MICA/NKG2D-Mediated Immunogene Therapy of Experimental Gliomas

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ABSTRACT

The failure of conventional cancer therapy renders glioblastoma an attractive target for immunotherapy. Tumor cells expressing ligands of the activating immunoreceptor NKG2D stimulate tumor immunity mediated by natural killer (NK), γδ T, and CD8+ T cells. We report that human glioma cells express the NKG2D ligands MICA, MICB, and members of the UL16-binding protein family constitutively. However, glioma cells resist NK cell cytolysis because of high MHC class I antigen expression. Plasmid-mediated or adenovirus-mediated overexpression of MICA in glioma cells enhances their sensitivity to NK and T-cell responses in vitro and markedly delays the growth of s.c. and intracranial LN-229 human glioma cell xenografts in nude mice and of SMA-560 gliomas in syngeneic VMDk mice. Glioma cells forming progressive tumors after implantation of stably MICA-transfected human LN-229 cells lost MICA expression, indicating a strong selection against MICA expression in vivo. Rejection of MICA-expressing SMA-560 cells in VMDk mice resulted in prolonged animal survival to a subsequent challenge with wild-type tumor cells. Finally, the growth of syngeneic intracranial SMA-560 tumors is inhibited by peripheral vaccination with adenovirus-mediated, MICA-infected irradiated tumor cells, and vaccination results in immune cell activation in the NK and T-cell compartments in vivo. These data commend MICA immunogene therapy as a novel experimental treatment for human malignant gliomas.

INTRODUCTION

Glioblastoma, the most frequent malignant primary brain tumor, carries a poor prognosis, with a median survival after resection, radiotherapy, and chemotherapy of 12 months (1). The lack of effective immune responses to glial tumors inside the central nervous system has been attributed to the immune-privileged status of the brain conferred by the blood-brain barrier, the lack of conventional lymphatics, and the local release of immunosuppressive factors (2–4). However, both lymphocytes and macrophages infiltrate malignant gliomas, indicating the potential for lymphocyte homing and presentation of processed tumor antigens (2).

NKG2D is a C-type, lectin-like homodimeric receptor expressed by human natural killer (NK), γδ T, and CD8+ αβ T cells (5). In the mouse, NKG2D expression is found on NK cells, activated CD8+ T cells, and lipopolysaccharide-stimulated macrophages (6). Ligation of NKG2D may be a critical activating pathway to provide tumor surveillance (7–9). NKG2D interacts with ligands that are not constitutively expressed but are inductively expressed, including human MHC class I chain-related A (MICA) and MICB, distant cell stress-inducible homologs of MHC class I (10–12). The tissue distribution of MIC molecules is highly restricted to intestinal epithelia, but they are frequently expressed in epithelial tumors (11, 13). A second family of human NKG2D ligands (NKG2DL), designated UL16-binding proteins (ULBP), ULBP2, and ULBP3, has been characterized (14–16). Although no murine MIC molecules have been found, there are mouse orthologs of ULBP, the retinoic acid early inducible gene-1 products (RAE-1). The minor histocompatibility antigen H60 is another murine ortholog of ULBP that interacts with HLA-A, -B, -C, or -G antigens, and a heterodimer formed by the C-type lectin CD94/NKG2A that interact with HLA-E (20–24). Because a decrease or loss of MHC class I antigen expression may accompany neoplastic transformation, NK cells represent a natural defense to sense and selectively eliminate abnormal cells (25–27). This NK-mediated cytolytic activity must be down-regulated in the brain to prevent autoimmune damage because of low expression levels of MHC class I antigens on glial cells and a lack of MHC class I antigen expression on neurons (28, 29).

We show here that malignant glioma cells, in contrast to nonneoplastic astrocytic cells, express the NKG2DL MIC, and ULBP, which is paralleled by an increase of MHC class I expression, counteracting the activating potential of NKG2DL. By ectopic overexpression of MICA, engagement of NKG2D overcomes the inhibitory effect of MHC class I expression, inducing potent glioma cell sensitization to NK cells and providing costimulation of TCR-dependent T-cell activation in vitro and in vivo. We also demonstrate the feasibility of MICA overexpression as a strategy to enhance tumor immune surveillance and to inhibit the growth of gliomas in human xenogeneic and murine syngeneic glioma models.

