Altered MicroRNA Expression after Infection with Human Cytomegalovirus Leads to TIMP3 Downregulation and Increased Shedding of Metalloprotease Substrates, Including MICA

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Proteolytic shedding of ligands for the NK group 2D (NKG2D) receptor is a strategy used by tumors to modulate immune recognition by NK cells and cytotoxic T cells. A number of metalloproteases, especially those of the a disintegrin and metalloprotease (ADAM) family, can mediate NKG2D ligand cleavage and this process can be modulated by expression of the thiol isomerase ERp5.

In this article, we describe that an increased shedding of the NKG2D ligand MICA is observed postinfection with several strains of human CMV due to an enhanced activity of ADAM17 (TNF-α converting enzyme) and matrix metalloprotease 14 caused by a reduction in the expression of the endogenous inhibitor of metalloproteases tissue inhibitors of metalloproteinase 3 (TIMP3). This decrease in TIMP3 expression correlates with increased expression of a cellular miRNA known to target TIMP3, and we also identify a human CMV–encoded microRNA able to modulate TIMP3 expression. These observations characterize a novel viral strategy to influence the shedding of cell-surface molecules involved in immune response modulation. They also provide an explanation for previous reports of increased levels of various ADAM17 substrates in the serum from patients with CMV disease. Consistent with this hypothesis, we detected soluble MICA in serum of transplant recipients with CMV disease. Finally, these data suggest that it might be worthwhile to prospectively study ADAM17 activity in a larger group of patients to assay whether this might be a useful biomarker to identify patients at risk for development of CMV disease. The Journal of Immunology, 2014, 193: 1344–1352.
metalloprotease 17 (ADAM17) and matrix metalloprotease 14 (MMP14) in HCMV-infected cells associated with a reduced expression of the endogenous metalloprotease inhibitor tissue inhibitors of metalloproteinase 3 (TIMP3), and we demonstrate that the HCMV-encoded microRNA US25-2-3p is able to modulate TIMP3 expression. These observations may explain previous reports documenting increased levels of a range of ADAM17 substrates, including TNFRs (16, 17), VCAM1, and ICAM-1 (18, 19), in the serum of patients with CMV disease.

**Materials and Methods**

**Abs and inhibitors**

mAb 1H10, MICA-specific (20) (a kind gift of Dr. A. Brooks, University of Melbourne, Melbourne, VIC, Australia), was purified from hybridoma supernatant by chromatography on protein G-Sepharose. mAb specific for MHC (clone 236511) and biotinylated goat anti-MICA and goat anti-MICB polyclonal Abs were purchased from R&D Systems. The ICAM-1-specific mAbs 51-5 (Ab-R6.5) (21) and RR 1/1 (22) were a kind gift of Prof. J.M. Casasnovas (Centro Nacional de Biotecnología/Consejo Superior de Investigaciones Científicas, Madrid, Spain); mAb 15.2 was purchased from Santa Cruz Biotechnology. Abs specific for ADAM17 (cytoplasmic tail), MMP14 (cytoplasmic tail), and β-actin were purchased from Sigma. The anti-HCMV Ab was purchased from Dako (CCH2) and Millipore (IET2). Polyclonal Ab specific for ERp5 was a gift of Prof. Jon Gibbins (University of Reading, Reading, U.K.).

The protease inhibitors leupeptin and pepstatin A were bought from Sigma, whereas BB94 (Batinamast) was purchased from Tocris Biosciences. TIMP-1, -2, and -3 were prepared as described previously (23, 24) and used in cell culture at a final concentration of 500 nM.

**Cells and viruses**

The generation and culture of the U373 cell lines expressing MICA*019 and MICB have been described previously (20, 25). For transfection of U373/MICA*019 cells, a construct expressing the N-terminal domain of MICA (MICA*019 cells were transfected with this mixture using Lipofectamine 2000. Stable transfectants were prepared by culture in selective medium (1 µg/ml puromycin; Calbiochem).

HCMV RK-13 cells (99–9% CD34–CD56+) were isolated from peripheral blood using a negative selection kit (Miltenyi Biotec) and expanded in media (RPMI 1640 medium with 10% human serum) containing rIL-2 and 500 nM anti-TNFα. Cells were washed extensively with PBS and recultured in fresh medium containing 500 nM anti-TNFα and rhIL-2 (26). After 48 h, expanded cells were transfected with the plasmids pCMVR8.91 and media (RPMI 1640 medium with 10% human serum) containing rIL-2 and blood using a negative selection kit (Miltenyi Biotec) and expanded in stocks of these viruses were prepared and titered on immortalized cells and this virus was grown and titered using Vero cells.

