Malignant Glioma Cells Counteract Antitumor Immune Responses through Expression of Lectin-Like Transcript-1

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Abstract
Glioblastoma, one of the most lethal tumors, is paradigmatic for tumor-associated immunosuppression. Lectin-like transcript-1 (LLT1) is a newly identified ligand for the inhibitory natural killer (NK) cell receptor CD161. Here, we report that glioma cells express LLT1 mRNA and protein in vitro and in vivo, whereas expression levels in normal brain are low. LLT1 expression in human gliomas increases with the WHO grade of malignancy. We further show that transforming growth factor-β (TGF-β) up-regulates the expression of LLT1 in glioma cells. Small interfering RNA (siRNA)-mediated down-regulation of LLT1 in LNT-229 and LN-248 cells promotes their lysis by NK cells. Thus, LLT1 acts as a mediator of immune escape and contributes to the immunosuppressive properties of glialoma cells.[Cancer Res 2007;67(8):3540–4]

Introduction
Glioblastoma is the most malignant intrinsic brain tumor. Glioma cells express many molecules that interact with cells of the innate and adaptive immune system. However, the activatory natural killer (NK) and costimulatory T-cell ligands that are expressed by glioma cells are overridden by numerous inhibitory signals, including the master immunosuppressive molecule transforming growth factor-β (TGF-β) (1–3). Glioma cells show high surface expression of classic MHC class I molecules and a significant expression of the nonclassic MHC molecules HLA-E and HLA-G (4, 5). These ligands contribute to the immune escape phenotype of gliomas by interacting with different NK cell receptors (6). We recently described regeneration and tolerance factor as another molecule that may mediate immune escape of gliomas by inhibition of NK and T cells (7).

The administration of drugs directed against defined molecular targets such as the TGF-β receptor has yielded promising results in experimental glioma models (8). The identification of further mechanisms of tumor-induced immune inhibition is therefore critical to find potential targets for an effective antitumor immune therapy.

Lectin-like transcript-1 (LLT1; alternative name, CLEC2D) is similar to the mouse Clr molecules and is expressed on cells of lymphocytic origin (9, 10). LLT1 is a ligand for CD161 (alternative name, NKR-P1A; ref. 11, 12). Human CD161 is a C-type lectin receptor expressed on most human NK cells, CD1c-restricted NKT cells and a small subset of T cells (13–15). The lytic activity of NK cells is negatively regulated via CD161 expression mediated by IL-12 (16, 17). Expression and functional activity of LLT1 in tumor cells have not been examined. Here, we show that LLT1, expressed by gliomas, contributes to tumor-associated immunosuppression by affecting the lytic activity of NK cells.

Materials and Methods

Cells and reagents. The human glioma cell lines were provided by Dr. N. de Tribolet (Lausanne, Switzerland; ref. 18). All primary glioblastoma cells were established from freshly resected tumors and used during the first passages. The cells were maintained in DMEM containing 10% FCS (Biochrom KG, Berlin, Germany) and penicillin (100 IU/mL)/streptomycin (100 μg/mL; Life Technologies, Karlsruhe, Germany). Cell culture supernatants were concentrated with the Centriplus centrifugal filter device YM-3 (Millipore, Eschborn, Germany). For transient transfections, 2 × 106 glioma cells were seeded in a six-well plate and transfected with 20 nmol/L of either LLT1 small interfering RNA (siRNA1): 5′-UUAAGGGCUCAUAAUGAAUGUCUC-3′ and 5′-GAGCAUUCAUAUAAAGCUCUUCUUC-3′; LLT1 siRNA2: 5′-UUCAGAUGGUGUUGUCUUCCUUC-3′ and 5′-GGAGAAGACAUAAACACCAUGUA-3′, or irrelevant GL3 control siRNA: 5′-CUUAGGGCUUGAUCAUGUGUGAAG(dTdT)-3′ and 5′-UCAGAAGAUCACCCUGUAAG(dTdT)-3′, using TransIT-TKO Transfection Reagent (Mirus, Madison, WI). The generation of stable LNT-229 TGF-β1/2 siRNA cells has been described (3). Anti-LLT1 antibody (clone 4C7) was purchased from Abnova (Taipei City, Taiwan), immunoglobulin G1 (IgG1) isotype-matched antibody from BD PharMingen (Heidelberg, Germany). Biotinylated rabbit anti-mouse antibody was from DAKO (Hamburg, Germany), streptavidin-APC was from BD PharMingen, FITC-labeled anti-CD161 was from BD PharMingen, FITC-labeled anti-CD161 was from Serotec (Düsseldorf, Germany), anti-human CD3-APC, CD56-PE, and isotype controls were from Immunotools (Friesoythe, Germany), and goat anti-mammalian β-actin (I-19) was from Santa Cruz Biotechnology (Santa Cruz, CA). Becombinant human TGF-β was purchased from R&D Systems (Wiesbaden, Germany). The TGF-β receptor I kinase inhibitor SD-208 was kindly provided by Scios Inc. (Fremont, CA).