MATERIALS AND METHODS

Cell Lines and Transfectants. The human Sv-FHAS astrocytic cell line was provided by D. Stanimirovic (Institute of Biological Sciences, National Research Council of Canada, Ontario, Ottawa, Canada). The human malignant glioma cell lines were provided by Dr. N. de Tribolet (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland). Primary glioblastoma cells were established from freshly resected tumors, cultured in monolayers, and used between passages 4 and 9 (30). The murine glioma cell lines GL261 and SMA-560 were gifts of X. O. Breakefield (Harvard Medical School, Boston, MA) and D. D. Bigner (Duke University Medical Center, Durham, NC). The cells were maintained in DMEM supplemented with 2 mm l-glutamine (Life Technologies, Inc., Paisley, United Kingdom), 10% FCS (Biokom KG, Berlin, Germany), and penicillin (100 IU/mlstreptomycin (100 μg/ml) (Gibco). NKL cells, kindly provided by M. J. Robertson (Indiana University School of Medicine, Indianapolis, IN), COS-7, YAC-1, and K562 cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 medium supplemented with 15% FCS, 2 mm l-glutamine, 1 mm sodium pyruvate, and penicillin (100 IU/mlstreptomycin (100 μg/ml). Transfections with cDNA clones, RSV.5neo, RSV.5neo-MICA*01 (10, 11) or RSV.5neo-
PatMIC1 (31) were conducted using FuGENE6 (Roche, Mannheim, Germany) and selection with G418 (1 mg/ml). PatMIC1 is the chimpanzee homolog of MICA, which shows 87% homology and was used as a negative control in some experiments (31). Stable, positive isolates were identified by flow cytometry and, to speed up the selection process, were sorted using a FACS Vantage (Becton Dickinson, Heidelberg, Germany).

Reverse Transcription (RT)-PCR. Total RNA was prepared using RNeasy (Qiagen, Hilden, Germany) and transcribed according to standard protocols. The conditions for all standard PCR were: 5 min at 95°C, 30 cycles of 1 min at 95°C, 1 min at 65°C, and 1 min at 72°C, using the following PCR primers: H60: sense 5′-GATGGTACAGACTCTCTAAGT-3′ and antisense 5′-CAGACCTCCTGTTTCAGAATTG-3′ (554 bp); RAE-1: sense 5′-ATGGCAGGCACAAGTGATCC-3′ and antisense 5′-CACATCGCAAGAATGCAAATATG-3′ (748 bp); MICA: sense 5′-GTGGCCAGGCTCTCAGTCTGAG-3′ and antisense 5′-GTGGACCTCCTTTGGTTGCTGAAG-3′ (321 bp); and antisense 5′-TGAGGGTGCTGATATGAAGTTTG-3′ and antisense 5′-CAGGAGGGATCTGAGTGGTTTCC-3′ (635 bp); MICB: sense 5′-GGGGTATCTTTGAAGTGCC-3′ and antisense 5′-GTCAAGGACAGCTGCTGATTGTGCC-3′ (690 bp); ULBP2: sense 5′-CTAGGCCAGGATGCTTTGGA-3′ and antisense 5′-TGAGGGTGGTGGCCATGCTTTGGA-3′ (319 bp); ULBP3: sense 5′-CTGCAGAGCAGATGCTTTGGA-3′ and antisense 5′-TGAGGGTGGTGGCCATGCTTTGGA-3′ (327 bp); ULBP6: sense 5′-CTCAGCGTCACTGGTGGTCTGAG-3′ and antisense 5′-TGAAGCGGTGTTGCTGATTG-3′ (321 bp); β-actin: sense 5′-AGCGATGTCAGTAGCCT-3′ and antisense 5′-TTTGGATTTGCAAGACGATTG-3′ (232 bp).

Generation of Monoclonal Antibodies. The mouse P815 mastocytoma cell line was transfected using FuGENE6 with full-length cDNA of NK2DL. MICA*01, MICA*04, MIBC*02, ULBP2, ULBP6, or ULBP3 in RSV.5 neo (15). Transfectants were selected with G418. BALB/c mice were immunized either with a mixture of MICA*01-, MICA*04-, and MIBC*02-, or ULBP1-, ULBP2-, and ULBP3-expressing P815 cells. Splenocytes of immunized mice were fused with P3 × 63Ag8.653 myeloma cells. Hybridoma supernatants were tested by indirect immunofluorescence using a FACS Calibur (Becton Dickinson) for selective binding to P815-NKG2DL transfectants and to COS cells transiently transfected with the various NK2DL cDNAs. Hybridomas producing NK2DL-specific mAbs were subcloned twice, and immunoglobulins were isolated using an isotyping kit (Roche). BAMO-1 (IgG1) and BAMO-2 (IgG2a) recognize MICA and MICB; ABO-1 (IgG1) is MICA specific; BMO-1 (IgG1) is MICB specific; AUMO-1 (IgG1) is ULBP1 specific; BUMO-2 (IgG1) ULBP2 specific; and CUMO-1 (IgM) is ULBP3 specific (32).

