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CUTTING EDGE

Cutting Edge: Down-Regulation of MICA on Human Tumors by Proteolytic Shedding

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The immunoreceptor NKG2D stimulates tumor immunity through activation of CD8 T cells and NK cells. Its ligand MICA has been shown to be broadly expressed on human tumors of epithelial origin. MICA expression correlates with an enrichment of Vδ1 T cells in tumor tissue. We report that human tumor cells spontaneously release a soluble form of MICA encompassing the three extracellular domains, which is present at high levels in sera of patients with gastrointestinal malignancies, but not in healthy donors. Release of MICA from tumor cells is blocked by inhibition of metalloproteinases, concomitantly causing accumulation of MICA on the cell surface. Shedding of MICA by tumor cells may modulate NKG2D-mediated tumor immune surveillance. In addition, determination of soluble MICA levels may be implemented as an immunological diagnostic marker in patients with epithelial malignancies. *The Journal of Immunology*, 2002, 169: 4098–4102.

While the activity of NK cells is regulated by signals from activating and inhibitory receptors (1, 2), effector function of CTL requires engagement of the clonotype-specific TCR by cognate Ag. Despite this fundamental difference, NK cells and CTL share receptor/ligand systems involved in initiation and regulation of cellular immunity. Among these are members of the Ig superfamily like CD28, the TNF superfamily (e.g., 4-1BB/CD137), and the C-type lectin-like receptors like NKG2A/CD94. Recently, the C-type lectin-like receptor NKG2D has been demonstrated to play an important role in the activation of NK cells and costimulation of CD8 $\alpha\beta$ T cells (3).

Among its ligands is the MHC class I-related, stress-inducible surface glycoprotein MICA[2] that exhibits a highly restricted expression in vivo (4–6). Interestingly, MICA is broadly expressed on epithelial tumors and is associated with increased frequencies of tumor-infiltrating Vδ1 γδ T cells (7). Engagement of NKG2D by MICA expressed on transfectants and tumor cell lines triggers NK cells and Vδ1 γδ T cells and costimulates CD8 $\alpha\beta$ T cells (4, 8, 9). The structure of MICA is similar to the protein fold of MHC class I, with an $\alpha1\alpha2$ platform domain and a membrane-proximal Ig-like $\alpha3$ domain (5). MICA and its close relative MICB, which also serves as a ligand for NKG2D, are both polymorphic (10) and the polymorphism has been shown to affect the affinity for NKG2D (11). More recently, the UL16-binding proteins (ULBP) have been identified as additional ligands of NKG2D (11, 12). Their expression in vivo has yet to be explored.

In the mouse, which lacks MHC class I chain (MIC) genes, a family of proteins structurally related to ULBP, the retinoic acid-early (RAE-1) molecules function as ligands for NKG2D (13, 14). RAE-1 expression has been shown to be induced by carcinogens and to stimulate antitumor activities of γδ T cells (15). Furthermore, in mice 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 a role for NKG2D in the tumor immune surveillance (16, 17).

Several membrane-bound molecules among other protein families such as the Ig-like and the TNF superfamily have been shown to be released as a soluble form. Release of the molecules affects cell-cell interactions by reduction of ligand densities and distally modulates effector cells bearing the respective receptor (18–20). In this study, we describe that MICA is released as a soluble form from the cell surface of tumor cells and can be detected at high levels in sera of patients with gastrointestinal malignancies.

Materials and Methods

Cells

The cell lines HeLa, HCT116, HT29, LX1, PC3, and SKBR3 were cultured in 10% FCS-IMDM. C1R cells were cultured in 10% FCS-RPMI 1640 and C1R-transfectants in 10% FCS-RPMI 1640 with 1.8 mg G418/ml (PAA Laboratories, Linz, Austria). For the production of soluble MICA-containing culture supernatants, cells were grown in IMDM without any additives for 48 h.

