GDF-15 Contributes to Proliferation and Immune Escape of Malignant Gliomas

Patrick Roth¹,², Markus Junker³, Isabel Tritschler², Michel Mittelbronn⁴, Yvonne Dombrowski⁵, Samuel N. Breit⁵, Ghazaleh Tabatabai¹,², Wolfgang Wick²,⁶, Michael Weller¹,², and Jörg Wischhusen²,³

Abstract

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**Experimental Design:** The expression of GDF-15 in glioma cell lines was assessed by quantitative reverse transcriptase-PCR and immunoblot. GDF-15 levels in situ and in the peripheral blood of glioma patients were examined by immunohistochemistry and enzyme-linked immunosorbent assay, respectively. The effects of short hairpin RNA-mediated GDF-15 inhibition on proliferation and immunogenicity of SMA-560 glioma cells were investigated by [methyl-³H]thymidine incorporation and immune-mediated target cell lysis. The impact of GDF-15 on glioma growth in vivo was assessed in syngeneic mice.

**Results:** GDF-15 is expressed by gliomas of different WHO grades as assessed by immunohistochemistry. The high expression of GDF-15 in tumor tissue translates into elevated GDF-15 serum levels in glioblastoma patients compared with healthy controls. GDF-15 mRNA and protein are also detectable in human and mouse glioma cells in vitro. Silencing of GDF-15 by RNA interference reduces the proliferation of malignant glioma cells. Immunologically, the depletion of glioma-derived GDF-15 enhances the susceptibility of mouse glioma cells towards syngeneic natural killer cells and splenocytes. This results in a reduced in vivo tumorigenicity and increased T-cell infiltration of GDF-15–deficient glioma cells in syngeneic mice.

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(GDF)-15 (11) in human and murine glioma cells. GDF-15, also known as macrophage inhibitory cytokine 1 (MIC-1; ref. 12), placental TGF-β (PTGF-β; ref. 13), placental bone morphogenetic protein (PLAB; ref. 14), prostate-derived factor (PDF; ref. 15), nonsteroidal anti-inflammatory drug-activated gene (NAG-1; ref. 16), and PL74 (17), is a divergent member of the TGF-β superfamily that displays similarity with both bone morphogenetic proteins (31-35% homology) and classical TGF-β isoforms (~25% homology). It is synthesized as proprotein (279 amino acids) that is secreted and proteolytically processed to yield the mature homodimeric cytokine (2 × 112 amino acids). Intriguingly, the cleaved prodomain can bind to the extracellular matrix (18). Thus, both the prodomain and the mature protein could exert biological functions.

Under physiologic conditions, low to moderate levels of GDF-15 are expressed in most healthy tissues, including brain (11, 19), liver, breast, colon (20), and bone marrow. However, much higher levels of GDF-15 are found in placenta (21), where it may help to prevent miscarriage (22). Additionally, GDF-15 overexpression has been described in numerous malignancies, including breast, colorectal, pancreatic, and prostate cancer (23, 24). In melanoma, higher GDF-15 expression is seen in metastasis tissue than in primary tumor (25). Likewise, glioma patients with high cerebrospinal fluid GDF-15 levels show shorter survival (26).

Functionally, GDF-15 may exert very heterogeneous functions in tumors. It protects prostate cancer cells against the cytotoxic effects of docetaxel and mitoxantrone (27). However, it can also act as a downstream mediator against the cytotoxic effects of docetaxel and mitoxantrone (28). A tumor suppressor function of GDF-15 became evident when C57BL/6J-Apc+/− mice were crossed with mice that expressed a GDF-15 transgene. In this spontaneous model for intestinal neoplasia, GDF-15 overexpression reduced the formation of adenomas and polyps by about 60% (29). Likewise, in a nude mouse glioma model, GDF-15–transfected LN-Z308 cells lost their tumorigenicity (30). In contrast, GDF-15–overexpressing DU145 prostate cancer cells grew normally in nude mice. Interestingly, the high levels of GDF-15 were linked to tumor-related cachexia in these animals, which correlates with clinical data from prostate cancer patients (31). Thus, the effect of GDF-15 on tumor growth seems to depend on the tumor stage and type. One major problem with respect to the cited studies is that they all investigated effects caused by artificial overexpression of GDF-15, which may not be observed at endogenous GDF-15 levels. Moreover, the potential impact of GDF-15 on antitumor immunity has not been addressed so far. Thus, we set out to investigate whether endogenous GDF-15 levels affect glioma growth in vitro and in vivo in the presence of a fully functional murine immune system.