The generation and culture of the U373 cell lines expressing MICA*019 and MICB was a gift from Dr. Thomas Mertens, Institute of Virology, Ulm, Germany), and Toledo, Spain. These reactions produced single PCR amplicons of the expected length and were analyzed with SDS2.2 sequence detection systems. These reactions produced single PCR amplicons of the expected length and were analyzed with SDS2.2 sequence detection systems.

**Quantitative PCR**

Total RNA, isolated using TRizol (Invitrogen), was reverse transcribed using random hexamers and 100 U Superscript II RTase (Invitrogen). For quantitative PCR analysis, 2.5 µl cDNA (10-fold dilution series) was mixed with primers and SYBR Green PCR Master Mix (BD Biosciences). Each gene was analyzed in triplicate. Forty cycles of PCR were done with 30 s of denaturing at 95°C, 30 s of annealing at 60°C, and 1 min of PCR at 72°C. Reactions were run on an Applied Biosystems Prism 7900HT. The sequences of the primers used for each target gene are shown in Table I. These reactions produced single PCR amplicons of the expected length and melting temperature, as assessed by dsDNA melting curve analysis. Data were analyzed with SDS2.2 sequence detection systems.

**Lentiviruses**

Lentiviruses were generated by transfection of 293T cells with the vector conferring resistance to puromycin, then U373/MICA*019 MICA*019 cells with a construct expressing the N-terminal domain of MICA were used as a negative control. The luciferase reporter vector containing the full-length 3′-untranslated region (UTR) of TIMP3 (bp 1866–5486) has been described previously (29) and was a gift from Dr. Samson T. Jacob (Ohio State University). The sequence containing the putative binding sites for the peroxidase substrate system (ABTS, Roche). The absorbance was measured at 410 nm with a reference wavelength of 490 nm. Under these conditions, the cutoff for detection of recombinant soluble (s)MICA and sMICB (R&D Biosciences) was around 100 pg/ml, and the ELISA absorbance values were directly proportional to the concentration of sMICB protein over the range of 1 to 100 ng/ml.

**SDS-PAGE and Western blot**

Infected cells were lysed in 0.5 ml lysis buffer (50 mM Tris, 150 mM NaCl, pH 7.5, 1% NP-40 with protease inhibitors [leupeptin and pepstatin A]) and centrifuged to pellet nuclei. Adherent monolayers of mock or infected cells were washed extensively in fresh medium with PBS and recultured in fresh medium (no serum) for 24 h, to recover soluble proteins. Cell culture media were centrifuged twice for 10 min at 300 × g to remove cell debris, and then centrifuged for 30 min at 10,000 × g and 2 h at 100,000 × g sequentially to remove membrane fragments and microvesicles. Soluble proteins were recovered from the 100,000 × g supernatant by TCA precipitation. The pellets were solubilized in reducing SDS-PAGE sample buffer and analyzed by Western blot.

Samples were resolved by SDS-PAGE on 10 or 12% gels and transferred to Immobilon-P (Millipore). The membrane was blocked using 5% nonfat dry milk in PBS-0.1% Tween 20. MICA proteins were detected by incubating the membrane with biotinylated goat polyclonal anti-MICA Ab, whereas ICAM-1 was detected using the mAb 15.2. Bound Abs were visualized using HRP-conjugated Ab (HRP) and the ECL Plus system (GE Pharmaceuticals). Quantitative analysis of the Western blot data was done using ImageJ software (W.S. Rasband; ImageJ, 1997–2008; National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/).

**Flow cytometry**

The effect of HCMV infection on the expression of cell-surface molecules was evaluated by flow cytometry. U373 cells were infected with AD169 using a low multiplicity of infection (MOI). Three days postinfection, either BB94 or vehicle control was added to the cells, and the incubation continued for another 2 d. The cells were then detached by incubation in PBS 1% BSA, 5 mM EDTA, 0.05% sodium azide (PBA), spun down, washed once with PBS containing 1% BSA, and stained with PE-conjugated mAb to MICA, ICAM-1, MHC class I, or LFA-3. Cells were then washed, fixed with 2% paraformaldehyde in PBS, permeabilized with 0.5% saponin, and stained intracellularly with Alexa 488–conjugated mAb specific for HCMV (clone 5B1; Millipore). Finally, cells were washed and the samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson), comparing the levels of expression on the infected (CMV Ag–positive) and noninfected (CMV Ag–negative) U373 cells.

For staining NKGD2 and CD56 on NK cells exposed to supernatants from fibroblasts or HCMV-infected cells, 105 NK cells were preincubated in PBA, and then NKGD2 and CD56 expression were visualized using PE-labeled NKGD2 or CD56-specific mAbs (Biologend).