Production of Soluble mNK2GD in Insect Cells. Recombinant soluble mNK2GD lacking the NH2-terminal cytoplasmic region and transmembrane domain was produced in Sf9 insect cells (In Vitrogen, Karlsruhe, Germany) using the BAC-to-BAC system (Life Technologies). The expression construct included an N-terminal FL-A (M12) signal sequence (15). Transfections were done using the insect cell line High Five (Invitrogen). Recombinant soluble mNK2GD was purified from culture supernatant by affinity chromatography on a Nickel-chelating Sepharose (BD PharMingen) and size-exclusion filtration.

Monoclonal Antibodies and Flow Cytometry. Cell surface expression of MHC class I antigens and NK2GD was assayed with the following monoclonal antibodies (mAbs): W6/32, IgG2a pan anti-HLA-A, -B, -C, -E, -G (Biozol, Munich, Germany); anti-H-2K (BD Pharmedics, Heidelberg, Germany); anti-H-2D (Caltag Laboratories, Hamburg, Germany); and M585, IgG1 anti-NK2GD (kindly provided by Immunex, Seattle, WA). IgG1, IgG2a, and IgM isotype-matched antibodies were used as controls (BD Pharmedics). For the mNK2GD binding assays, mAb anti-FLAG M2 (5 µg/ml; Sigma) was mixed with soluble mNK2GD (3 µg/ml) before addition to cells. COS-7 cells were transiently transfected with neo-control, MICA*01, or PatMIC1. MICA/B and PatMIC1 expression levels were detected by flow cytometry using the mAb AMO-1. Interactions of human MICA*01 and chimpanzee PatMIC1 with mouse NK2GD were analyzed by staining with flagged soluble mouse NK2GD and anti-FLAG mAb. Glioma cells were detached using Accutase (PAA, Vienna, Austria), preincubated in PBS with 2% BSA, and incubated with the specific antibody or matched mouse immunoglobulin isotype (10 µg/ml) as a control for 30 min on ice. Specific binding was detected with phycoerythrin-conjugated goat anti-mouse IgG (Sigma). Cell surface expression of CD16 (FcγRIII) was assayed with the mAb 3G8, IgG1 anti-CD16-CyChrome (BD Pharmedics). Flow fluorescence was measured in a Becton Dickinson FACScalibur. Specific fluorescence indexes (SFIs) were calculated by dividing mean fluorescence obtained with specific antibody by mean fluorescence obtained with control antibody.

Adenoviral Constructs and Transduction. Replication-deficient recombinant adenoviruses (Ad5DeI.3) that had the E1A region replaced by the full-length MICA*01 cDNA (Ad-MICA) were constructed using the AdEasy System, kindly provided by Bert Vogelstein (The Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD). Recombinant adenoviral particles were produced in 293 cells (ATCC CRL-1573), and transgene expression was assessed by RT-PCR. Infection of target cells with recombinant viruses was accomplished by exposing cells to different concentrations of adenovirus in PBS containing 10% glycerol for 15 min, followed by the addition of serum-containing medium for 2 days.

Purification of PBL and Isolation of NK and T Cells. Peripheral blood mononuclear cells were derived from healthy donors by density gradient centrifugation (Biocol, Biobrom KG) and depletion of plastic-adherent cells. Peripheral blood lymphocytes (PBLs) were cultured on irradiated RPMI 8866 feeder cells to obtain polyclonal NK cell populations. To further enrich NK cells, PBLs were sorted by immunomagnetic depletion using Dynabeads (NK Cell Negative Isolation kit; Dynal, Oslo, Norway). CD3+CD56+ cells were used for cytotoxicity assays. To obtain purified T cells, fresh PBLs were depleted of B cells and monocytes using the LymphoKwik T reagent (One Lambda, Inc., Canoga Park, CA).

Mouse Lymphocyte Isolation. Murine NK cells were prepared from splenocytes from CD1-deficient BALB/c nude mice or MVMd mice. NK cells were positively selected using DX5 mAb-coupled magnetic beads with the corresponding column system (Milenyi Biotech, Bergisch Gladbach, Germany). Polyclonal mouse NK cells were cultured with mouse IL-2 (5000 units/ml; Preprectone, London, United Kingdom) for at least 10 days before use in cytotoxicity assays.

