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 subcutaneous 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 (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, γδ 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-NKG2D,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: M585 IgG1 anti-NKG2D (kindly provided by Amgen, Thousand Oaks, CA), BAM01 IgG1 anti-MICA/B, BAM03 IgG1 anti-MICA/B, AM01 IgG1 anti-MICA, BM02 IgG1 anti-MICB (29), HIITA 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 isotype-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 cytotoxicity assays. To obtain purified CD8+ T cells, fresh PBLs were sorted by immunomagnetic CD8 MACS beads (Miltenyi Biotech, Bergisch Gladbach, Germany).

**Cell Lines and Transfectants.** The human SF-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 gliblastoma 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% fetal calf serum (FCS; Biochrom KG), and penicillin (100 IU/ml)/streptomycin (100 µg/ml; 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.), NKLs, 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 mM l-glutamine (Gibco Life Technologies, Inc.), 2 mM L-glutamine (Gibco Life Technologies, Inc.), and 10% FCS. The human malignant glioma cell lines were provided by Dr. N. de Tribolet, Germany).

**Immunoblot.** Protein extracts were electrophoresed on 8% to 12% SDS-PAGE gels under reducing conditions and transferred to nitrocellulose (Schleicher & Schuell, Dassel, Germany). The human malignant glioma cell lines were provided by Dr. N. de Tribolet, Germany).

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release was determined by adding 2% Nonidet P-40. The percentage of 51Cr release was calculated as follows: 100 × ([experimental release – spontaneous release]/[maximum release – spontaneous release]).

Glioma Spheroids. Multicellular glioma spheroids were obtained by seeding glioma cell transfectants (4 × 10⁴ cells per mL) in 96-well plates that were base-coated with 1.0% Noble Agar (Difco Laboratories, Detroit, MI) prepared in Dulbecco’s modified Eagle’s medium and culturing for 4–5 days until spheroids had formed. The extracellular matrix gel was prepared by mixing collagen I solution (Vitrogen 100; Cohesion, Palo Alto, CA) and minimal essential medium at a 8:1 ratio at 4°C, supplementing with fibronectin (10 μg/mL), and adjusting the pH by the addition of NaOH/NaHCO₃. This solution (400 μL) was added into 24-well plates, and spheroids of defined size were implanted into the gel. After gelation at 37°C, the gel was overlaid with 400 μL of complete medium. Photographs were taken after 0, 24, 48, 72, and 96 hours. The mean radial distance of 10 randomly selected glioma cells that had migrated from the tumor spheroid into the gel matrix was measured every 24 hours and expressed in relation to the mean radial distance at 0 hours.

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 × 10⁴ cells per mL) were added in 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 experiments 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 glioma cells (5 × 10⁴ cells per mL) in 96-well plates that were base-coated with 1.0% Noble Agar (Difco Laboratories, Detroit, MI) prepared in Dulbecco’s modified Eagle’s medium and culturing for 4–5 days until tumors had formed. The extracellular matrix gel was prepared by mixing collagen I solution (Vitrogen 100; Cohesion, Palo Alto, CA) and minimal essential medium at a 8:1 ratio at 4°C, supplementing with fibronectin (10 μg/mL), and adjusting the pH by the addition of NaOH/NaHCO₃. This solution (400 μL) was added into 24-well plates, and spheroids of defined size were implanted into the gel. After gelation at 37°C, the gel was overlaid with 400 μL of complete medium. Photographs were taken after 0, 24, 48, 72, and 96 hours. The mean radial distance of 10 randomly selected glioma cells that had migrated from the tumor spheroid into the gel matrix was measured every 24 hours and expressed in relation to the mean radial distance at 0 hours.

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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 NKGD2 Expression on CD8⁺ T and Natural Killer Cells from Glioma Patients and Down-regulation of NKGD2 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 expressed on glioma cells or NKGD2 expressed on immune cells might be functionally impaired in glioma patients. We therefore examined NKGD2 expression on CD8⁺ T (CD3⁺CD8⁺) and NK (CD3⁻CD56⁺) cells from peripheral blood of glioma patients (n = 7) and controls (n = 17). NKGD2 expression levels were significantly lower in CD8⁺ T cells and NK cells of 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 NKGD2 expression levels, freshly isolated and untreated CD8⁺ T (CD3⁺CD8⁺) or NK cells (CD3⁻CD56⁺) were incubated with glioma cell SN and then subjected to flow cytometry. LN-308 SN markedly reduced NKGD2 expression in a concentration-dependent manner (Fig. 1B and C).