Reagents

Anti-mouse IgG2a-HRP was from purchased Southern Biotechnology Associates (Birmingham, AL). The goat anti-mouse FITC conjugate and the goat anti-mouse HRP conjugate were obtained from Jackson ImmunoResearch (West Grove, PA). *N*-glycanase was obtained from New England Biolabs (Beverly, MA). For inhibition of metalloproteinases, (*N*-4-hydroxy-*N*-1-[(1*S*)-2-(methylamino)-2-oxo-1-(phenylmethyl) ethyl]-2-(2-methylpropyl)-(2*R*)-butanediamide) was prepared and used as described previously (21). Pefabloc SC (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride) was purchased from Roche (Mannheim, Germany).

MICA transfectants

The human B cell line C1R and the mouse mastocytoma cell line P815 were stably transfected with full-length cDNA encoding MICA*01, MICA*04, and MICB*01 in RSV.5 neo by electroporation or with Fugene 6 (Roche), respectively, according to standard protocols. The MIC cDNAs

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[2] Abbreviations used in this paper: MICA, MHC class I chain-related gene A; MICB, MIC gene B; ULBP, UL16-binding protein; RAE-1, retinoic acid early inducible gene 1; sMICA, soluble MICA; PNGase F, peptide:*N*-glycanase F; MMPI, matrix metalloproteinase inhibitor; MMP, matrix metalloproteinase.

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have been described previously (8). Transfectants obtained after G418 selection were sorted using a Vantage cytometer (BD Biosciences, Mountain View, CA), if necessary.

*Production of soluble MICA*04 in* Escherichia coli

Soluble MICA was produced and purified as previously described (11). In brief, the cDNA encoding the MICA*04 ectodomain (Glu1 through Lys276) in pET20b (Novagen, Madison, WI) was introduced in *E. coli* and MICA*04 production was induced by addition of isopropyl β-D-thiogalactoside. Inclusion bodies were solubilized and successively dialyzed against decreasing concentrations of urea. Refolded MICA*04 was purified by gel filtration, dialyzed against PNEA (50 mM PIPES, pH 7.0, 0.15 M NaCl, 1 mM EDTA, and 0.02% NaN_3), and eventually examined by SDS-PAGE and immunoblotting.

Monoclonal Abs

MIC-specific mAb were raised by immunizing BALB/c mice repeatedly with a mixture of P815-MICA*01, P815-MICA*04, and P815-MICB*01 transfectants according to standard procedures. Hybridoma supernatants were screened by flow cytometry using MICA-transfected C1R cells and hybridoma producing C1R-MICA-specific Abs were subcloned twice. Abs were purified by affinity chromatography on a protein A-Sepharose column. The two mAb clones AMO-1 and BAMO-1 were of isotype IgG1, mAb BAMO-3 was IgG2a.

Flow cytometry

Cells were incubated with the anti-MICA mAb or mouse IgG1 at 10 μg/ml and then, after washing, with goat anti-mouse FITC conjugate (1:100) as secondary reagent. Cells were counterstained with propidium iodide for dead cell exclusion. Samples were analyzed on a FACScan (BD Biosciences).

ELISA

For the detection of soluble MICA (sMICA), two anti-MICA mAb binding to different MICA domains were implemented. Plates were coated with the capture anti-MICA mAb AMO-1 at 2 μg/ml in PBS, then blocked by addition of 100 μl of 15% BSA for 2 h at 37°C and washed. Afterward the standard (recombinant MICA*04 in 7.5% BSA-PBS) and the samples were added and the plates were incubated for 2 h at 37°C. For analysis of patient samples, sera were diluted 1:10 in 5% BSA prior to addition to the plates. After incubation, plates were washed and the detection mAb BAMO-3 at 5 μg/ml in 7.5% BSA-PBS was added for 2 h at 37°C. Plates were then washed and anti-mouse IgG2a-HRP (1:8000 in 7.5% BSA-PBS) was added for 1 h at 37°C. Plates were then washed and developed using the Tetramethylbenzidine Peroxidase Substrate System (KPL, Gaithersburg, MD). The absorbance was measured at 450 nm. Results are shown as means with SD of triplicates.