Cytotoxicity Assay. Cytotoxicity was assayed in 4-h 51Cr release assays in the absence or presence of various amounts of soluble mNK2GD. The concentrations for the masking experiments were 10 µg of mAb/ml and 20 µg/ml for soluble mNK2GD. The 51Cr release assay was performed using 2000 51Cr-labeled targets/well. Effector and target cells were incubated at various E/T ratios for 4 h. Spontaneous release of 51Cr was determined by incubating the target cells with medium alone. Maximum release was determined by adding NP40 to a final concentration of 2%. The percentage of 51Cr release was calculated as follows: 100 × (experimental release − spontaneous release)/[maximum release − spontaneous release].

T-Cell Reaction against Allogeneic Glioma Cells. To suppress LN-229 glioma cell proliferation, the cells were irradiated at 100 Gy. HLA-A2-positive T cells or peripheral blood mononuclear cells (105/well) were cocultured with 104 irradiated LN-229 HLA-A2-negative glioma cells in 96-well plates in triplicates. After 4 days the cells were pulsed for 24 h with [methyl-3H]-thymidine (0.5 Ci/ml; Amersham, Braunschweig, Germany). After harvesting with a Perkin-Elmer 1450 Microbeta Plus Liquid Scintillation Counter, for the priming assays, nonirradiated glioma cells were incubated with peripheral blood mononuclear cells or T cells for specific priming and clonal expansion. Lymphocytes were harvested on day 5 and used as effectors in a 4-h 3H release cytotoxicity assay. Mock-transfected LN-229.neo cells or LN-229 cells transfected with MICA*01 or PatMIC1 were used as targets.

Interleukin (IL)-2 Release Assay. T cells (1 × 105/well) isolated at day 10 after tumor inoculation from spleens of vaccinated or control animals were stimulated ex vivo with irradiated SMA-560 cells (1 × 105). T-cell supernatants from triplicate wells were collected and pooled 48 h later. IL-2 release was determined by ELISA (R&D Systems, Minneapolis, MN).

Mice and Animal Experiments. Athymic CD1-deficient BALB/c nude mice were purchased from Farron Laboratories (Sulzfeld, Germany), and VMDk mice were purchased from the TSE Research Center (Berkshire, United Kingdom). Mice of 6–12 weeks of age were used in all experiments. The experiments were performed according to NIH guidelines, Guide for the Care and Use of Laboratory Animals. Groups of 4–6 mice were injected s.c. in the right flank with transfected LN-229 tumor cells or uninfected or infected SMA-560 glioma cells (1 × 105 cells) in 0.1 ml of PBS, as indicated. Mice were examined regularly for tumor growth using a metric caliper and killed when tumors reached >12-mm diameter. Mice were anesthetized by i.p. injection of 7% chloral hydrate before all intracranial procedures. For intracranial implantation, the mice were placed in a stereotactic fixation device.
Expression of NKG2DL and MHC Class I Antigens by Human Glioma Cells. To investigate the expression of the NKG2DL MICA, MICB, ULBP1, ULBP2, and ULBP3 by glioma cells, 12 glioma cell lines and primary cultures were assessed by RT-PCR (Fig. 1). MICA and ULBP1, ULBP2, and ULBP3 mRNA were expressed by almost all cell lines and most of the primary glioma cell cultures. MICB was not detectable in U251MG or U373MG cells and several primary glioma cell cultures. The nonneoplastic Sv-FHAS cell line did not express any NKG2DL, as determined by RT-PCR and flow cytometry. Sv-FHAS cells showed low MHC class I antigen expression (Table 1).

Stable Transfection and Adenoviral Gene Transfer of MICA into Glioma Cells Stimulate NK Cytolytic Activity. To determine whether increased NKG2DL expression confers additional stimulatory signals for NK cells, we generated stable MICA*01 and PatrMIC1 [chimpanzee MIC homolog (31)] transfectants of the LN-229 and Sv-FHAS cell lines (Fig. 2, A and B). PatrMIC1 was included in this study to serve as a negative control for the nude mouse model in vivo (see below). Enhanced MICA or PatrMIC1 expression rendered cells susceptible to lysis by human NKL effector cells, suggesting that ectopic MIC expression overcomes the inhibitory effect of MHC class I antigens (Fig. 2, A and B, lower panels). Similar results were obtained with freshly isolated polyclonal NK cells (data not shown). For a gene therapeutic approach, we generated an adenoviral vector encoding MICA*01 (Ad-MICA). Infection of LN-229, primary glioma cells (Fig. 2C), or other glioma cell lines (data not shown) with Ad-MICA resulted in a marked increase in MICA expression. Adenoviral MICA gene transfer also promoted NK-mediated cytolysis (Fig. 2C, lower panel).