**ELISA**

At the indicated time points, mock-infected or HCMV-infected cells were washed and then incubated in complete medium for 2–5 h at stated in the figure legends. Supernatants were collected, centrifuged, to remove cellular debris, and the supernatant proteins detected by ELISA. In experiments using chemical or protein inhibitors, the cells were pretreated with the different compounds for 30 min, washed, and then incubated in medium, again supplemented with the inhibitor, for periods between 2 and 5 h. Shed MIC and ICAM-1 proteins were detected using sandwich ELISAs. Plates were coated with purified mAb: 1H10 for MICA, clone 236511 for MICB, and R6.5 for ICAM-1. After incubation overnight at 4°C, the plates were washed with PBS-PBS for 2 h at 37°C; then the samples were added and incubated overnight at 4°C. Bound proteins were detected using biotinylated goat anti-MICA, biotinylated goat anti-MICB, or biotinylated RR1/1 mAb followed by streptavidin-HRP (Amersham) and developed using the peroxidase substrate system (ABTS, Roche). The absorbance was measured at 410 nm with a reference wavelength of 490 nm. Under these conditions, the cutoff for detection of recombinant soluble (s)MICA and sMICB (R&D Biosciences) was around 100 pg/ml, and the ELISA absorbance values were directly proportional to the concentration of sMICB protein over the range of 1 to 100 ng/ml.
the HCMVUS25-2-3p miRNA was deleted from this construct by digestion with EcoRI and NdeI, treatment with Klenow, and religation. The short TIMP3 3′-UTR segment (bp 1822–2220) was generated by PCR using the oligos

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Oligo Sequence</th>
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<tr>
<td>ADAM9</td>
<td>5′-GTCTCCTTGAGGCAAAAGGAC-3′</td>
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<tr>
<td>ADAM10</td>
<td>5′-AGGACACCTTGGGCCAAAC-3′</td>
</tr>
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<td>ADAM12</td>
<td>5′-CTCTTGCTTGAGTGGTGTGC-3′</td>
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<td>5′-AGTTAGAGCCGATTTGCCTG-3′</td>
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<td>MMP14</td>
<td>5′-CCGTCCACAAGCAATGAGT-3′</td>
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<tr>
<td>TIMP1</td>
<td>5′-TCATCATCCACCCCTTACCTT-3′</td>
</tr>
<tr>
<td>TIMP2</td>
<td>5′-TCTCAGCTGTCCCGGTCGCCCG-3′</td>
</tr>
<tr>
<td>TIMP3</td>
<td>5′-TCGCAATCCTCCAGGCTATC-3′</td>
</tr>
</tbody>
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Degranulation assay

For degranulation assays quantifying cell-surface CD107a expression, 1 × 10^5 resting NK cells were washed twice in PBS and added to 2 × 10^5 target cells in 500 μl complete medium. Cells were spun down for 3 min at 300 rpm and incubated for 2 h at 37°C in 5% CO2. Thereafter, the non-adherent cells were recovered, and the adherent monolayers were washed once in ice-cold PBA. The recovered cells were mixed, spun down, and stained with FITC-conjugated anti-CD56 and allophycocyanin-conjugated anti-CD107a mAbs for 45 min on ice. The recovered cells were mixed, spun down, and adherent cells were recovered, and the adherent monolayers were washed once in ice-cold PBA. The recovered cells were mixed, spun down, and stained with FITC-conjugated anti-CD56 and allophycocyanin-conjugated anti-CD107a mAbs for 45 min on ice. The cells were washed, resuspended in PBA, and analyzed by flow cytometry.

Patient samples

The serum samples used in this study were obtained from transplant recipients attending the Hospital Universitario Gregorio Marañon who experienced episodes of active CMV infection. The diagnosis of CMV disease was based on observation of clinical signs or symptoms attributable to this microorganism, and CMV infection was confirmed by detection of virus either by antigenemia or using a plasma real-time PCR assay. Control samples were obtained from transplant recipients who were free of CMV disease and attending the hospital for routine follow-ups after transplantation. Informed consent was obtained from all subjects. The study protocol was conducted according to the ethical guidelines of the 1975 Declaration of Helsinki and approved by the research ethics committee of the Gregorio Marañon Hospital.