Immunoblot analysis

Cell supernatants were concentrated ~10-fold and separated on 12% SDS-PAGE gels. Where indicated, samples had been treated before separation with peptide:*N*-Glycanase F (PNGaseF) for 1 h at 37°C according to the manufacturer's instructions (New England Biolabs). Gels were blotted to Hybond-ECL membranes (Amersham, Little Chalfont, U.K.), blocked with PBS containing 10% nonfat dried milk and 5% BSA, and then analyzed with 1 μg/ml anti-MICA mAb BAMO-1. Binding of BAMO-1 was detected with an HRP-labeled goat anti-mouse HRP conjugate and chemiluminescence reagent (NEN Life Science Products, Boston, MA).

Results and Discussion

To study the expression and release of MICA by human tumor cells, we generated a panel of mAb specific for human MIC molecules. The mAb AMO-1, BAMO-1, and BAMO-3 markedly stained P815-MICA*01 and P815-MICA*04 transfectants as well as COS cells transiently transfected with MICA*01 and MICA*04, but not the respective mock-transfected controls (data not shown). Similarly, all three mAb strongly bound to C1R cells stably transfected with MICA*01 or MICA*04. None of the three mAb revealed any allelic preference in binding to these two fairly divergent MICA allelic variants as determined by FACS analysis (data not shown). Analysis of MICB*02-transfected C1R revealed strong binding of mAb BAMO-3 and BAMO-1, but not of mAb AMO-1. Thus, mAb BAMO-3 and BAMO-1 detect both human MIC species (MICA and MICB), while mAb AMO-1 is exclusively specific for MICA (data not shown). The MICA specificity of the mAb was further validated by ELISA. All three mAb bound to soluble recombinant MICA*04, produced in *E. coli*, in a concentration-dependent manner, but not to BSA as control protein, demonstrating that MICA itself harbors the epitopes of AMO-1, BAMO-1, and BAMO-3 and that these are independent of glycosylation. Furthermore, the epitope of BAMO-3 could be attributed to the MICA α3 domain due to its binding to ULBP2α1α2 MICAα3 hybrid molecules expressed on transfected COS cells. Conversely, no binding was seen for BAMO-1 and AMO-1, and competition with soluble NKG2D for binding to C1R-MICA*01 cells suggested that their epitopes localize to the α1α2 domain (data not shown). The anti-MICA mAb AMO-1 and the anti-MICA/B-specific mAb BAMO-3 were implemented to establish a highly sensitive sandwich ELISA for sMICA. Recombinant sMICA*04 was used as a standard and detected at concentrations below 100 pg/ml (Fig. 1*A*). Only background signals were obtained with control proteins (BSA and HLA-A2 tetramer, respectively) at 0.1 μg/ml.

With this ELISA we analyzed supernatants from C1R transfectants that strongly express MICA at the cell surface. In fact, high levels of sMICA were detected in supernatants of C1R-MICA*01 cells and C1R-MICA*04 cells after 24 h of incubation (Fig. 1*B* and data not shown). In contrast, supernatants of mock-transfected C1R cells that constitutively express MICA at low levels (data not shown) contained much less sMICA (~0.5 ng/ml). Since many epithelial tumor cell lines have been reported to express MICA at various levels on the cell surface (6–8), we analyzed supernatants of the carcinoma cell lines HCT116 (colon), HeLa (cervix), HT29 (colon), SKBR3 (mamma), LX1 (lung), and PC3 (prostate) for sMICA. Except for SKBR3, we detected significant levels of sMICA in the supernatants of all cell lines ranging from 0.4 to 3.5 ng/ml (Fig. 1*B*).