For blocking experiments with mAbs, we first ruled out that a potential lysis seen in the presence of mAbs is attributable to antibody-dependent cellular cytotoxicity (ADCC). Unlike fresh polyclonal NK cells isolated from PBLs, the NKL cell line used here does not express FcγRIII (CD16), which mediates ADCC (Fig. 3A; Ref. 35). LN-229.neo transfectants were sensitized to NK cytolysis by treatment with the anti-MHC class I mAb W6/32 (Fig. 3B), confirming that classical MHC class I antigens protect LN-229.neo cells from NK cytolysis. Anti-MICA/B (BAMO-1) or anti-NKG2D (M585) mAb had no effect on cytolysis in LN-229.neo cells. However, these mAbs either attenuated (anti-MICA) or blocked (anti-NKG2D) the sensitization mediated by anti-MHC class I mAb. The superior effect of anti-NKG2D compared with anti-MICA reflects the composition of the system of a single receptor (NKG2D) in NK cells but multiple ligands (MIC and ULBP molecules) expressed by LN-229 cells (Fig. 1; Table 1). Soluble mouse MICA (mNKG2D) attenuated the anti-MHC class I mAb-mediated sensitization as effectively as anti-MICA, suggesting that murine NKG2D interacts with some human NKG2DL. NK cytolysis of LN-229 MICA targets pretreated with anti-MICA or anti-NKG2D mAb was reduced (Fig. 3C), demonstrating that enhanced cytolysis of LN-229.MICA targets is attributable to MICA overexpression, overruling MHC class I antigen inhibitory signals. Similar results were obtained with LN-229.PatrMIC1 transfectants (Fig. 3D).

Costimulatory Functions of MICA-transfected Glioma Cells. Allostimulation assays were performed to analyze whether MICA/NKG2D interactions promote the induction of T-cell responses. LN-229.MICA or LN-229.PatrMIC1 cells were substantially more potent stimulators of T-cell proliferation than LN-229.neo cells (Fig. 4A). mAb masking of MICA abrogated the augmentation of T-cell proliferation mediated by MICA but had no effect when T cells were coincubated with LN-229.neo cells (Fig. 4B). Additional evidence for the costimulatory potential of NKG2D for T cells was obtained by rechallenging primed T cells. T cells were primed for 4 days with LN-229.neo, LN-229.MICA, or LN-229.PatrMIC1 cells and then used as effectors against fresh LN-229.neo, LN-229.MICA, or LN-229.PatrMIC1 cells. No target cell lysis was seen when LN-229.neo

Fig. 1. NKG2DL expression in human glioma cells. NKG2DL (MICA, MICB, ULBP1, ULBP2, ULBP3) mRNA expression was assessed by RT-PCR in human glioma cell lines (A) and primary glioma cells (B). β-actin was used as a standard.

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cells, LN-229.MICA, or LN-229.PatrMIC1 cells were used as targets for LN-229.neo-primed T cells (Fig. 4, C–E). In contrast, LN-229.MICA- or LN-229.PatrMIC1-primed T cells efficiently killed LN-229.MICA, or LN-229.PatrMIC1 cells with comparable efficacy, indicating that NKG2D ligation by MICA or PatrMIC1 acts as a costimulatory signal in the priming phase of T cells (Fig. 4, C–E).