Real-time PCR. Total RNA was prepared using the RNeasy system (Qiagen, Hilden, Germany) and transcribed according to standard protocols. For real-time PCR, cDNA amplification was monitored using SYBRGreen chemistry on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany). The conditions were 40 cycles, 95°C/15 s, 60°C/1 min, using the following specific primers for 18S: 5′-CGGCTAC-CACATCCAAAGGAA-3′ and 5′-GCTGGAATTCACGGCTGCT-3′; for LLT1: 5′-TGGCCAGAAGCTGGATGTG-3′ and 5′-AACTTGAGCAAGATGAGCATC-3′. For data analysis, threshold cycles (Ct) for 18S (reference) and LLT1 (sample) were determined in duplicates. We chose cDNA of non-neoplastic SV40-FHAS cells or normal brain tissue as a calibrator tissue (100%) and determined the relative change (rI) in copy numbers according to the formula rI = 2(ΔΔCt) (ΔΔCt = (Ct 18S calibrator tissue – Ct 18S glioma tissue) – (Ct LLT1 glioma – Ct LLT1 18S glioma)).

Immunoblot. Cellular soluble proteins (30 μg per lane) were separated on 12% acrylamide gels (Bio-Rad, Munich, Germany). After transfer to nitrocellulose (Bio-Rad), the blots were blocked in PBS containing 5% skim milk and 0.05% Tween 20 and incubated with antibodies to LLT1 or β-actin.

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Visualization of protein bands was accomplished using horseradish peroxidase (HRP)-coupled secondary antibodies (Sigma, Deisenhofen, Germany) and enhanced chemiluminescence (Amersham, Braunschweig, Germany).

Figure 1. Human malignant glioma cells express LLT1 in vitro. A, LLT1 mRNA expression was assessed by quantitative reverse transcription-PCR. B, LLT1 protein levels in whole cell lysates were assessed by immunoblot using β-actin as a reference. C, LLT1 protein levels exposed at the cell surface were measured by flow cytometry. Thick line, LLT1; dotted line, isotype control. D, SFI values for primary glioblastoma cell cultures.

Figure 2. Human gliomas express LLT1 in vivo. A, paraffin-embedded tissue sections of normal human brain gray and white matter, gliomas of different grades of malignancy, and human plasmacytoma were stained with mAb 4C7 or isotype control antibody. While the IgG control did not result in any labeling on tissue sections (insets), both membrane and cytoplasmic staining patterns were observed in WHO grade II (middle right), III (bottom left) and IV (bottom right) gliomas. Only low LLT1 immunoreactivity was detected in the gray (top right) and white (middle left) matter of normal human brain, whereas human plasmacytoma, used as a positive control, showed intense labeling (top left). Original magnification, ×400. B, LLT1 expression levels in normal human brain gray and white matter and gliomas of different grades of malignancy were quantified. Scoring was based on the percentage of positive cells (see Materials and Methods). C, LLT1 mRNA expression levels of fresh glioblastoma tissue were analyzed by real-time PCR (one representative of three independent experiments is shown).
Flow cytometry. Approximately $10^6$ glioma cells were detached using Accutase (PAA, Vienna, Austria), blocked with 2% FCS in PBS, and incubated for 30 min on ice using anti-LLT1 monoclonal antibody (mAb) or matched isotype control antibody (5 μg/mL). This was followed by a biotinylated rabbit anti-mouse antibody and streptavidin-APC. Fluorescence was detected in a CyanADP flow cytometer (DakoCytomation, Hamburg, Germany). Specific fluorescence indexes (SFI) were calculated by dividing the mean fluorescence obtained with the specific antibody by the mean fluorescence obtained with the control antibody.