To characterize the molecular nature of sMICA, concentrated supernatants of C1R transfectants and HCT116 were subjected to immunoblot analysis. In C1R-MICA*04 supernatants, a smear in the range between 50 and 60 kDa was detected (Fig. 1*C*). Accordingly, in a previous report, membrane-bound MICA immunoprecipitated from C1R transfectants resulted in diffuse signals that had been attributed to heavy glycosylation of MICA, which has eight potential *N*-linked glycosylation sites in its three extracellular domains (6). After PNGaseF treatment of C1R-MICA*04 supernatants, two distinct sMICA species, centered around the size of recombinant sMICA*04 (~33 kDa), could be detected, suggesting that processing may be complex, involving one or more sheddases. Similar results were obtained with supernatants of C1R-MICA*01 transfectants (data not shown). Similarly, when supernatants of C1R-neo cells were investigated after PNGaseF treatment, we detected a weak signal ~33 kDa (Fig. 1*C*). With HCT116 supernatants, a smear similar to C1R-MICA*04 supernatants was detected, which, after deglycosylation, changed into a distinct band of ~33 kDa (Fig. 1*C*). Taken together, these results demonstrate that MICA is spontaneously released as a soluble, heavy glycosylated form from tumor cell lines expressing endogenous or transfected MICA. Since 1) the size of the released protein corresponds to the size of the three extracellular domains of MICA (i.e., the size of recombinant MICA*04), 2) it is released from MICA cDNA-transfected cells as well as from cells constitutively expressing MICA, and 3) because it is detected by mAb that recognize the α1/α2 and α3 domains, respectively, we conclude that sMICA encompasses the entire MICA ectodomain.

Since it has been shown for various proteins that their membrane-bound form is cleaved by proteases and released as a soluble

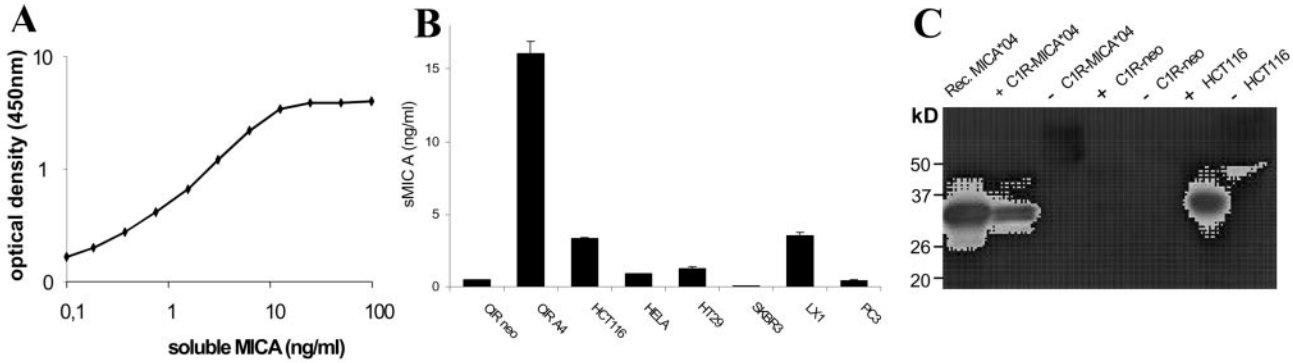

FIGURE 1. sMICA is released by tumor cells. *A*, Sandwich ELISA. Serial dilutions of recombinant MICA*04 were analyzed using mAb AMO-1 and BAMO-3 followed by anti-mouse IgG2a-HRP. The means of four replicates are shown. *B*, Supernatants of tumor cell lines were investigated for levels of sMICA. The data shown are means of triplicates with SD of one representative experiment from a total of four. *C*, Immunoblot analysis of sMICA. PNGaseF-treated (+) or untreated (−) culture supernatants were separated by SDS-PAGE, transferred to nitrocellulose membranes, and then probed with anti-MICA mAb BAMO-1 followed by goat anti-mouse IgG-HRP conjugate.