Results

HCMV infection triggers shedding of MICA and MICB proteins

It has been reported that MICA expression on U373 cells was reduced, by an unknown mechanism, postinfection with the HCMV strain AD169 that does not express UL142 (30). We hypothesized that increased shedding of MICA could underlie this phenomenon, and therefore compared the amount of soluble MICA released to the supernatant by mock-infected U373 cells expressing the MICA*019 allele, or cells infected with either HSV-1 or HCMV AD169. Increased amounts of MICA were found in the supernatants of HCMV-infected cells compared with either mock-infected or HSV-1–infected U373 cells (Fig. 1A). This enhanced release of MICA from infected cells was evident early and increased as infection progressed (Fig. 1B). MICA released from the infected cells was soluble and comprised multiple species of truncated protein (Supplemental Fig. 1). Enhanced MICA shedding was observed postinfection with several strains of HCMV (Fig. 1C), implying that this phenomenon is conserved. Finally, HCMV infection was also associated with augmented release of another NKG2D ligand, MICB (Fig. 1D). The shedding of ULBP2 from cells infected with AD169 was also increased, but this did not reach statistical significance (data not shown). However, interpretation of these experiments is complicated because the UL16 glycoprotein of HCMV will modulate the expression of ULBP1 and ULBP2, but not MICA, on the infected cell and could conceivably modulate the release of these molecules (31).

Shedding of MICA after HCMV infection is mediated by metalloproteases

Prior studies have shown that proteolytic release of MICA/B molecules from tumor cells depends on the action of metalloproteases including ADAM9, ADAM10, and ADAM17 (32–34), as well as MMP14 (35). Thus, HCMV-infected U373 cells were incubated with inhibitors of serine and cysteine proteinases (leupeptin), aspartate proteinases (pepstatin) and BB94 that inhibit matrix, and some ADAM family metalloproteases. BB94, but not other inhibitors, dramatically decreased the shedding of MICA from AD169-infected U373/MICA cells (Fig. 2A). Treatment with this inhibitor had no effect on cell viability, suggesting that the reduction in the levels of released sMICA was specifically due to the inhibition of sheddases. No HCMV metalloprotease has been identified, so these data suggested that the increased shedding of MICA after HCMV infection was due to viral modulation of host metalloprotease expression or activity. The ability of TIMPs to inhibit this phenomenon was assayed to address the question of which metalloprotease(s) mediated shedding of MIC proteins from HCMV-infected cells. TIMPs are the major cellular inhibitors of metalloproteases and inhibit, with varying efficiencies, active forms of all the MMPs studied and some of the ADAMs (36). Of the various metalloproteases reported to cleave MICA/B from tumor cells, the enzymatic activity of ADAM9 is not regulated by TIMPs (37), ADAM 10 is inhibited by both TIMP1 and TIMP3, whereas ADAM17 is inhibited by TIMP3 only and MMP14 is inhibited by both TIMP2 and TIMP3 (36). Addition of TIMP-3, but not TIMP-1, to AD169-infected U373/MICA cells reduced the shedding of soluble MICA markedly (Fig. 2B). The inhibition of MICA release by TIMP2, although weak, was reproducible. These data strongly suggest an important role for ADAM17 in mediating MIC shedding from HCMV-infected cells and that MMP14 could also play some role in this process. Consistent with these observations, experiments using U373 cells expressing MICA mutants, previously shown to be fully, or partially, resistant to cleavage by metalloproteases (32), showed markedly reduced shedding of these molecules during CMV infection (Fig. 2C).

HCMV infection is not associated with changes in processing of ADAM17 or expression of ERp5

The identification of ADAM17 and MMP14 as sheddases for MICA in HCMV-infected cells posed the question of how viral infection could lead to an increase in the activity of these enzymes in infected cells. ADAM17, like most metalloproteases, is synthesized as a proprotein that is activated by proteolytic removal of the prodomain (38). However, no differences between mock- and HCMV-infected cells, in either level of expression or the ratio of proprotein to mature ADAM17 protein, was noted in experiments.
of Western blot analysis, where two bands corresponding to the precursor and mature (active) forms of ADAM17 could be detected (Fig. 3A). These data suggest that HCMV infection affects neither the expression nor the processing of ADAM17. Similarly, no differences in the processing or expression of MMP14 were noted when mock- and HCMV-infected cells were compared (Fig. 3B). Shedding of MICA has also been shown to depend on the thiol isomerase ERp5 (39); however, again there were no obvious differences in the processing or expression of MMP14 when mock- and HCMV-infected cells were compared (Fig. 3B).