Immunohistochemistry. A total of 166 brain tumors, including 36 WHO grade II, 51 grade III astrocytomas, and 79 glioblastomas (WHO grade IV) were examined. Normal human brain gray and white matter was used as control tissue. LLT1 immunohistochemistry was done on formalin-fixed and paraffin-embedded samples using the Benchmark immunohistochemistry system (Ventana, Strasbourg, France). Endogenous peroxidase was blocked with 3% H$_2$O$_2$ in methanol for 14 min. Anti-LLT1 antibody (1:20) or isotype control antibody was applied for 32 min. Because LLT1 is highly expressed on activated lymphoid cells, we used plasmacytoma tissues as a positive control (19). Blockers for avidin and biotin were applied followed by an incubation with I-View-Biotin Ig (Ventana). For 3,3'-diaminobenzidine (DAB) visualization, the sections were incubated with I-View HRP-conjugated streptavidine for 8 min and then with DAB/H$_2$O$_2$ for 8 min. The sections were incubated with a copper enhancer, washed, counterstained with hematoxylin, and mounted. Expression levels were quantitated by two investigators, taking into account the percentage of positive cells. On the chosen scale, 0 signifies the absence of a staining, 1 corresponds to single positive tumor cells in a focal pattern, 2 denotes positive tumor cells in a diffuse pattern, 3 indicates up to 20% of positive tumor cells, 4 was allotted when the percentage was between 20% and 50%, 5 was given for >50% of positive tumor cells.

Purification of peripheral blood lymphocytes and isolation of NK cells. Peripheral blood lymphocytes (PBL) were obtained by density gradient centrifugation (Biocoll; Biochrom). They were maintained in medium supplemented with recombinant human TGF-$\beta_2$ (10 ng/mL) or glioma cell supernatants for 48 h as indicated and analyzed for CD3, CD56, and CD161 expression by flow cytometry. For the generation of polyclonal NK cells, PBL depleted of monocytic cells by adherence were cultured on irradiated (30-Gy) RPMI 8866 feeder cells. Cytotoxicity was assessed in 4 h $^{51}$Cr release assays with $10^5$ $^{51}$Cr-labeled targets per well at various effector/target (E:T) ratios. Spontaneous $^{51}$Cr release was determined by incubating the target cells with medium alone. To obtain the maximum $^{51}$Cr release, NP40 (2%) was added. After coincubation for 4 h, supernatant was transferred to a Luma-Plate 96 (Packard, Dreieich, Germany), dried, and measured. The percentage of $^{51}$Cr release was calculated as follows: 100 × [(experimental release – spontaneous release) / (maximum release – spontaneous release)].

Statistics. Where indicated, significance was analyzed using the two-tailed Student’s $t$ test. For the analysis of the immunohistochemical score values, we used ANOVA. For gliomas and for the comparison of gray and white matter, matched-pairs analysis was done using the one-sample $t$ test. The score means are given together with their 95% confidence intervals (95% CI). For adjustment of the $P$ values due to multiple testing, we used the method of Bonferroni-Holm. JMP IN was used for statistical analysis ($*$, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Results

Human malignant glioma cells express LLT1 in vitro and in vivo. Real-time PCR showed elevated LLT1 mRNA levels in all examined glioma cell lines compared with cDNA of the SV40-transformed non-neoplastic astrocytic cell line SV-FHAS. Highest mRNA levels were found in U87MG, LNT-229, and LN-308 cells. Conversely, LLT1 expression was rather low in D247MG and T98G cells, but still higher than in control cells (Fig. 1A). Immunoblot analysis allowed the detection of LLT1 protein in almost all glioma cell lines. Similar to the mRNA data, LLT1 protein levels were lowest in D247MG and T98G cells, but still higher than in control cells (Fig. 1A). Immunoblot analysis allowed the detection of LLT1 protein in all 12 glioma cell lines. Similar to the mRNA data, LLT1 protein levels were lowest in SV-40-FHAS cells (Fig. 1B). Flow cytometry revealed cell surface expression of LLT1 in most glioma cell lines, with absent to low expression in A172, U373MG, and SV40-FHAS cells. Highest mRNA levels were found in U87MG, LNT-229, and LN-308 cells. Conversely, LLT1 expression was rather low in D247MG and T98G cells, but still higher than in control cells (Fig. 1B). Flow cytometry revealed cell surface expression of LLT1 in most glioma cell lines, with absent to low expression in A172, U373MG, and SV40-FHAS cells. LN-308 and LNT-229 cells showed the highest LLT1 levels (Fig. 1B). LLT1 expression was also found on the surface of all primary glioma cell cultures investigated (Fig. 1D).
To assess the expression of LLT1 in vitro, gliomas of different WHO grades were examined (Fig. 2A and B). In human gray and white matter tissue specimens, LLT1 expression was almost restricted to small bipolar elongated cells with thin processes, most likely microglial cells (arrows). In gliomas, cytoplasmic and cell surface staining was observed. LLT1 protein levels were homogeneous within one tumor entity and increased with malignancy from WHO grades 2 to 4. The statistical evaluation of LLT1 levels in normal brain and gliomas of different grade of malignancy is summarized in Fig. 2B. Real-time PCR revealed that LLT1 mRNA expression was elevated in fresh glioblastoma biopsies when compared with normal human brain (Fig. 2C).