form, we investigated whether there is a similar mechanism for the release of MICA. C1R-MICA*04, HeLa, and HCT116 were cultured for 24 h in the presence of a broad serine protease inhibitor (Pefabloc) and a metalloproteinase inhibitor (matrix metalloproteinase inhibitor (MMPI)). Subsequently we investigated the levels of sMICA in the culture supernatants detectable by ELISA. The levels of sMICA were compared using Student's *t* test; results with a *p* value below 0.05 were considered to be statistically significant. In the absence of any compound, supernatants of MICA*04-transfected C1R cells contained ~30 ng/ml sMICA. Addition of the Pefabloc serine protease inhibitor did not result in significant changes of detectable levels of sMICA, whereas treatment of cells with MMPI markedly reduced the release of sMICA. A concentration of 0.5 μM MMPI in the culture medium caused a statistically significant reduction of sMICA in the C1R-MICA*04 culture medium (Fig. 2*A*). With HeLa and HCT116 cells, respectively (Fig. 2, *B* and *C*), the levels of spontaneously released sMICA in the culture medium were lower (2.5 and 7.3 ng/ml, respectively) compared with those seen with the C1R-MICA*04 transfectants. Again, the addition of the serine protease inhibitor did not cause relevant changes of sMICA levels, whereas addition of MMPI concentration-dependently reduced the levels of sMICA in the cell supernatants. With both HeLa and HCT116 cells, concentrations of 1 μM MMPI or more caused a statistically significant reduction of the levels of sMICA. Similar effects of the MMPI were observed after 48 h of incubation. Treatment with both protease inhibitors had no effect on tumor cell growth or viability, thus the reduction in the levels of released sMICA observed with the MMPI was due to the inhibition of sheddases and not due to a toxic effect of the compounds (data not shown).

It has been shown that expression of ligands for NKG2D is associated with malignant transformation (15), and MICA expression was described for many epithelial tumors. This has led to the implication that the NKG2D/NKG2DL system may play an important role in immune surveillance of tumors (17). In fact, two recent reports described that tumor cells highly expressing NKG2DL were rejected by NK cells and CD8 $\alpha\beta$ T cells and stimulated tumor immunity against the parental cell lines in a mouse model (16, 22). It was shown that the immune response observed in these studies was critically dependent on the expression levels of NKG2DL on the tumor cells. Therefore, reduction of MICA expression by release from the cell surface would potentially reduce immunogenicity of tumor cells. To investigate whether the effect of sheddases on MICA surface expression mirrored the changes in the levels of sMICA in the culture supernatants, C1R-MICA*04 cells, HCT116 cells, and HeLa cells were cultured in fresh culture medium for 24 h in the presence of protease inhibitors. MICA surface levels of treated and untreated cells were determined by FACS using the MICA-specific mAb AMO-1. While inhibition of serine proteases did not result in significant changes of MICA surface expression on C1R-MICA*04 transfectants, addition of MMPI to the culture medium increased levels of membrane-bound MICA in a concentration-dependent manner (~1.5-fold increase in mean fluorescence; Fig. 2*D*). The cell lines HeLa and HCT116 showed lower levels of constitutive cell surface MICA expression (Fig. 2*D*), which also were increased by addition of MMPI to the culture medium (~2- and 3-fold, respectively), but not by Pefabloc. The MMPI used in these experiments has been reported previously (21) to inhibit a sheddase-like activity. Matrix metalloproteinases (MMP) are structurally related endopeptidases capable of degrading extracellular matrix and are involved in tissue morphogenesis, repair, and angiogenesis, but have also been shown to play an important role in the pathophysiology of tumors. Direct evidence for the involvement of MMP in tumor growth has been provided by several studies and especially in cancers of the gastrointestinal tract high levels of MMP are related to poor survival (for review, see Ref. 23). Degradation of stromal extracellular matrix and basement membranes due to activity of MMP is believed to contribute to tumor angiogenesis and invasion capacity in vivo. (23). There is also accumulating evidence for immunomodulatory functions of MMP. For example, TNF mediates systemic functions following shedding from the cell surface due to the activity of MMP (18), while MMP-mediated conversion of membrane-bound Fas ligand (CD178) to its soluble form has been shown to be associated with down-regulation of its proapoptotic activity (19). In the case of MICA, reduction of surface expression on tumor cells by MMP lowers the levels of NKG2DL capable of inducing a cellular antitumor response by cytotoxic lymphocytes and may provide a mechanism for the cells to escape local immune surveillance by limiting activating signals to the host. In fact, it has been shown that expression levels of NKG2DL on the surface of target cells critically determine the outcome of NKG2D-mediated immune responses. Cell stress-induced increase of MICA expression on tumor cells renders these more susceptible to lysis by Vδ1$\gamma\delta$ T cells (8), and an enhanced NKG2DL expression triggers NK cells overcoming inhibitory signals by MHC class I molecules (4, 12). Furthermore, it has been shown that there is a clear correlation of NKG2DL surface levels on NKG2DL-transduced tumor cell lines with regard to their capacity to stimulate tumor immunity in vivo (16).

