL expression may be regulated on a post-transcriptional level (23, 29, 30). In our study, the up-regulation of NKG2DL mRNA was paralleled by a marked increase of total, soluble and surface MIC molecules. Flow cytometric analysis of HepG2 and Hep3B cells revealed an up-regulation of MICA/B surface expression following VPA treatment that correlated with the changes in total MIC protein and with the results of the immunofluorescence analysis. Because tumor cells have been shown to shed and release MIC molecules from the cell surface (34, 37, 43), we determined the levels of soluble MIC (sMIC) protein in the culture supernatants of hepatoma cells in the presence or absence of VPA. Both sMICA and sMICB were present at constitutively low levels, which extends the available data regarding tumor entities that release sMIC molecules to hepatocellular carcinoma. Treatment with VPA significantly increased the levels of sMIC.

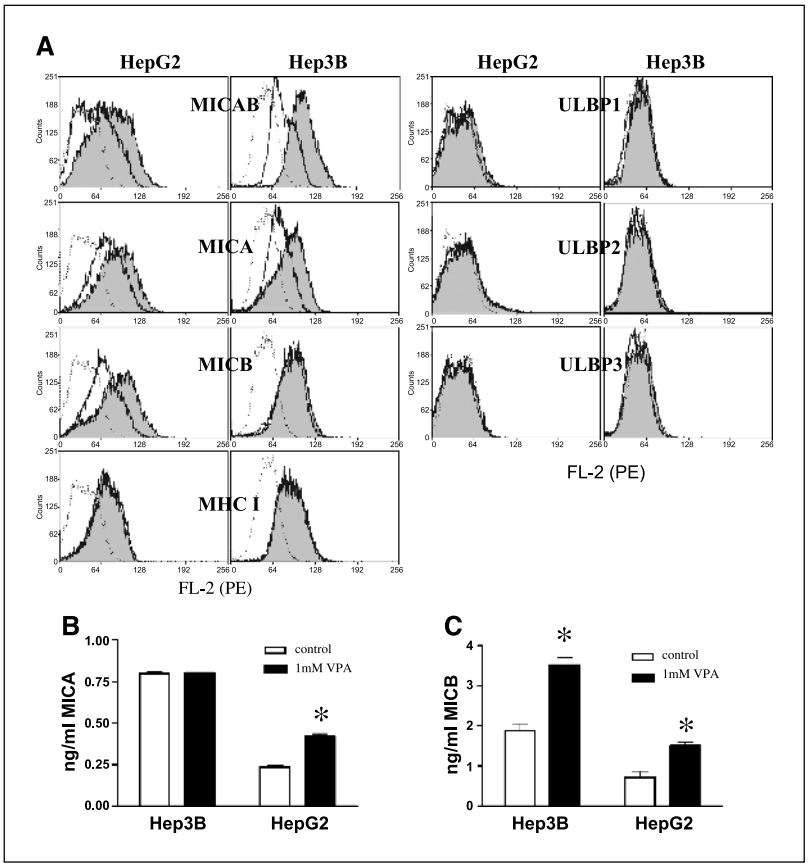

Figure 2. VPA-mediated alterations in NKG2DL surface expression and release. *A,* HepG2 and Hep3B cells were cultured for 48 hours with 1 mmol/L VPA or control medium prior to flow cytometric analysis of NKG2DL and MHC class I surface expression. *Open histograms,* staining of untreated cells; *shaded histograms,* expression of VPA-treated cells and dotted lines are isotype control stainings. *B* and *C,* HepG2 and Hep3B cells were cultured at 10^6 cells/cm^2 in medium with (1 mmol/L VPA) or without (*control*) VPA. After 48 hours, supernatants were analyzed for soluble MICA (*B*) or MICB (*C*) by ELISA as described in Materials and Methods. *Columns,* mean of three independent experiments done in triplicate; *bars,* SD. *, $P < 0.05$, significant changes in the amount of soluble MIC proteins compared with untreated samples.