**FIGURE 1.** HCMV infection is associated with increased shedding of MICA proteins. (A) Infection with HCMV, but not HSV-1, leads to an increase in the release of soluble MICA. U373MICA*019 cells were either left uninfected or infected with HSV-1 or AD169 (MOI 10). One day, for mock- and HSV-1–infected cells, and 4 d, for mock- and AD169-infected cells, postinfection the cells were washed and incubated in fresh medium for 5 h. Cell-free supernatants were prepared and shed MICA measured by ELISA. The data have been normalized to the amount of MICA shed by the mock-infected cells in each incubation period. (B) MICA shedding increases through infection. Cells were infected with AD169 at MOIs of either 5 (+++) or 2 (++), or left uninfected. At the indicated times the cells were washed and cultured in fresh medium for 5 h before ELISA analysis. MICA shedding was significantly increased at every time point (p < 0.05). (C) Infection with multiple isolates of HCMV leads to increased MICA shedding. Four days postinfection, cells, uninfected or infected with the indicated virus (MOI 5), were washed, then cultured in fresh medium for 5 h. (D) HCMV infection also leads to increased MICB shedding. U373MICA*019 cells and U373MICB cells were either infected with AD169 or left uninfected. Four days postinfection, the cells were washed, cultured in fresh medium for 5 h, and shed MICA, MICB, and ULBP2 analyzed by ELISA. The increased shedding of MICA and MICB induced by infection with AD169 was statistically significant. Data shown represent the mean ± SD of at least three experiments. *p < 0.05, ***p < 0.01.

HCMV infection leads to a reduced expression of the endogenous inhibitor of metalloproteases TIMP3

Quantitative RT-PCR experiments confirmed that HCMV infection had no significant effect on the levels of expression of a number of metalloproteinases implicated in shedding of MICA molecules. These data did, however, reveal that HCMV infection triggered a clear downregulation of TIMP3, an endogenous regulator of metalloprotease activity (Fig. 4A). Consistent with previous microarray experiments (40), infection with HSV was not associated with significant changes in TIMP3 expression (data not shown). These data suggest that the enhanced shedding of MICA by HCMV-infected cells could be because of a reduction in expression of endogenous inhibitors of metalloproteases. To test this hypothesis, U373 cells expressing MICA*019 were further transfected with a construct expressing the N-terminal domain of TIMP-3 (nTIMP3), where the inhibitory activity of this molecule for metalloproteases resides (24). Overexpression of nTIMP3 markedly reduced the enhanced release of MICA seen after HCMV infection (Fig. 4B). These data support the idea that the increase in enzyme activity is due to the reduced expression of the TIMP3 inhibitor.

The hypothesis that HCMV infection is associated with a general increase in ADAM17 activity was further tested by examining the effect of infection on the shedding of other ADAM17 substrates. U373 cells express high levels of cell-surface ICAM-1, whose

**FIGURE 2.** The increased shedding of MICA proteins on HCMV infection depends principally on ADAM-family metalloproteases. (A) MICA shedding after HCMV infection is blocked by BB94 (p < 0.01), but not leupeptin or pepstatin A. U373 MICA*019 cells were infected with AD169 at MOIs of either 5 (+++) or 2 (++), or left uninfected. On day 4 postinfection, the cells were washed and cultured in fresh medium supplemented with the indicated inhibitor. (B) Incubation with TIMP2 or TIMP3 significantly reduces MICA shedding from HCMV-infected compared with uninfected cells. U373 MICA*019 cells were infected with AD169 or left uninfected. Four days postinfection, the cells were washed and cultured in fresh medium supplemented with the indicated inhibitor. Data shown represent the mean ± SD of at least three experiments. *p < 0.05, ***p < 0.01.

Mutations that render MICA molecules resistant to shedding by metalloproteases also block the increased shedding triggered by HCMV infection. U373 cells transfected with MICA*019, MICA*001, and two previously described mutants of the MICA*001 allele, 1D and 2D, were either left uninfected or infected with HCV AD169. On day 4 postinfection, the cells were washed and cultured in fresh medium for 5 h. At the end of this incubation, cell-free supernatants were prepared and the levels of soluble MICA molecules were measured using a capture ELISA. Given the wide variation in the amounts of basal shedding of MICA molecules by the different transfectants, the data are presented as the fold increase in shedding induced by HCMV infection. These data are the mean ± SD of three experiments. *p < 0.05, ***p < 0.01.
**FIGURE 3.** HCMV infection does not cause changes in the processing or expression of ADAM17, MMP14, or ERp5. U373 MICA*019 cells were infected with AD169 (MOI 5) or left uninfected. Day 4 postinfection, cell lysates were prepared. (A) Western blot analysis to assay the levels of expression and processing of ADAM17 in mock- and HCMV-infected cells, and β-actin was used as a loading control. In four experiments, the ratio of pro to mature ADAM17 protein was 0.64 for the mock-infected cells and 0.68 for the AD169-infected cells; this difference was not statistically significant. (B) Western blot analysis of MMP14 processing and expression in mock- and HCMV-infected cells. The ratio of pro to mature MMP14 protein was 2.8 for the mock-infected cells and 3.0 for the AD169-infected cells (four experiments); this difference was not significant. (C) Western blot analysis of the effect of HCMV infection on expression of the ERp5 molecule (three experiments). No significant difference was noted between infected and control cells.