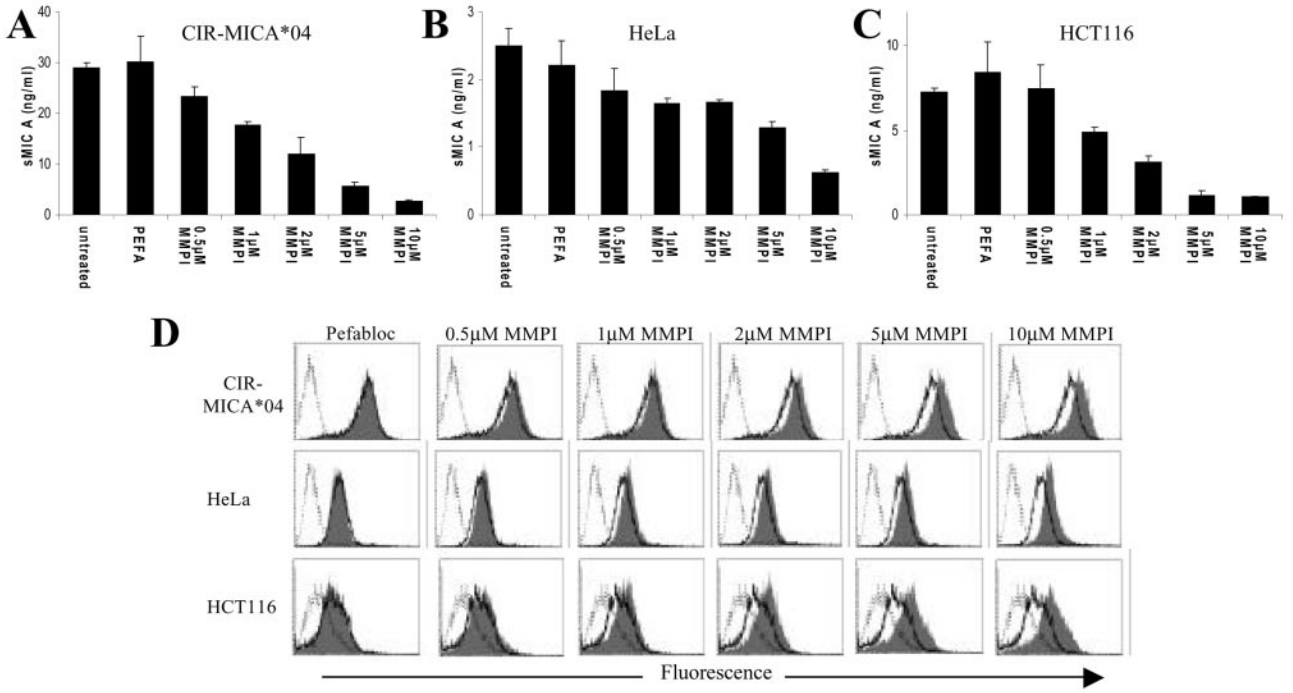

FIGURE 2. Modulation of MICA surface expression and cleavage by MMP. Release of MICA: cells were incubated for 24 h in the presence of the indicated compounds, then the supernatants were harvested and investigated by ELISA. The data shown are means of triplicates with SD of one representative experiment from a total of four. *A,* C1R-MICA*04 cells; *B,* HeLa cells; and *C,* HCT116 cells. Surface expression: cells were cultured in the presence or absence of the indicated compounds. After 24 h, cells were stained for MICA and investigated by FACS. Open peaks show MICA expression on untreated cells, shaded peaks represent MICA expression on cells after treatment, and dotted lines show staining with the isotype control. Cells were treated as indicated in the panels and as described in *Materials and Methods* (*D*). Pefabloc was used at 0.4 mM.

To investigate the role of shedding of MICA as a potential immune escape mechanism of human tumors in vivo, we analyzed sMICA levels in sera of patients with gastrointestinal malignancies. Serum samples of healthy volunteers and patients were collected and assayed by ELISA for sMICA. All investigated sera of healthy volunteers contained low levels of sMICA ranging between 0.6 and 1.0 ng/ml, with mean and median values of 0.8 and 0.8 ng/ml, respectively, which is close to the detection limit of the ELISA (Fig. 3). Sera from patients with stomach carcinoma showed levels of sMICA ranging between 1.9 and 11.3 ng/ml, with a mean of 5.5 ng/ml and a median of 5.1 ng/ml. In patients with colon carcinoma, the range of sMICA was between 2.0 and 7.9 ng/ml, the mean being 5.5 ng/ml and the median 5.9 ng/ml. The highest levels of sMICA were detected in sera from patients with