RNA Interference Targeting Transforming Growth Factor-β Enhances NKG2D-Mediated Antiglioma Immune Response, Inhibits Glioma Cell Migration and Invasiveness, and Abrogates Tumorigenicity In vivo

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ABSTRACT

Transforming growth factor (TGF)-β is the key molecule implicated in impaired immune function in human patients with malignant gliomas. Here we report that patients with glioblastoma, the most common and lethal type of human glioma, show decreased expression of the activating immunoreceptor NKG2D in CD8+ T and natural killer (NK) cells. TGF-β is responsible for the down-regulation of NKG2D expression in CD8+ T and NK cells mediated by serum and cerebrospinal fluid of glioma patients in vitro. Moreover, TGF-β inhibits the transcription of the NKG2D ligand MICA. Interference with the synthesis of TGF-β1 and TGF-β2 by small interfering RNA technology prevents the down-regulation of NKG2D on immune cells mediated by LNT-229 glioma cell supernatant and strongly enhances MICA expression in the glioma cells and promotes their recognition and lysis by CD8+ T and NK cells. Furthermore, TGF-β silencing results in a less migratory and invasive glioma cell phenotype in vitro. LNT-229 glioma cells deficient in TGF-β exhibit a loss of subcortical and orthotopic tumorigenicity in nude mice, and NK cells isolated from these mice show an activated phenotype. RNA interference targeting TGF-β1/2 results in a glioma cell phenotype that is more sensitive to immune cell lysis and less motile in vitro and nontumorigenic in nude mice, strongly confirming TGF-β antagonism as a major therapeutic strategy for the future treatment of malignant gliomas.

INTRODUCTION

Glioblastoma, the most frequent intrinsic malignant brain tumor, carries a poor prognosis with a median survival time of 12 months (1). The infiltration of malignant gliomas by lymphocytes and macrophages confirms a potential for lymphocyte homing and presentation of processed tumor antigens (2). Unfortunately, because gliomas grow progressively and eventually kill their host, the immune system clearly fails to mount an effective immune response against these tumors. The lack of effective immune responses to gliomas has been attributed to the immune-privileged status of the brain conferred by the blood–brain barrier and to the local release by glioma cells of soluble immunosuppressive factors such as transforming growth factor (TGF)-β (2–4). The synthesis of TGF-β by glioma cells has been amply documented in analyses of glioma cell lines in vitro (5–7), cerebrospinal fluid (CSF) samples (8, 9), and cyst fluids ex vivo (10, 11) as well as human glioma specimens (12, 13).

TGF-β interferes with antitumor immune responses through the inhibition of maturation and antigen presentation by dendritic cells and by inhibiting the activation of T and natural killer (NK) cells (14). Furthermore, TGF-β may act directly as a tumor progression factor. Increased production of TGF-β occurs in various tumor types and correlates with tumor grade (15). TGF-β influences proinvasive functions that enable the general spreading of cancer cells by regulating the expression, secretion, or activity of matrix metalloproteinases (MMPs) by endothelial cells and tumor cells, creating an environment that favors angiogenesis, cell growth, motility, and survival (16). MMPs are proteolytic enzymes that shape the cellular microenvironment. Compared with normal tissue, their expression and activation are increased in almost all human cancers (17). In particular, MMP-2 and MMP-9 are highly expressed in human gliomas (18).

NKG2D is a C-type lectin-like homodimeric receptor expressed by human NK, y6 T and CD8+ αβ T cells (19). Ligation of NKG2D stimulates tumor immune surveillance (20–23). NKG2D interacts with ligands that are not constitutively but inducibly expressed by cell stress, including human MICA and MICB, distant homologs of major histocompatibility complex class I (24–26). The tissue distribution of MIC molecules is physiologically restricted to intestinal epithelia, but these molecules are frequently expressed in epithelial tumors and gliomas (23, 25, 27). MIC engagement of NKG2D triggers NK cells and costimulates antigen-specific effector T cells. NKG2D ligands are therefore induced self-ligands and represent molecular markers that flag stressed, transformed, or infected cells for killing by NK and CD8+ T cells (28).