**FIGURE 4.** A marked reduction in the expression of TIMP3 underlies the increased activity of metalloproteases in HCMV-infected cells. (A) U373 MICA*019 cells were infected with AD169 (MOI 5) or left uninfected. Day 4 postinfection, total RNA was extracted from the cells, and quantitative RT-PCR was used to assay the levels of expression of the indicated metalloproteases and TIMPs. The results are expressed as Log10RQ normalized to the levels of expression of 18S mRNA. Data presented are the mean ± SD of three experiments. Inset shows Western blot analysis of 100,000 g supernatants of control and HCMV-infected cells also showed the marked increase in ICAM-1 shed from virus-infected cells and confirmed that this material was of a lower m.w. than the full-length ICAM-1 protein present in cell lysates (Fig. 4C, inset), consistent with the soluble species of ICAM-1 being produced by proteolytic cleavage.

**TIMP3 downregulation is mediated by cellular and HCMV-encoded microRNAs**

The real-time quantitative PCR data on the marked downregulation of TIMP3 after HCMV infection confirmed earlier observations of TIMP3 downregulation after HCMV infection of fibroblasts and macrophages (42, 43). Reduced expression of TIMP3 is found in multiple forms of human cancer and can be mediated by increased promoter methylation (44); however, no significant changes in methylation of the TIMP3 promoter were reproducibly detected after HCMV infection (Supplemental Fig. 2). Multiple cellular microRNAs have also been shown to modulate TIMP3 expression in cancer (29, 45–47), and the expression of one of these miRNAs, miR-17p, is significantly increased 72 h after HCMV infection (48, 49) and could contribute to TIMP3 downregulation. However, bioinformatics analysis suggested that TIMP3 could also be a target for the HCMV-encoded miRUS25-2-3p, and cotransfection of a plasmid expressing this miRNA with a reporter vector expressing the luciferase gene coupled to the full-length TIMP3 3′-UTR confirmed that TIMP3 expression was significantly downregulated in the presence of this miRNA (Fig. 5A). Coexpression of miRUS25-2-3p with a shortened version of the TIMP3 3′-UTR containing two putative binding sites for miRUS25-2-3p also resulted in a significant reduction in luciferase activity, but this reduction was less marked than for the full-length UTR (Fig. 5A), suggesting that there may be other, as yet unidentified sites for binding of this miRNA in the TIMP3 mRNA. This idea is consistent with the observation that coexpression of miRUS25-2-3p with a mutated version of the TIMP3 3′-UTR from which two putative miRNA binding sites had been deleted could still induce a mild reduction in luciferase activity. These data confirmed that expression of HCMV miRUS25-2-3p destabilized the TIMP3 transcript leading to a reduction in expression. Strikingly, the amount of
soluble MICA shed from U373 cells overexpressing this miRNA was increased 2- to 3-fold (Fig. 5B), whereas transfection with a vector expressing a control shRNA had no effect on MICA shedding. This observation supports the hypothesis that the reduced expression of TIMP3 contributes to the increase in metalloprotease activity associated with HCMV infection.

Inhibition of metalloproteases augments NK recognition of HCMV-infected target cells

One obvious question arising from these data is whether the enhanced shedding of metalloprotease substrates such as MICA and ICAM-1 contributes to protect infected cells from NK cell killing. Mock and 3-d HCMV-infected U373 cells were either left untreated or were treated overnight with an inhibitor of metalloproteases, BB94, to test this idea experimentally. These cells were then cultured with NK cells for 2 h, and degranulation of these NK cells was measured by flow cytometry for CD107a expression. Incubation of HCMV-infected cells with the metalloprotease inhibitor was associated with a clear increase in CD107a mobilization on NK cells exposed to these targets (Fig. 6A). This increased degranulation correlated with a significantly increased expression of MICA on HCMV AD169-infected U373 cells cultured with BB94 (Fig. 6B).

Treatment with the metalloprotease inhibitor had no significant effect on the number of U373 cells expressing HCMV Ags (data not shown). Medium was recovered from control or infected U373 cells and centrifuged to remove cellular debris such as membrane fragments from dead cells, to assess directly whether the increased shedding of MICA postinfection with AD169 could affect NKG2D expression (33). NK cells were then cultured overnight in different dilutions of this medium, and the level of expression of the NKG2D receptor was assayed by flow cytometry. To assess the role of soluble MICA in this process, we added an mAb specific for MICA (25) to some aliquots of the supernatants taken from mock and infected cells to neutralize MICA in these preparations. The results from these experiments clearly show that culture in medium from AD169-infected U373 cells led to a specific decrease in NKG2D receptor expression that was blocked by the inclusion of an MICA-specific mAb in the culture (Fig. 6C).