Glioma-Derived Transforming Growth Factor-β1 and -β2 Down-Regulate NKGD2 Gene Transcription. As part of our efforts to elucidate the mechanism of glioma-induced loss of NKGD2 expression on immune cells, we noted that TGF-β1 and TGF-β2 down-regulated NKGD2 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-

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. Exogenous TGF-β treatment of the NK effector cells had no effect on NK cell-mediated glioma cell killing and inhibits NKG2D-mediated T-cell costimulation. A NKL cells untreated or pretreated with TGF-β (10 ng/mL) were used in a standard 4-hour 51 Cr release assay, using LNT-229.MICA stable transfectants or mock transfectants (LNT-229.neo) as target cells. Data are expressed as specific lysis at different E:T ratios. B Purified CD8+ T cells were cultured with precoated CD3 mAb (OKT3) and immobilized control IgG, NKG2D mAb, or CD28 mAb (2 μg/mL) in the absence or presence of TGF-β (10 ng/mL) for 96 hours. Cultures were pulsed with [methyl-3H]thymidine for the last 16 hours. Data represent mean ± SD and are expressed in cpm.

Fig. 2. TGF-β inhibits NKG2D expression. A. freshly isolated PBLs were untreated (filled profiles) or treated with TGF-β1 or TGF-β2 (10 ng/mL) for 48 hours (open profiles) and subjected to flow cytometry for NKG2D expression on gated CD8+ T or NK cells. The SFI values for NKG2D expression are indicated. B–D. Freshly isolated CD8+ T cells (B) or NKL cells (C) were treated with TGF-β1 (③) or TGF-β2 (④) at increasing concentrations for 48 hours or (D) increasing lengths of time (NKL) at 10 ng/mL. E. NKL cells were incubated with LN-308 SN in the presence of control IgG or anti–TGF-β mAb (10 μg/mL). F. NKG2D mRNA expression was assessed in NKL cells exposed to LN-308 glioma SN (1:4) or TGF-β1 and TGF-β2 (10 ng/mL) for 48 hours. G. Diluted serum (1:10) or CSF (1:4) from a glioma patient was added to NKL cells for 48 hours in the presence of control IgG or anti–TGF-β (10 μg/mL). **, P < 0.01; *, P < 0.05; t test.

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Fig. 3. TGF-β impairs NK cell-mediated glioma cell killing and inhibits NKG2D-mediated T-cell costimulation. A. NKL cells untreated or pretreated with TGF-β (10 ng/mL) were used in a standard 4-hour 51 Cr release assay, using LNT-229.MICA stable transfectants or mock transfectants (LNT-229.neo) as target cells. Data are expressed as specific lysis at different E:T ratios. B. Purified CD8+ T cells were cultured with precoated CD3 mAb (OKT3) and immobilized control IgG, NKG2D mAb, or CD28 mAb (2 μg/mL) in the absence or presence of TGF-β (10 ng/mL) for 96 hours. Cultures were pulsed with [methyl-3H]thymidine for the last 16 hours. Data represent mean ± SD and are expressed in cpm.

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The release of TGF-β1 and TGF-β2 in SN of pSUPER-puro-TGF-β1 and pSUPER-puro-TGF-β2 stably transfected LNT-229 cells (TGF-β1 siRNA) or mock transfectants (control) was monitored by immunoblot. B, Intracellular TGF-β signaling was assessed in untreated or TGF-β1 (5 ng/mL, 16 hours)-treated control cells (C) or TGF-β1 siRNA cells (D). Luminescence counts for pGL2-3TP-Luc and pGL3b-TGF-β1-Luc were divided by the counts obtained from cotransfected pRL-CMV. Their ratio is given as relative luciferase activity (mean ± SD; t test, **P < 0.01). C, NKL cells were incubated with glioma cell SN (1:1) of control or TGF-β1 siRNA cells for 48 hours and analyzed for MICA expression by flow cytometry. D, MICA expression at the cell surface of control or TGF-β1 siRNA cells untreated or treated with TGF-β2 (10 ng/mL) for 7 days was analyzed by flow cytometry. SFI values are indicated in the top right corner. The expression of MICA in cell lysates of control or TGF-β1 siRNA cells was monitored by immunoblot. An actin control was included to verify equal amounts of loaded protein for the cell lysates. MICA mRNA expression was assessed in control or TGF-β1 siRNA cells untreated or treated with TGF-β2 (10 ng/mL) for 48 hours by real-time PCR. E, Control or TGF-β1 siRNA cells untreated or treated with anti-MICA (BAMO1; 10 μg/mL) were used as target cells in a standard 4-hour 51Cr release assay using NKL cells as effectors. Data are expressed as specific lysis at different E:T ratios.