Here we identify TGF-β as a central molecule regulating NKG2D-mediated immune escape of human glioma cells by down-regulating NKG2D expression in CD8+ T and NK cells and down-regulating MICA expression in glioma cells. Furthermore, TGF-β serves as an important autocrine factor that regulates glioma proliferation, motility and invasiveness. Silencing of TGF-β expression by small interfering RNA (siRNA) technology blocks these critical features of malignancy in vitro and abrogates glioma cell tumorigenicity in vivo.

MATERIALS AND METHODS

Patient Characteristics. We studied peripheral blood mononuclear cells from patients with glioblastoma (five males and two females; median age, 56 years; age range, 48–71 years) who had not received radiotherapy, chemotherapy, or glucocorticoids for 12 weeks. The glioma patients were compared with a group of 17 age- and sex-matched healthy donors (controls) without neurologic disease or any other known disease. CSF was obtained from glioma patients or patients with other neurologic diseases as part of the routine diagnostic work-up. The study was performed according to a protocol approved by the University of Tübingen Medical School Ethics Committee.

Monoclonal Antibodies and Flow Cytometry. Neutralizing pan–anti-TGF-β1,2,3 monoclonal antibody [mAb (1D11, IgG1)] was from R&D Systems (Wiesbaden, Germany). Cell surface expression of MICA/B, NKG2D, CD3, CD8, and CD56 was assessed with the following mAbs: 5M85 IgG1 anti-NKG2D (kindly provided by Amgen, Thousand Oaks, CA), BAM01 IgG1 anti-MICA/B, BAM03 IgG1 anti-MICA/B, AM01 IgG1 anti-MICA, BMO2 IgG1 anti-MICB (29), HIT3a IgG2a anti-CD3-fluorescein isothiocyanate-
nate, HIT8a IgG1 anti-CD8-phycoerythrin (PE), and B159 IgG1 anti-CD56-PE (all from BD PharMingen, Heidelberg, Germany). Biotin-conjugated rabbit antimouse IgG (Dako, Hamburg, Germany), streptavidin-APC (BD PharMingen), and PE-conjugated goat antimouse IgG (Sigma, Deisenhofen, Germany) were used for detection. Conjugated and unconjugated IgG1 and IgG2a iso-
type-matched mAbs were used as controls (BD PharMingen). Peripheral blood lymphocyte (PBLs) or glioma cells detached using Accutase (PAA, Wien, Austria) were preincubated in PBS with 2% bovine serum albumin and incubated with the specific mAb or matched mouse immunoglobulin isotype (5 µg/mL) for 30 minutes on ice. Specific binding was detected with the specific conjugate or by using a secondary conjugated antibody. Fluorescence was measured in a Becton Dickinson FACScalibur. Specific fluorescence index (SFI) values were calculated by dividing mean fluorescence obtained with specific antibody by mean fluorescence obtained with control antibody. Purification of Peripheral Blood Lymphocytes and Isolation of Natural Killer and T Cells. PBLs were prepared by density gradient centrifugation (Biocoll; Biochrom KG, Berlin, Germany) and depletion of plastic-adherent mononuclear cells. 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 cyto-
toxicity assays. To obtain purified CD8+ T cells, fresh PBLs were sorted by immunomagnetic CD8 MACs beads (Miltenyi Biotec, Bergisch Gladbach, Germany).

Cell Lines and Transfectants. The human SV-FHAS astrocytic cell line was provided by D. Stanimirovic (Institute for Biological Sciences, 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 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 2 mmol/L L-glutamine (Gibco Life Technologies, Inc., Paisley, United Kingdom), 10% fetal calf serum (FCS; Biochrom KG), and penicillin (100 IU/mL)/streptomycin (100 µg/mL; Gibco Life Technologies, Inc.). NLK cells, kindly provided by M. J. Robertson (Indiana University School of Medicine, Indianapolis, IN; ref. 31), and YAC-1 cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 supplemented with 15% FCS, 2 mmol/L L-glutamine (Gibco Life Technologies, Inc.), and 10% FCS. PBLs were prepared by density gradient centrifugation (SFI) values were calculated by dividing mean fluorescence obtained with specific mAb over that of isotype-matched mAbs as controls (BD PharMingen, Heidelberg, Germany) with or without anti-
MICA, 5'CCCTACGAGAAGGGGTG3' (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1 µg/mL in

B cells after activation with plate-

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mAb sc-146 goat anti–TGF-β1, sc-90 goat anti–TGF-β2 (Santa Cruz Biotechnol-

ogy), Ab-3 mouse anti–MMP-2, Ab-7 mouse anti–MMP-9, or 113-5B7 mouse anti–MT1-MMP (Oncogene, San Diego, CA) were used at 1 µg/mL in PBS containing 0.05% Tween 20 and 1.3% skim milk. Labeling was visualized using enhanced chemiluminescence (ECL; Amersham, Braunschweig, Germany).