**MICA Expression Delays Growth of Glioma Xenografts in Nude Mice.** We next sought to examine the immune-stimulatory capacity of MICA-overexpressing glioma cells in murine in vivo models. We first tested the physical and functional engagement of MICA*01 and PatrMIC1 by mouse NKG2D (mNKG2D). Interaction of MICA*01 and PatrMIC1, respectively, with mNKG2D, was assessed by using soluble mNKG2D. COS-7 cells transiently transfected with either MICA*01 or PatrMIC1 cDNA stained with the mAb AMO-1, but only MICA*01-transfected COS cells bound soluble mNKG2D (data not shown). We concluded that mNKG2D interacts with human MICA*01 but not with PatrMIC1. PatrMIC1 differs from MICA and MICB in several positions directly involved in NKG2D interaction, which may selectively interfere with binding of mNKG2D (10–12). Accordingly, NK cell cytotoxicity assays using polyclonal mouse NK cells as effector cells revealed marked lysis of human MICA*01-transfected COS cells, whereas only low level H-2Db expression (Fig. 6B). SMA-560 cells, whereas GL261 showed only a weak expression. H60 was only expressed by SMA-560 and not by GL261 cells, consistent with the report that C57BL/6 mice do not express H60 (Ref. 8; Fig. 6A). Soluble mNKG2D was more prominently bound by SMA-560 than GL261 cells, indicating higher expression levels of mNKG2DL in SMA-560 cells. Whereas SMA-560 cells expressed both MHC class I antigens H-2K<sup>d</sup> and H-2D<sup>d</sup>, which are potential ligands for inhibitory Ly49 receptors, GL261 cells showed only low level H-2D<sup>b</sup> expression (Fig. 6B). SMA-560 cells thus resemble the phenotype of human glioma cell lines and were used in further animal experiments. When SMA-560 cells were infected with Ad-MICA, MICA expression increased in a multiplicity of infection (MOI)-dependent manner: the SFI values were 6.6 at 100 MOI, 12.2 at 300 MOI, and 22.8 at 1000 MOI (Fig. 7B). Similar to LN-229 cells, the growth of SMA-560 cells was unaffected by forced MICA expression (Table 2). The functional significance of increased MICA expression on mouse glioma cells after Ad-MICA infection was confirmed in cytotoxicity assays using syngeneic VMDk NK cells as effectors (Fig. 6C). Blocking experiments with anti-MICA mAb and soluble mNKG2D demonstrated that the increased killing of Ad-

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**Table 1 Flow cytometric analysis of MHC class I antigens and NKG2DL expression by human malignant glioma cell lines, non-neoplastic Sv-FHAS astrocytes, and primary glioma cell cultures.** The cells were stained with the indicated mAb or isotype-matched Ig. Expression was quantified as SFI values (mean fluorescence specific mAb /mean fluorescence isotyp control).
Fig. 2. Ectopic MICA*01 and PatrMIC1 expression by glioma cells promote NK cell-mediated cytolysis. LN-229 (A) or Sv-FHAS (B) cells transfected with MICA*01 or PatrMIC1 were assessed for MICA (mAb BAMO-1) or PatrMIC1 (mAb BAMO-2) expression (filled profiles) as compared with mock transfectants (neo-control) by flow cytometry. Open profiles show the labeling with isotype-matched immunoglobulin. The SFI values are indicated in the upper right of each panel. C, LN-229 or TU113 primary glioma cells were infected with Ad-MICA or Ad-dE1 (300 MOI) and assessed for MICA expression accordingly. In the lower panels of A–C, the cell lines were tested as targets for human NKL cells in 51Cr-release assays. Data are expressed as specific lysis at different E:T ratios (means; bars, SD). t test: *, P < 0.05; **, P < 0.01.
MICA-infected SMA-560 cells was attributable to MICA expression. Attenuation of the constitutive lysis of control-infected SMA-560 cells by mNKG2D probably reflects the activation by endogenous NKG2DLs such as RAE-1 and H60 (Fig. 6D). Accordingly, neutralization of MICA was less effective in inhibiting lysis of Ad-MICA-infected cells than mNKG2D.

MICA Expression in SMA-560 Glioma Cells Delays Tumor Growth. To analyze the immune-stimulatory capacity of MICA-transduced SMA-560 tumor cells in vivo, VMDk mice were inoculated s.c. with syngeneic tumor cell transductants. There was no difference between the in vivo growth of Ad-dE1-infected and unin-
MICA/NKG2D-MEDIATED THERAPY OF GLIOMAS

DISCUSSION

This study investigates the potential of NKG2DL to promote an immune response to glioblastoma. We show that NKG2D-mediated