LLT1, but not its receptor CD161, is regulated by TGF-β in vitro. Next, we assessed a possible effect of TGF-β on the expression of CD161 on NK and T cells. Freshly isolated PBL were incubated with medium alone, TGF-β2 (10 ng/mL), or a mixture of fresh medium and glioma cell supernatants (1:1). Cells were stained after 48 h with CD3, CD56, and CD161 antibodies to examine CD161 expression on NK and T cells. No consistent shifts in CD161 expression were seen either on NK (Fig. 3A) or T cells (data not shown) exposed to TGF-β or glioma cell supernatants. We further hypothesized that TGF-β might influence the expression of LLT1 on glioblastoma cells. siRNA technology was used to stably down-regulate TGF-β1 and TGF-β2 expression in LNT-229 glioma cells (3). LNT-229 stTGF-β1/2 cells showed a markedly decreased surface expression of LLT1. A similar decreased expression of LLT1 was seen when LNT-229 cells were treated for 72 h with the TGF-β receptor I kinase inhibitor SD-208 (Fig. 3B).

siRNA-mediated down-regulation of LLT1 enhances NK cell-mediated lysis of glioma cells. To assess a functional role of LLT1, RNA interference was used to suppress LLT1 expression in LNT-229 and LN-428 cells. Two LLT1-specific target sequences were used. After 72 h, LLT1 cell surface expression was reduced by ~60% to 80% in LNT-229 and LN-428 glioma cells when compared with cells transfected with an irrelevant scrambled sequence (Fig. 3C). On a functional level, LNT-229 siLLT1 and LN-428 siLLT1 glioma cells were significantly more susceptible to NK cell lysis than control cells (Fig. 4C and D).

Discussion

The ability of glioma cells to suppress the initiation of an effective antitumor immune response is mediated by various factors. Here, we characterize the expression and functional activity of a novel candidate molecule that could be a target for further therapeutic strategies. We report the expression of LLT1 by glioma cells in vitro, using real-time PCR, immunoblot, and flow cytometry (Fig. 1). The fact that LLT1 cell surface expression determined by flow cytometry did not exactly parallel the LLT1 levels found in whole cell lysates might be due to post-transcriptional regulation by cleavage from the cell surface and subsequent degradation. Surface expression of LLT1 was also detectable on primary glioblastoma cells. More importantly, LLT1 expression in vivo, assessed by immunohistochemistry, was detectable in the majority of the glioma specimens examined (Fig. 2). The hypothesis that the immunosuppressive capacities of glomas increase with malignancy is supported by our finding that the expression of LLT1 in human brain tumors increases with WHO grade of malignancy. However, no significant statistical differences were observed between WHO grades III and IV tumors. Overall, the expression of LLT1 may support a selection process favoring the survival of less immunogenic tumor cells.

Our study further confirms the hypothesis that TGF-β is a key player among the immune escape mechanisms in glioblastoma. A knockdown of TGF-β in the glioma cell line LNT-229 led to a down-regulation of cell surface LLT1 (Fig. 3B). This effect was also observed after exposure to the TGF-β receptor I kinase inhibitor SD-208 (Fig. 3B). In contrast, TGF-β did not affect the expression of the LLT1 receptor CD161 on NK or T cells (Fig. 3A). These findings are supported by the observation that the CD161 expression on NK cells of glioblastoma patients does not differ from that of healthy control donors (data not shown). Using two different LLT1-specific siRNA-sequences, we show that LLT1 expressed on glioma cells suppresses NK cell-mediated target cell lysis (Fig. 4), most likely through the interactions with its receptor CD161 (12). The relevance of LLT1/CD161 interactions for the inhibition of T cell–mediated antitumor responses is less clear because only a small population of T cells expresses CD161 (11) and because CD8+ T cells with high expression of CD161 are thought to be anergic.
However, a very small population of T cells with intermediate expression of CD161 can secrete IFN-γ (15), but it remains unclear whether these cells play a decisive role in the initiation or maintenance of tumor-directed T cell response. Nevertheless, the reported defects in the antigen-processing machinery of glioma cells (20) might preclude effective T cell responses, thus emphasizing the critical importance of NK cells for glioma immunosurveillance. Of note, treatment strategies that aim at the inhibition of TGF-β signaling in glioma gain further support from the finding that LLT1 is, at least partially, regulated in a TGF-β-dependent manner. The fact that LLT1 expression can be found on primary glioma cell cultures as well as on glioma tissue specimens suggests that this inhibitory pathway might be of considerable significance in vivo and might become a therapeutic target in the future.

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