Zymography. The activities of MMP-2 and MMP-9 were analyzed using SDS-PAGE gels containing 0.1% gelatin (w/v) and 10% polyacrylamide (w/v; Bio-Rad, Munich, Germany). Coomassie Brilliant Blue staining and subse-
quent destaining with glacial acid result in decreased staining at the level of the electrophoretic migration of MMP-2 and MMP-9.

Real-Time Polymerase Chain Reaction. Total RNA was prepared using RNAeasy (Qiagen, Hilden, Germany) and transcribed according to standard protocols. Complementary DNA amplification was monitored using SYBR Green chemistry on the ABI PRISM 7000 Sequence Detection System (Ap-
plied Biosystems, Weiterstadt, Germany). The conditions for all polymerase chain reactions (PCRs) were 40 cycles of 95°C for 15 seconds and 60°C for 1 minute using the following specific primers (forward and reverse): 18S, 5’-CCGGCTTACACATCAAGGAA-3’ and 5’-GCTGGAAATACCGCGGCCT-3’; NGK2D, 5’-TCTCGACAGCTGGGATG-3’ and 5’-GACATCTTTTGTTGCAATGC-3’; and MICA, 5’-CCTTGCCCCATGCACTGAC-3’ and 5’-CCTCTGGAGCTCCCTGCG-3’. Data analysis was done by using the ΔCt method for relative quantification. Briefly, threshold cycles (Ct values) for 18S rRNA (reference) and NGK2D or MICA (sample) were determined in duplicates. We arbitrarily defined the values obtained for untreated cells as the standard value (100%) and determined the relative change (Δr) in copy numbers according to the formula Δr = 2−[(Ct Sample−Ct Reference)/Ct Standard reference].

Transforming Growth Factor-β Bioassay. Levels of bioactive TGF-β were determined using a modification of the CCL64 bioassay (33). Briefly, 105 L929 cells were adhered to 96-well plates for 24 hours. After removal of regular medium, the cells were exposed to glioma cell SN, serum, or CSF from glioma patients or normal controls diluted in serum-free medium for 72 hours. Viable cell counts were obtained by crystal violet staining.

Transforming Growth Factor-β Reporter Assays. Intracellular TGF-β signaling was assessed by reporter gene activity, using the pGL2-3TP-Luc (34) reporter gene plasmid (J. Massague, Memorial Sloan-Kettering Cancer Center, New York, NY), which contains a synthetic promoter composed of a TGF-β-responsive plasmogen activator inhibitor 1 promoter fragment in-
serted downstream of three phorbol ester-responsive elements. For assessment of TGF-β1 transcription, a pGL3b-TGF-β1-Luc construct containing the TGF-β1 5’-flanking sequence (from −453 to +1 bp; ref. 35) was used (C. Weigert, Division of Endocrinology, Tübingen, Germany). Cells were cotransfected with a 10-fold excess of the specific reporter and pRLE-CMV plasmid (Clontech, Palo Alto, CA) at 32 hours after transfection, TGF-β1 (5 ng/mL) was added for 16 hours. The respective activities of firefly and Renilla reniformis luciferase were determined sequentially in a LumatPlus (EG&G Berthold, Pforzheim, Germany) luminometer, using the firefly dual luminescence reporter gene assay (Perkin-Elmer, Rodgau-Jüge-
sheim, Germany). Background was subtracted from all values, and the counts obtained from the measurement of firefly luciferase were normalized with respect to pRL-CMV.