s.c. Vaccination with MICA-expressing Glioma Cells after Tu-

Fig. 5. Ectopic MICA expression delays the growth of s.c. and intracerebral human glioma xenografts in nude mice. A, LN-229 transfectants were tested as targets for BALB/c nude mice NK cells in a 51Cr-release assay (means; bars, SD). t test: **, P < 0.01. B, the growth of s.c. LN-229.PatrMIC1 or LN-229.MICA tumors was monitored every 2 days (mean; bars, SD). t test: **, P < 0.01. C, LN-229.PatrMIC1 (solid line) or LN-229.MICA*01 (broken line) cells (5 × 10⁶) were inoculated intracerebrally in BALB/c nude mice. Survival data for five animals per group are shown, evaluated by the Kaplan-Meier method (log-rank test, P < 0.01). D, at 25 days after injection of tumor cells, the mice were killed, and freshly isolated tumor cells from s.c. tumors were analyzed for PatrMIC1 in PatrMIC1 tumors (left) or MICA in MICA tumors (right) by flow cytometry. Representative tumor isolates are shown as gray profiles, the preincubation levels as black profiles, and an isotype control as open profiles.
fected SMA-560 cells. Conversely, MICA*01 expression by SMA-560 glioma cells resulted in a substantial delay of tumor growth in a MOI-dependent manner (Fig. 7A). Of note, 2 of 6 animals in the groups challenged with 300 and 1000 MOI-infected glioma cells, respectively, developed no tumor. Irrespective of the initial level of MICA expression before inoculation, glioma cells harvested from progressive tumors at day 10 after inoculation were devoid of MICA (Fig. 7B). To address whether prior immunization with glioma cells that express MICA induces protective immunity to wild-type glioma cells, mice that had previously rejected MICA-transduced tumor cells were rechallenged with wild-type SMA-560 cells 10 weeks after the first exposure in the opposite flank. Wild-type SMA-560 cells grew progressively in naïve VMDk mice but were rejected by mice that had been exposed previously to the MICA-transduced tumor cells (Fig. 7C).

Proliferation (cpm)

Table 2. MICA gene transfer does not modulate glioma cell growth as assessed by [3H]thymidine incorporation in vitro

[3H]thymidine incorporation was measured after 24 h by liquid scintillation counting. Data are expressed as cpm ± SD.

<table>
<thead>
<tr>
<th>Proliferation (cpm)</th>
<th>LN-229</th>
<th>SMA-560</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental</td>
<td>1802 ± 401</td>
<td>3078 ± 523</td>
</tr>
<tr>
<td>PatrMIC1 (stable)</td>
<td>1766 ± 431</td>
<td>3153 ± 500</td>
</tr>
<tr>
<td>MICA (stable)</td>
<td>1722 ± 386</td>
<td>3122 ± 489</td>
</tr>
<tr>
<td>Ad-dE1 (300 MOI)</td>
<td>3153 ± 500</td>
<td>3122 ± 489</td>
</tr>
<tr>
<td>Ad-MICA (300 MOI)</td>
<td>3122 ± 489</td>
<td>3122 ± 489</td>
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NK cell triggering induced by NKG2DLs expressed on glioma cells is overruled by MHC class I antigens that engage inhibitory receptors on NK cells. Gene transfer-mediated ectopic expression of MICA on human and murine glioma cells permits their in vitro immune recognition and lysis and in vivo growth inhibition, despite a high level MHC class I antigen expression. These observations provide a rationale for MICA-based immunogene therapy of gliomas.

Malignant transformation of glial cells is associated with an NKG2DL expression, i.e., of MICA/B and ULBP molecules, at the cell surface (Fig. 1; Table 1). However, the NK cell inhibitory potential of MHC class I antigens expressed on human glioma cells masks the activating signals of NKG2DL. The interaction of effector NKG2D with endogenous tumor MIC and ULBP molecules promotes NK cell-mediated tumor cell lysis in vitro when MHC class I antigens are masked (Fig. 3B). NK cell-mediated tumor cell lysis was also achieved when MICA expression was enhanced via plasmid transfection or adenoviral gene transfer (Figs. 2 and 3C; Ref. 27). Thus, glioma cells outbalance activating ligand expression by inhibitory MHC class I antigens and prevent NK cell lysis. This concept is supported by a highly significant correlation between MHC class I antigen expression and NKG2DL expression on human glioma cells when the SFI values for the NK cell inhibitory MHC class I antigens were correlated with a sum score of the SFI values for MICA, MICB, ULBP1, ULBP2, and ULBP3 (t test, r² = 0.6523, P = 0.0015).