rectum carcinoma; the range was between 1.6 and 17.1 ng/ml, with a mean of 6.5 ng/ml and a median of 5.4 ng/ml. The presence of sMICA in patient sera was validated by immunoblot analysis of sera following PNGaseF treatment, which revealed a band comparable to that of sMICA in culture supernatants (data not shown). The differences of the levels of sMICA in sera of healthy donors and patients with stomach carcinoma, colon carcinoma, and rectal carcinoma were statistically significant ($p < 0.001$) as determined by Student's *t* test. This strong correlation of tumor incidence and elevated sMICA levels clearly suggests that MICA is released at significant amounts from tumor cells in vivo. Elevated levels were detected in all patient sera regardless of presumed allelic MICA differences. The impact of cleavage of membrane-bound MICA to its soluble form on cell-cell interactions and the effect of sMICA at distal sites, which might account for some of the pathophysiology of malignant diseases, require further elucidation.

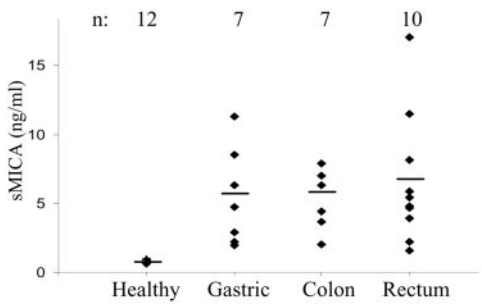

FIGURE 3. Levels of sMICA in sera of patients and healthy donors. Serum samples from patients with different gastrointestinal malignancies and healthy volunteers were investigated by ELISA using sMICA*04 as standard. The data shown are means of triplicates. *n*, number of donors in each group; —, mean of all measurements in the respective group.

In particular, the influence of sMICA on NKG2D-bearing effector cells is currently under study. It will also be important to determine whether the levels of sMICA in patient sera correlate to progress and outcome of malignancies and whether serum levels of MICA can be used as a tumor marker.

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