T-Cell Costimulation Assay. T-cell proliferation was measured using freshly isolated peripheral blood CD8+ T cells after activation with plate-
bound mAbs. mAbs were plate-bound overnight in 96-well flat-bottomed maxisorb plates. T cells were stimulated with solid-phase anti-CD3 (OKT3); G. Jung, Institute for Cell Biology, Tübingen, Germany) with or without anti-
CD28 (9.3, G. Jung), anti-NKG2D (M585), or control IgG (2 µg/mL). Cultures were pulsed with [methyl-3H]thymidine (1 µCi; Amersham) on day 3 and collected 16 hours later using a cell harvester (Tomtec, Halden, CT). Incorpora-
ted radioactivity was determined in a Wallac 1450 Microbeta Plus Liquid Scintillation Counter.

Cytotoxicity Assay. Cytotoxicity was assessed in 4-hour 51Cr release as-
says in the absence or presence of various mAbs or soluble mNKG2D. The concentrations for the masking experiments were 10 µg/mL for mAbs and 20 µg/mL for soluble mNKG2D. The 51Cr release assay was performed using 2,000 51Cr-labeled targets per well. Effector and target cells were incubated at various effector to target (E:T) ratios for 4 hours. Spontaneous 51Cr release was determined by incubating the target cells with medium alone. Maximum

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release was determined by adding 2% Nonidet P-40. The percentage of \(^{51} \text{Cr}\) release was calculated as follows: 100 × (experimental release − spontaneous release)/maximum release − spontaneous release).

Glioma Spheroids. Multicellular glioma spheroids were obtained by seed-
growing glioma cell transfectants (4 × 10\(^6\) cells per mL) in 96-well plates that were base-
coated with 1.0% Noble Agar (Difco Laboratories, Detroit, MI) before use in cytotoxicity assays.

Matrigel Invasion Assays. Invasion in vitro was measured in Boyden chamber
assays (BD Biosciences, Heidelberg, Germany). Briefly, the glioma cells were harvested in enzyme-free cell dissociation buffer (Gibco Life Technologies, Inc., Karlsruhe, Germany). The cell suspensions (200 μL: 2.5 × 10\(^6\) cells per mL) were added to triplicates to each Matrigel-coated Transwell insert. NIH 3T3-conditioned medium (500 μL) was used as a chemoattractant in the lower wells. After 20 hours of incubation, the cells on the lower side of each membrane were fixed in methanol at 4°C, stained with toluidine blue, and sealed on slides. Photographs of representative microscopic fields were taken at ×200 magnification. Quantification of cell invasion was expressed as the mean count of stained cells in five random fields of each membrane.

Mice and Animal Experiments. Athymic CD1 nude mice were purchased
from Charles River Laboratories (Sulzfeld, Germany). Mice used in all exper-
iments were 6 to 12 weeks of age. The experiments were performed according to the NIH Guide for the Care and Use of Laboratory Animals. Groups of four to six mice received subcutaneous injection in the right flank with transfected LNT-229 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 in diameter. Mice were anesthetized by an intraperitoneal injection of 7% chloral hydrate before all intracranial procedures. For intracranial implantation, the mice were placed in a stereotactic fixation device (Stoelting, Wood Dale, IL). A burr hole was drilled in the skull 2 mm lateral to the bregma. The needle of aHamilton syringe (Hamilton, Darmstadt, Germany) was introduced to a depth of 3 mm. LNT-229 glioma cells (5 × 10\(^6\)) in a volume of 2 μL of PBS were injected into the right striatum. The mice were observed daily and sacrificed when neurologic symptoms developed.

Mouse Lymphocyte Isolation. Murine NK cells were prepared from splenocytes of CD1 nude mice by positive selection using DX5 mAb-coupled
magnetic beads with the corresponding column system (Miltenyi Biotech) before use in cytotoxicity assays.

Statistics. Where indicated, analysis of significance was performed using the two-tailed Student’s t test; \( P < 0.05 \) was considered significant, and \( P < 0.01 \) was considered highly significant (Excel, Microsoft, Seattle, WA).

Evaluation of survival patterns in mice bearing intracerebral gliomas was performed by the Kaplan-Meier method (36). \( P \) values were evaluated by the Mantel log-rank test (37).