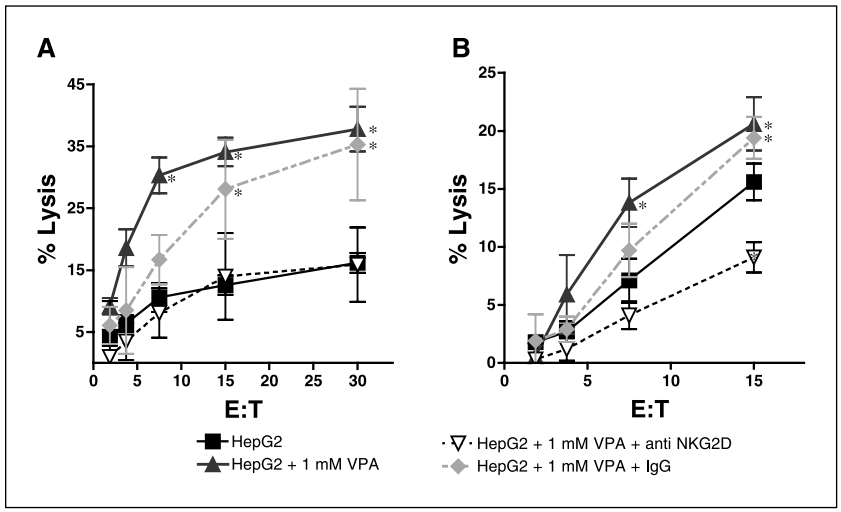

Figure 3. Enhanced NK cell mediated lysis of hepatoma cells following VPA treatment. HepG2 cells were incubated for 48 hours with 1 mmol/L VPA or control medium before the cytotoxicity assay using either (*A*) NK-92 cells or (*B*) primary NK cells from healthy donors. Where indicated, NK cells were preincubated with 10 μg/mL anti-NKG2D or mouse IgG1 as control. Cellular cytotoxicity assays were done three times with NK92 and primary NK cells, respectively. *Points,* means of triplicates of one representative experiment; *bars,* SD. *, $P < 0.05$, significant differences compared with untreated HepG2 cells at each E/T ratio.

Interestingly, in HepG2 cells, VPA increased MICA and MICB expression both in cell-bound and soluble form, whereas in Hep3B cells an increase in cell surface MICA and no changes in sMICA versus unchanged MICB surface expression and increased sMICB could be observed. Whereas the increase in sMIC protein in HepG2 cells parallels the changes in total protein and surface expression, the data obtained with Hep3B indicate that alterations in cell surface and soluble MICA and MICB do not necessarily correlate. MIC molecules are released from the cell surface of tumor cells due to the activity of metalloproteinases, which can be inhibited by addition of a broad-spectrum metalloprotease inhibitor (43). However, the specific protease responsible for shedding of either MIC molecule remains to be identified. Possibly, different sheddases might be responsible for the release of the two MIC molecules, which could explain the results obtained with Hep3B cells.

In previous studies, the observed NKG2D-mediated activation of cytotoxic lymphocytes was critically dependent on NKG2DL expression levels at the cell surface (31, 32). Therefore, release of sMIC is considered as a tumor immune-escape mechanism by diminishing NKG2DL density on the tumor cell surface and as a mode of T-cell silencing leading to endocytosis and degradation of NKG2D (37). Therefore, enhanced release of sMIC could counteract, at least in part, the beneficial effects of enhanced MIC surface expression following treatment with VPA. Investigations of sMIC levels in serum of hepatocellular carcinoma patients and potential NKG2D down-modulation on their cytotoxic lymphocytes should clarify this subject and are currently under study.

We next determined the significance of enhanced NKG2DL surface expression following VPA treatment for priming hepatocellular carcinoma cells for NK cell cytotoxicity using NK-92 cells and primary NK cells. NK-92 is a NK cell line that has already entered clinical trials evaluating adoptive immunotherapy of malignancies, because it was shown to exhibit substantial antitumor activity against leukemia and melanoma *in vitro* as well as in xenografted SCID mice (44). In our study, *NK cells* killed untreated hepatocellular carcinoma cells, and this lysis was significantly enhanced following treatment of cells with VPA. The cytotoxocity was critically dependent on NKG2D/NKG2DL interaction, because it was abolished by addition of anti-NKG2D. This shows that NKG2DL expression renders hepatocellular carcinoma cells susceptible for NK cell–mediated killing that can be markedly enhanced by VPA treatment. Theoretically, an induction of MIC proteins in healthy, nonmalignant cell types could interfere with the NK cell–based antitumor effect of VPA treatment. However, we did not detect a relevant expression or up-regulation of MICA or MICB in PHH suggesting that VPA is able to specifically up-regulate "danger signals" for the innate immune system in malignant cells. To our knowledge, this is the first report of any therapeutic compound that is potentially able to differentially modulate NKG2DL in malignant and nonmalignant cell types.