Increased levels of soluble MICA molecules are present in the serum of transplant recipients diagnosed with CMV disease

Overall, the previous data demonstrate that HCMV infection modulates the expression of the metalloprotease regulator TIMP3, leading to an increase in the shedding of various metalloprotease substrates including MICA/B and ICAM-1. Previous studies have documented that samples of serum from patients with CMV disease contain increased levels of various ADAM17 substrates including TNFRs (16), VCAM1, and ICAM-1 (18, 19); thus, we examined whether there was any relationship between CMV infection and MICA/B shedding in vivo. For these experiments, serum samples from a cohort of heart transplant recipients who experienced clinical signs and disease consequent to active CMV infection were analyzed. The samples of serum were collected on or within 2 d of diagnosis. Control serum samples were from heart transplant recipients with no evidence of CMV-related disease. Soluble MICA molecules could be detected in the serum of 7 of 12 patients with acute CMV disease (average, 152 ng/ml; Fig. 7). In contrast, soluble MICA was detected in only one of the CMV transplant recipients. For a few patients it was possible to analyze samples of serum collected after antiviral therapy had resolved the episode of CMV-related disease. The amount of soluble MICA in these serum samples was reduced to undetectable levels (Supplemental Fig. 3), suggesting that the presence of soluble MICA in patient serum correlates with episodes of CMV infection and disease, and support the idea that the association between active HCMV infection and MICA/B shedding characterized in vitro occurs in vivo.

Discussion

These experiments demonstrate that infection in vitro with HCMV leads to enhanced shedding of MICA and MIB. The observation that increased levels of soluble MICA can also be found in the serum of recipients of heart transplants who have experienced development of CMV-related disease suggests that this phenomenon can also occur in vivo. Indeed, it is at least possible that the increased levels of soluble MICA detected in the serum of some HIV-infected individuals (14, 15) might be related to concurrent CMV infection. The shed MICA can modulate the expression of NKG2D on NK cells, and treatment of infected cells with metalloprotease inhibitors is associated with significant increases in cell-surface MICA expression and NK cell recognition. The enhanced release of MICA molecules by metalloproteases seen after HCMV infection activity does not reflect alterations in the expression or processing of sheddases. Instead, HCMV infection triggers a marked decrease in the expression of the endogenous metalloprotease inhibitor TIMP3, thus indirectly increasing sheddase activity toward MICA and other substrates. Silencing of TIMP3 expression occurs in multiple types of human cancer (44); thus, these data suggest that the expression of TIMP3, as well as ERp5 and sheddases, by any given tumor will be an important determinant of whether proteolytic release of soluble NKG2D ligands will be a feature of the pathogenesis of that tumor. The observation of TIMP3 downmodulation also provides an expla-
nation for previous reports documenting that samples of serum from patients with CMV disease contain increased levels of various ADAM17 substrates including TNFRs (16, 17), VCAM1, and ICAM-1 (18, 19).

Increased methylation of the Timp3 promoter is known to mediate downmodulation of TIMP3 expression in cancer, but in our experiments, HCMV infection was not associated with increased methylation. TIMP3 expression is also known to be modulated by a number of cellular microRNAs, and expression of one cellular miRNA, mIR-17p, known to regulate TIMP3 expression (47) is increased after HCMV infection (48, 49). In addition, bioinformatics analysis identified potential binding sites for the HCMV-encoded mIR-US25-2-3p in the 3’-UTR of TIMP3, and reporter gene assays clearly established that this viral miRNA can also downregulate TIMP3 expression. These observations therefore suggest that the TIMP3 mRNA could be attacked by two microRNAs, one cellular and one viral, because the putative binding sites for these miRNAs do not overlap.

HCMV mIR-US25-2-3p is encoded between the US24 and US26 genes and forms the 3’ arm of the premiR-US25-2 stem-loop structure, and is expressed both early and late in infection (49). Overexpression of mIR-US25-2-3p has been shown to reduce the replication of a number of DNA viruses, including HCMV (50), an effect that depends on knockdown of eukaryotic translation initiation factor 4A1 (eIF4A1), a direct target of mIR-US25-2-3p (51).