RESULTS

Reduced NKG2D Expression on CD8\(^+\) T and Natural Killer
Cells from Glioma Patients and Down-regulation of NKG2D Expression Mediated by Glioma Cell Supernatant. The constitutive presence of MIC at the surface of fresh primary human glioma cells (23) in the apparent absence of relevant tumor immunity suggests that MIC expression on glioma cells or NKG2D expression on immune cells might be functionally impaired in glioma patients. We therefore examined NKG2D expression on CD8\(^+\) T (CD3\(^+\)CD8\(^+\)) and NK (CD3\(^-\)CD56\(^+\)) cells from peripheral blood of glioma patients (\( n = 7 \)) and controls (\( n = 17 \)). NKG2D expression levels were significantly lower in CD8\(^+\) T cells than those of NK cells in untreated, steroid-free glioma patients than those of controls (mean CD8\(^+\): 11.8 versus 21,

\( P = 0.004 \); mean NK: 12 versus 16.5, \( P = 0.02 \); Fig. 1A). To investigate whether the SN of glioma cell cultures down-regulates NKG2D expression levels, freshly isolated and untreated CD8\(^+\) T (CD3\(^+\)CD8\(^+\)) or NK cells (CD3\(^-\)CD56\(^+\)) or NKL cells were incubated with glioma cell SN and then subjected to flow cytometry. LN-308 SN markedly reduced NKG2D expression in a concentration-dependent manner (Fig. 1B and C).

Glioma-Derived Transforming Growth Factor-\( \beta 1 \) and -\( \beta 2 \) Down-Regulate NKG2D Gene Transcription. As part of our efforts to elucidate the mechanism of glioma-induced loss of NKG2D expression on immune cells, we noted that TGF-\( \beta 1 \) and TGF-\( \beta 2 \) down-regulate NKG2D expression in freshly isolated CD8\(^+\) T and NK cells as well as in NKL cells (Fig. 2A–C; refs. 38 and 39). In contrast, interleu-

Fig. 1. Reduced expression of NKG2D on CD8\(^+\) T and NK cells in glioma patients and down-regulation of NKG2D expression mediated by glioma cell SN. A. Freshly isolated PBLs from glioma patients (\( n = 7 \)) or controls (\( n = 17 \)) were examined for NKG2D expression at the cell surface by three-color flow cytometry with gating on CD3\(^+\)CD8\(^+\) T cells or CD56\(^+\) NK cells. B. Freshly isolated PBLs were untreated (filled profiles) or exposed to LN-308 glioma cell SN (1:4, open profiles) for 48 hours and analyzed for NKG2D expression. The SFI values for NKG2D expression are indicated. C. CD8\(^+\) T cells freshly isolated by MACS beads (■) or NKL cells (□) were examined accordingly, using increasing SN concentrations. Data are expressed as individual SFI values. *, \( P < 0.05 \); **, \( P < 0.01 \) (t test).
kin-10 had no such effect (data not shown). The TGF-β-mediated downregulation of NKG2D expression was concentration and time dependent (Fig. 2B). Because glioma cell SN, notably from LN-308 cells, contains large amounts of TGF-β1 and TGF-β2 (7), we next asked whether TGF-β mediated the effects of glioma SN on NKG2D expression. Anti–TGF-β mAb nullified the inhibitory effects of glioma cell SN on NKG2D expression (Fig. 2E), indicating that TGF-β is the principle soluble factor that reduces NKG2D levels at the surface of immune effector cells. Furthermore, real-time PCR analysis showed a reduction of NKG2D mRNA levels in NKL cells at 48 hours after exposure to glioma cell SN, TGF-β1, or TGF-β2 (Fig. 2F). Further evidence for a decisive role of TGF-β was gained from the coincubation of NKL cells with sera or CSF samples from glioma patients. Both body fluids reduced NKG2D expression in NKL cells. The inhibition of NKG2D expression by paired serum (TGF-β1, 40 ng/mL) and CSF (TGF-β2, 2.2 ng/mL) samples from a glioma patient was reversed by pan–anti-TGF-β (Fig. 2G). The restoration of NKG2D expression was incomplete in serum, suggesting the presence of other modulators of NKG2D expression in these samples (4).

Transforming Growth Factor-β Inhibits Natural Killer Cell-Mediated Glioma Cell Killing and T-Cell Costimulation. Next we assessed the functional role of the TGF-β–mediated reduction of NKG2D expression on NK cells by glioma cells. We performed 51 Cr release assays using immune effector cells pretreated with TGF-β.