The capability of VPA to mediate HDAC inhibition and to stimulate innate immunity is of great interest, because VPA is a well-established drug for long-term therapy of patients with convulsions and exhibits great advantages compared with other HDAC-I substances, particularly when side effects that include abdominal pain, cardiac toxicity, somnolence, confusion, vomiting, or peripheral edema are taken into account (9). Considering possible side effects of VPA in patients with hepatocellular

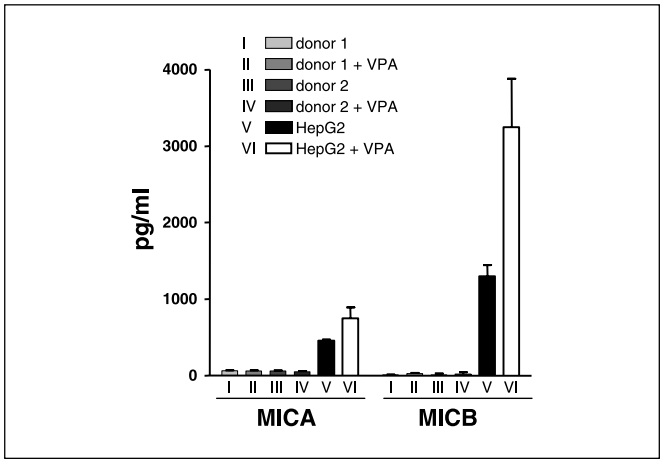

Figure 4. Sodium valproate (*VPA*) does not induce MIC expression in PHH. PHH from two different human donors and HepG2 cells were treated with medium containing 1 mmol/L VPA (donor 1 + VPA, donor 2 + VPA, HepG2 + VPA) or control medium (donor 1, donor 2, HepG2) for 48 hours. MICA and MICB protein levels were detected in whole cellular protein extracts by ELISA as described in Materials and Methods. Columns, means of triplicates of all experiments performed with each donor and HepG2 samples; *bars,* SD.

carcinoma which might have an additional viral infection of the liver such as hepatitis C or which present with significantly reduced liver function, it is noteworthy that the previously described hepatotoxicity of VPA seems to be an idiosyncratic reaction, especially in young patients on comedication. A study thoroughly investigating cases of fatal hepatotoxicity in the United States between 1978 and 1984 showed that the primary risk of fatal hepatic dysfunction in children receiving VPA occurred in the context of a polytherapy; the risk declined with age, was low in patients receiving VPA as monotherapy, and no fatalities occurred in patients above the age of 10 years receiving VPA as monotherapy (45). In addition, it was recently reported that VPA can be used safely in patients suffering from chronic hepatitis C by monitoring alanine aminotransferase levels (46). Another study compared pharmacokinetic differences of VPA in healthy adults, patients with alcoholic cirrhosis, and patients recovering from acute hepatitis. A slight impairment of the elimination of VPA was observed, but the authors assumed that VPA will not accumulate to an extent that toxic plasma levels are reached in patients with liver dysfunction (47). Taken together, these reports suggest that VPA might be well tolerated in patients with hepatocellular carcinoma under close monitoring of VPA and alanine aminotransferase serum levels. Moreover, in case of intolerable side effects of VPA treatment, other HDAC-I might exert similar therapeutic effects on hepatocellular carcinoma while exhibiting even better toxicologic profiles; this issue is currently under investigation.

Taken together, our results indicate that the mechanisms of VPA-mediated antitumor activity, which up to now have focused on the immediate effects of HDAC-I activity on tumor cell cycle, differentiation, and apoptosis have to be extended to VPA-induced recognition of tumor cells by NK cells. Strikingly, we found a differential regulation of MICA and MICB proteins in hepatoma cells and in primary liver cells. These results suggest that VPA might be able to prime tumor cells *in vivo* for NK cell lysis and even open up the possibility of an adoptive immunotherapy of hepatocellular carcinoma after VPA treatment using clonal NK cell lines, such as NK-92 (44, 48), which already has been employed in clinical trials.

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