Fig. 4. Altered in vitro phenotype of enhanced immunogenicity in TGF-β siRNA cells. A. The release of TGF-β1 and TGF-β2 in SN of pSUPER-puro-TGF-β1 and pSUPER-puro-TGF-β2 stably transfected LNT-229 cells (TGF-β1 siRNA) or mock transfectants (control) was monitored by immunoblot. B, Intracellular TGF-β signaling was assessed in untreated or TGF-β1 (5 ng/mL, 16 hours)-treated control cells (C) or TGF-β1 siRNA cells (D). Luminescence counts for pGL2-3TP-Luc and pGL3b-TGF-β1-Luc were divided by the counts obtained from cotransfected pRL-CMV. Their ratio is given as relative luciferase activity (mean ± SD; t test, **P < 0.01). C, NKL cells were incubated with glioma cell SN (1:1) of control or TGF-β1 siRNA cells for 48 hours and analyzed for MICA expression by flow cytometry. D, MICA expression at the cell surface of control or TGF-β1 siRNA cells untreated or treated with TGF-β2 (10 ng/mL) for 7 days was analyzed by flow cytometry. SFI values are indicated in the top right corner. The expression of MICA in cell lysates of control or TGF-β1 siRNA cells was monitored by immunoblot. An actin control was included to verify equal amounts of loaded protein for the cell lysates. MICA mRNA expression was assessed in control or TGF-β1 siRNA cells untreated or treated with TGF-β2 (10 ng/mL) for 48 hours by real-time PCR. E, Control or TGF-β1 siRNA cells untreated or treated with anti-MICA (BAMO1; 10 μg/mL) were used as target cells in a standard 4-hour 51Cr release assay using NKL cells as effectors. Data are expressed as specific lysis at different E:T ratios.
Fig. 5. Altered \textit{in vitro} phenotype of reduced malignancy in TGF-\beta siRNA cells. \textbf{A}. The growth of control or TGF-\beta/1/2 siRNA cells was assessed by \[^{\text{3}}\text{H}\text{] thymidine incorporation and measured in 96-well plates (5,000 cells per well) after a 16-hour incubation (cpm \pm SD).} \textbf{B}. The migratory and invasive properties of LNT-229 control and TGF-\beta/1/2 siRNA cells were examined in a glioma spheroid model (top panel) and in Matrigel invasion assays (bottom panel). \textbf{C}. MMP-2 (72 kDa) and MMP-9 (92 kDa) in the SN of control cells or TGF-\beta/1/2 siRNA were assessed by immunoblot, and specific activity was assessed by zymography.

Fig. 6. TGF-\beta/1/2 siRNA LNT-229 cells are nontumorigenic in nude mice and induce NK cell activation \textit{in vivo}. \textbf{A}. The growth of subcutaneous LNT-229 mock tumors (control) or TGF-\beta/1/2 siRNA tumors was monitored every 2 days. \textbf{B}. LNT-229 control or TGF-\beta/1/2 siRNA cells (5 \times 10^4) 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, \( P < 0.01 \)). \textbf{C}. At day 5, splenocytes were recovered from the differently treated animals. NK cells were isolated and used as effector cells in a \(^{\text{51}}\text{Cr 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 and the down-regulation of MICA expression in glioma cells. By promoting an up-regulation of NKG2D on immune cells paralleled by an increase of cell surface MICA expression through disinhibited transcription, glioma cells can be recognized efficiently by innate immune recognition via induced self-danger signals (28, 42). Moreover, TGF-β regulates the intrinsic malignant phenotype of glioma cells by enhancing proliferation, migration, and invasiveness, and these features of malignancy are also lost after TGF-β silencing.

We have shown previously that freshly isolated primary glioma cells exhibit low levels of NKG2D ligand expression (23). These data, the observation of reduced NKG2D expression in peripheral blood CD8+ T and NK cells (Fig. 1A), and the increase in TGF-β levels in sera and CSF of human glioblastoma patients (8, 9) all suggest that TGF-β might compromise NKG2D-mediated immune surveillance in patients with malignant gliomas. We showed that recombinant TGF-β mimicks the effects of glioma cell SN on NKG2D expression and that TGF-β was the principle molecule within the glioma cell SN that mediates the loss of NKG2D in immune cells (Figs. 1B and C and 2A–E). Real-time PCR indicated that the reduction of NKG2D mediated by TGF-β involved NKG2D gene transcription (Fig. 2F). It has been reported that systemic immune deficiency in cancer patients can be associated with circulating tumor-derived soluble MICA, which is released by tumor cells at high levels into the serum and binds to cell surface NKG2D, causing impairment of the responsiveness of tumor antigen-specific effector T cells (43). Although primary glioma cells and long-term glioma cell lines released soluble MICA into the cell culture SN, the soluble MICA levels in patient sera or CSF were below the detection limit of our enzyme-linked immunosorbent assay, and we failed to confirm that soluble MICA released by glioma cells down-regulates NKG2D (data not shown).

The disruption of the MICA/NKG2D recognition system by TGF-β not only involves the loss of NKG2D expression in effector cells, mediated in a paracrine fashion, but also involves an autocrine effect of TGF-β on the expression of the cognate ligand, MICA, on glioma cells. This was disclosed by TGF-β gene silencing, which resulted in a strong increase in MICA expression at the cell surface (Fig. 4D). Moreover, SN of TGF-β1/2 siRNA transfectants did not down-regulate NKG2D in immune effector cells (Fig. 4C), supporting the key role of TGF-β in down-regulating NKG2D (Fig. 1A). Consequently, reducing TGF-β bioavailability is a suitable means to enable the immune cell-mediated lysis of glioma cells (Fig. 4E). These antitumor effects might be further enhanced by a decrease in proliferation, migration, and invasiveness of glioma cells after silencing of TGF-β (Fig. 5). The autocrine regulation of MMP-2 and MMP-9 expression by TGF-β is most likely the cause for the inhibition of invasion and migration (Fig. 5B 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 invasion 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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