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the lysis of control-transfected LNT-229 cells but markedly reduced the specific lysis of stable LNT-229.MICA transfectants (ref. 23; Fig. 3A), indicating a specific effect of TGF-β on NGK2D-mediated glioma cell killing. To evaluate the significance of TGF-β for NGK2D-mediated CD8⁺ T-cell costimulation, we stimulated purified human CD8⁺ T cells with anti-CD3 mAb in combination with NGK2D mAb or CD28 mAb in the presence or absence of TGF-β. Whereas anti-CD3 mAb alone induced a moderate T-cell response, significant increases in T-cell proliferation were observed by inclusion of NGK2D mAb. This effect, however, was less potent than that of anti-CD28 mAb. The stimulatory activity of anti-NGK2D was reduced by TGF-β treatment of CD8⁺ T cells, whereas anti-CD28 stimulation was less affected by TGF-β, showing a prominent effect of TGF-β on NGK2D-mediated activation (Fig. 3B).

**Transforming Growth Factor-β Gene Silencing in Glioma Cells: In vitro Phenotype.** Having shown the decisive role of TGF-β in compromising NGK2D-mediated antitumor immune responses, we next pursued a possible therapeutic perspective by using siRNA technology to silence TGF-β1 and TGF-β2 gene expression in LNT-229 glioma cells. TGF-β1 protein was stably reduced by 95% and TGF-β2 protein was stably reduced by 99% with a combined approach using TGF-β1 and TGF-β2 siRNA target sequences (Fig. 4A). TGF-β1 siRNA only targeted TGF-β1 but not TGF-β2, and vice versa (data not shown). Accordingly, reporter gene assays using p3TP-Luc confirmed that intracellular TGF-β signaling was repressed in TGF-β1/2 siRNA cells. This effect was reversed by exogenous TGF-β1. The transcription of TGF-β1 itself was not impaired in the TGF-β1/2 siRNA cells, consistent with posttranscriptional mRNA degradation triggered by siRNA (Fig. 4B). SN of control transfectants downregulated NGK2D expression in NKL cells, whereas the SN of TGF-β1/2 siRNA cells left NGK2D levels unaltered (Fig. 4C). Furthermore, MICA expression was markedly increased on the cell surface of TGF-β1/2 siRNA cells and in cell lysates, and this was again diminished by exogenous TGF-β2. Real-time PCR showed a marked induction of MICA mRNA levels in TGF-β1/2 siRNA cells, whereas TGF-β2 reduced mRNA levels in control and TGF-β1/2 siRNA cells by ~50% (Fig. 4D). The changes in the expression of NGK2D and MICA resulted in the expected increase in immune-mediated lysis of TGF-β1/2 siRNA cells, in an anti-MICA mAb-sensitive manner (Fig. 4E). In addition to the enhanced immunogenicity, TGF-β siRNA cells exhibited an altered intrinsic tumor cell phenotype. Proliferation experiments showed a significant reduction in [³H]thymidine uptake as a measure of proliferation (Fig. 5A). Also, migratory and invasive properties were markedly impaired in TGF-β1/2 siRNA cells (Fig. 5B). This less motile and invasive phenotype might be explained by the suppression of MMP-2 and MMP-9 expression in TGF-β1/2.
Fig. 5. Altered in vitro phenotype of reduced malignancy in TGF-β siRNA cells. A. The growth of control or TGF-β1/2 siRNA cells was assessed by [3H]thymidine incorporation and measured in 96-well plates (5,000 cells per well) after a 16-hour incubation (cpm ± SD). B. The migratory and invasive properties of LNT-229 control and TGF-β1/2 siRNA cells were examined in a glioma spheroid model (top panel) and in Matrigel invasion assays (bottom panel). C. MMP-2 (72 kDa) and MMP-9 (92 kDa) in the SN of control cells or TGF-β1/2 siRNA were assessed by immunoblot, and specific activity was assessed by zymography.