Our experiments now show that mIR-US25-2-3p can directly regulate TIMP3 expression, and a key result is that expression of mIR-US25-2-3p in U373/MICA*019 cells leads to an increase in the release of soluble MICA molecules. The most straightforward explanation of this increased shedding is that expression of the microRNA downregulates TIMP3 favoring metalloprotease activity; however, it cannot be ruled out that expression of mIR-US25-2-3p might also modulate other factors to indirectly increase the release of MICA. Indeed, Qi et al. recently identified a number of other candidate mIR-US25-2-3p targets (51). Thus, the role of mIR-US25-2-3p in HCMV infection is likely to be complex, and expression of this viral miRNA could potentially modify multiple aspects of the interaction between the virus and host.

In this context, it is also worth mentioning that TIMP3, as well as inhibiting metalloproteases, can also mediate antiangiogenic and proapoptotic activities (36, 52); thus, the marked reduction in expression of this gene after HCMV infection may affect multiple aspects of the interaction between virus and host.
It is interesting that CMV infection actually induces expression of many molecules found, as soluble proteins, in the serum of CMV patients. HCMV infection has been associated with the induction of expression of ligands for the NKGD2 receptor (53), TNF-α receptors (54, 55), and markedly upregulated expression of a range of adhesion molecules, including ICAM-1 and VCAM-1 (56). It is tempting to speculate that the simultaneous induction, by HCMV infection, of the expression and shedding of multiple molecules able to modulate immune responses contributes to the secretion of host and virally encoded suppressive factors from CMV-infected cells that interfere with the development of immunity to the virus. This phenomenon may also partly underlie the reduced capacity for response of lymphocytes in patients with acute CMV infection (57, 58) that can lead to a transient immunosuppression and an increased risk for secondary infection (59).

Prior analyses have suggested that there may be some correlation between HCMV infection and disease in transplant recipients and the levels in patient serum of molecules such as VCAM, ICAM-1, or TNFFRs when assayed individually (16–19). Moreover, the idea that HCMV infection leads to an increase in the sheddase activity of ADAM17 and that this may be a factor underlying the increased release of all these different molecules suggests that it would be worthwhile investigating whether multiplex assay of a panel of ADAM17 substrates might have predictive value as a biomarker for patients at risk for development of CMV disease, and thus of potential use in selecting patients for prophylactic treatment.

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References

FIGURE 7. Transplant recipients who develop HCMV disease have increased levels of soluble MICA in serum. A capture ELISA was used to assay the levels of soluble MICA molecules present in the serum of transplant recipients who either had, or had not, experienced CMV infection. Bar indicates increased levels of soluble MICA in serum. A capture ELISA was used to assay. Transplant recipients who develop HCMV disease have increased levels of soluble MICA in serum. A capture ELISA was used to assay.


The MICA molecules shed by HCMV infected cells are soluble, truncated species of MICA protein.

Cells, either uninfected or infected with AD169 (MOI 5:1) were cultured for 3 days, washed and then cultured overnight in serum free medium. Soluble fractions of the supernatants were prepared by centrifugation at 100,000 xg. Proteins were recovered by TCA precipitation and then aliquots of the whole cell lysates and soluble protein preparations were separated on SDS-PAGE and analysed by western blot.
Supplementary Figure 2 HCMV infection produces no significant changes in methylation of the Timp3 promoter

U373 cells were either left uninfected or infected with HCMV for 3 days and then genomic DNA was isolated by standard procedures. Approximately 1 μg of DNA from control and infected cells was bisulphite-modified using the EZ DNA Methylation™ Kit (ZymoResearch), and analysed by PCR with primers that specifically amplify unmethylated (U) (5′-TTTTGTTTTGTTATTTTTGTTTTTGGTTTT-3′ and 5′-CCCCCAAAAACCCCACCTCA-3′), or methylated (Met) TIMP-3 promoter sequence (5′-CGTTTCGTTATTTTTGTTTTCGGTTTC-3′ and 5′-CCGAAAACCCCGCCTCG-3′) (Bachman et al, Cancer Res 59: 798, 1999). The PCR reactions were visualized on a UV transilluminator after electrophoresis on 6% nondenaturing polyacrylamide gels and staining with ethidium bromide.

Control unmethylated DNA was prepared by bisulphite modification of genomic DNA isolated from the lymphocytes of healthy donors. For the methylated DNA control, 2 μg of lymphocyte genomic DNA was treated with 10 U of M.SsI CpG Methylase (New England BioLabs Inc., Beverly, MA, USA) for 16 h at 37°C in a 50 μL reaction volume containing 160 μM S-adenosylmethionine and NEBuffer 2 before being bisulphite modified.
Supplementary Figure 3 Soluble MICA was not found in samples collected after the resolution of the HCMV infection. For six patients samples of serum collected after the resolution of the HCMV infection were available for ELISA assay of soluble MICA. These samples had been collected between 10 and 40 days after the detection of HCMV DNAemia or antigenaemia. No soluble MICA was detected in this set of samples.