One way of immune escape for glioma cells may be the expression of MHC class I antigens at a level that allows sufficient binding of NK inhibitory receptors and escape from NK-mediated killing. High expression of MHC class I antigens in human glioblastomas in vivo has been confirmed by several studies (37, 38). MHC class I down-regulation, conversely, is a hallmark of many peripheral cancers and decreases the susceptibility of tumor cells to lysis by cytotoxic T cells (26, 39). Possibly only those glioma cells that show an up-regulation of MHC class I antigens expression to compensate for the acquisition of activating NKG2DL expression during malignant transformation will survive. This selection may render tumor cells more vulnerable toward T cell-dependent elimination through tumor-associated antigen detection (26, 39). Because the brain lacks specific brain-associated lymphoid tissue, antigens that are introduced directly into the brain parenchyma exclusively elicit transient innate inflammatory immune responses but no adaptive immunity (40–44). However, if immunization is elicited by injecting the identical antigen outside the brain, the adaptive immune system becomes primed, and antigenic epitopes anywhere within the brain will be recognized as targets of either activated effector T cells, B cells, or antibodies (40, 42, 44). This immunological peculiarity may explain high expression levels of MHC class I antigens on glioma cells that could effectively present tumor-associated antigens to primed T cells. Because intracerebral T-cell priming is not found, the MHC class I antigens may only serve to suppress NK cell activation by NKG2DL.

The highly lethal nature of glioblastoma suggests that the levels of activating NKG2DLs expressed by glioma cells are too low to induce antitumor immunity. Thus, we concluded that immunity to gliomas may be boosted by engineering cells expressing high levels of activating NKG2DL. We noted that MICA gene transfer induces NK and T-cell responses to glioma cells in vitro (Figs. 2–4) and delays growth in a human xenograft and syngeneic mouse glioma model in vivo (Figs. 5–7). In this model, the in vivo selection for low MICA-expressing glioma cells strikingly demonstrated the NK cell activating potential of the NKG2D/NKG2DL system (Figs. 5D and 7B). Previously, it was shown that ligation of NKG2D on NK and T cells can promote subsequent T-cell immunity to parental tumors that lack NKG2DL, although T-cell priming was NK cell independent (8). However, a similar study did not detect such T cell-mediated memory
Fig. 7. MICA delays SMA-560 glioma growth in syngeneic mice. A, the growth of s.c. tumors formed by SMA-560 cells infected in vitro with Ad-dE1 (1000 MOI) or Ad-MICA (100, 300, or 1000 MOI) was monitored every 2 days. Two of 6 animals challenged with Ad-MICA-infected cells at 300 and 1000 MOI did not develop a measurable tumor. B, SMA-560 cells infected with Ad-MICA at increasing MOI were assessed for MICA expression before subcutaneous inoculation (black profiles). Open profiles show the labeling with isotype-matched immunoglobulin. Furthermore, glioma cells were freshly isolated from s.c. tumors at 10 days after inoculation and reanalyzed for MICA expression (gray profiles). C, VMDk mice that had previously rejected MICA-transduced SMA-560 cells were inoculated s.c. with wild-type SMA-560 glioma cells in the opposite flank. Primary exposure preceded the challenge by 10 weeks (means; bars, SD). t test: **, P < 0.01. D, 5 × 10^4 SMA-560 cells were inoculated intracerebrally in syngeneic VMDk mice (day 1). At days 3 and 8, the animals were vaccinated s.c. with 1 × 10^6 irradiated Ad-dE1- or Ad-MICA-infected (1000 MOI) or uninfected SMA-560 cells. The graph shows survival data for 6 animals/group, evaluated by the Kaplan-Meier method (log-rank test, P < 0.01). E and F, at day 10, splenocytes were recovered from the differently vaccinated animals, and T and NK cells were isolated. E, NK cells were used as effector cells in a 51Cr release assay using YAC-1 cells as targets (means; bars, SD). t test: *, P < 0.05; **, P < 0.01. F, isolated T cells were restimulated with irradiated wild-type SMA-560 cells, and IL-2 release was measured by ELISA 48 h later.
against the parental tumor in mice (7). Our data support that NKG2D engagement costimulates human CTL responses because prior immunization with tumor cells expressing the NKG2DL MICA induces protective immunity against a challenge with wild-type glioma cells in mice that had previously rejected MICA-transduced glioma cells (Fig. 7C: Ref. 8, 45). Finally, the peripheral vaccination with irradiated Ad-MICA-infected cells after the intracerebral implantation of wild-type tumor cells delayed the growth of syngeneic gliomas and promoted immune activation in the NK and T-cell compartments (Fig. 7, E and F, and Fig. 8). NKG2DL expression and consequent activation of NK cells and T cells may thus provide a novel therapeutic approach for the treatment of human gliomas. If the magnitude of the effect achieved with peripheral vaccination, i.e., a median prolongation of survival from 27.5 in control animals to 46 days in SMA-560.Ad-MICA-vaccinated animals, with 2 of 6 animals (33%) alive and asymptomatic after 90 days (Fig. 7D), could be transferred into the human situation, this would be a major advance, compared with all chemotherapy trials performed in the recent 20 years (46).

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