Fig. 6. TGF-β1/2 siRNA LNT-229 cells are nontumorigenic in nude mice and induce NK cell activation in vivo. A. The growth of subcutaneous LNT-229 mock tumors (control) or TGF-β1/2 siRNA tumors was monitored every 2 days. B. LNT-229 control or TGF-β1/2 siRNA cells (5 × 10⁴) were inoculated intracerebrally in CD1 nude mice. Survival data for six animals per group are shown, as evaluated by the Kaplan-Meier method (log-rank test, P < 0.01). C. At day 5, splenocytes were recovered from the differently treated animals. NK cells were isolated and used as effector cells in a ⁵¹Cr release assay using YAC-1 cells as targets.
siRNA cells, resulting in a 64% reduction in MMP activity by zymography (Fig. 5C). No such effect was observed for MT1-MMP (data not shown).

Transforming Growth Factor-β Gene Silencing in Glioma Cells: In vivo Phenotype. To assess whether the impaired immunogenicity and motility of TGF-β1/2–depleted cells resulted in a modulation of their tumorigenicity, we used a subcutaneous and an intracerebral glioma xenograft model. LNT-229 cells were injected subcutaneously into nude mice that possessed NK cells but lacked T cells, and tumor sizes were measured every 2 days. Mock transfectants grew rapidly to form compact tumors, whereas TGF-β1/2 siRNA transfectants did show some tumor growth between days 3 and 7 before the tumors were rejected (Fig. 6A). When LNT-229 cells were implanted stereotactically into the brains of nude mice, animals carrying mock transfectants developed neurologic symptoms and had to be sacrificed between days 34 and 41. In contrast, animals carrying TGF-β1/2 siRNA transfectants showed no neurologic symptoms after 90 days (Fig. 6B; log-rank test, P < 0.01). NK cells isolated from mice inoculated subcutaneously with the TGF-β1/2 siRNA-transfected glioma cells showed a substantially enhanced cytotoxic activity against YAC-1 target cells compared with NK cells from animals receiving mock transfectants (Fig. 6C), suggesting altered NK cell reactivity as a contributing mechanism mediating the antitumorigenic effects of RNA interference against TGF-β.

DISCUSSION

Among solid tumors, glioblastoma is paradigmatic for its immune-inhibitory properties that involve the expression of cell surface molecules such as HLA-G and CD70 as well as the release of soluble molecules such as TGF-β (2, 40, 41). TGF-β has been considered central to the malignant progression of glial tumors and immune dysfunction in human glioblastoma patients (2). Here we delineate a novel therapeutic approach to silence TGF-β gene expression using RNA interference that disrupts the immunosuppressive pathways mediated by TGF-β, specifically the down-regulation of NKG2D expression in CD8+ T and NK cells (Fig. 1A, B and C; ref. 44). Taken together, the significance of the biological effects of TGF-β were corroborated by the observation of a loss of tumorigenicity in vivo and enhanced NK cell activation when TGF-β1 and TGF-β2 gene expression were impaired using siRNA technology (Fig. 6). Because the TGF-β–depleted cells showed an initial proliferation in nude mice up to day 7 after inoculation (Fig. 6A), with subsequent elimination, an immune-mediated attack is likely, although the overall contribution of the intrinsic change in proliferation, migration, and invasiveness to the loss of tumorigenicity remains uncertain.

The general importance for TGF-β as a mediator of impaired antitumor immune surveillance is no longer disputed. The analysis of T cells expressing a dominant negative TGF-β receptor II transgene confirmed an inhibitory role of TGF-β in the generation of antitumor CD8+ T-cell responses (45). Such mechanisms might involve effects of TGF-β on costimulatory signals using NKG2D as the target molecule (Fig. 3B). Of note, the highly lethal nature of glioblastoma suggests that the levels of NKG2D expressed by immune cells or activating NKG2D ligand expressed by glioma cells in the current clinical setting are too low to induce antitumor immunity. Our previous studies had already indicated that the activation potential for immune cells depends on the level of NKG2D ligand expression on glioma cells (23). Furthermore, the inhibitory receptor CD94/NKG2A is induced by TGF-β and may thus potentiate the NK and CD8+ T-cell inhibition by glioma cells (46). TGF-β also reduces the expression of other NK cell activatory receptors (38). Collectively, these observations identify TGF-β as a principle therapeutic target for the biological treatment of glioblastoma and suggest that RNA interference targeting TGF-β in human tumors, including glioblastoma, should be further pursued as a therapeutic strategy.

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