Materials and Methods

Cells and reagents

All human glioma cell lines were kindly provided by Dr. N. de Tribolet (Lausanne, Switzerland) and have been characterized previously (32, 33). Primary glioblastoma cells were established from freshly resected tumor tissue, cultured in monolayers, and used between passages 4 and 9. SMA-560 glioma cells were obtained from D. D. Bigner (Duke University Medical Center, Durham, NC). The cells were maintained in DMEM containing 10% FCS (Biochrom KG) and penicillin (100 IU/mL)/streptomycin (100 μg/mL; Gibco). The pSUPERpuro constructs targeting nucleotides 722 to 740 of murine GDF-15 NM_011819.1 were cloned as previously described (33). For the generation of stable transfectants expressing short hairpin RNA against GDF-15, pSUPERpuro control or shGDF-15 plasmids were introduced using FuGene6 transfection reagent (Roche). The cells were selected in medium containing 2 μg/mL puromycin (Sigma). Anti-GDF-15 antibody was prepared as described (12, 34).

Quantitative reverse transcriptase-PCR

Total RNA was prepared using the RNeasy system (Quiagen) 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). The conditions for these PCR reactions were: 40 cycles, 95°C/15 seconds, 60°C/1 minute, using the following specific primers: 18S up: 5′-CGGCTACCATCCAGAAG-GA-3′ (nucleotides 450-469), 18S down: 5′-GCTGAATTACCGCCT-3′ (nucleotides 636-619); GDF-15 up: 5′-CCCTGACTCCGGATCCT-3′ (nucleotides 274-292), GDF-15 down: 5′-GAAAGAGCCGGTGAAG-G-3′ (nucleotides 400-383). Data analysis was done using the ΔΔCT method for relative quantification. Threshold cycles (Ct) for 18S rRNA (reference) and GDF-15 (sample) were determined in duplicates. We chose normal brain cDNA as calibrator tissue (100%) and determined the relative change (rI) in copy numbers according to the formula...
pure or diluted samples were applied for 2 hours at room temperature, and bound GDF-15 was detected with biotinylated anti-GDF-15 detection antibody (R&D Systems) followed by streptavidin-HRP (R&D Systems) and 3,3′,5,5′-tetramethylbenzidine (DAKO). The color reaction was stopped by the addition of 1 mol/L H₂SO₄, and absorbance was recorded in an ELISA reader (Tecan) at 450 nm. Using recombinant human GDF-15 as standard (R&D Systems), the assay was linear for GDF-15 concentrations between 0.1 ng/mL and 4 ng/mL.

For all other cytokine measurements, freshly isolated splenocytes (5 × 10⁶) from VM/Dk mice were stimulated with SMA-560 control or shGDF-15 cells (5 × 10⁵) for 3 days in 6-cm dishes. Supernatants were harvested and interleukin (IL)-2, IL-10, and IFN-γ concentrations were determined by ELISA according to the manufacturer’s protocol (eBioscience).

**Glioma cell proliferation**

SMA-560 control or shGDF-15 cells (5 × 10⁵) were plated in 96-well flat-bottomed plates and cultured in serum-free medium. Cultures were pulsed with [methyl-³²]H]thymidine (1 μCi; Amersham) on day 2 and collected 16 hours later using a cell harvester (Tomtec). Incorporated radioactivity was bound to a glass fiber filtermat (Wallac). The filtermat was wetted with Ultima Gold Scintillation Cocktail (Packard), and radioactivity was determined in a Wallac 1450 Microbeta Plus Liquid Scintillation Counter.

**Preparation of murine splenocytes and NK cells**

Splenocytes were prepared from VM/Dk mice. Murine NK cells were positively selected using DX5 monoclonal antibody–coupled magnetic beads with the corresponding column system (Miltenyi Biotech). Polyclonal mouse NK cells were cultured with mouse IL-2 (5,000 U/mL; PeproTech) for at least 10 days before being used as effector cells in cytotoxicity assays.

**Lysis assays**

NK cell cytotoxicity was assessed in 4-hour ⁵¹Cr release assays with 10⁶ ⁵¹CrO₄⁻²-labeled targets per well and various effector:target (E:T) ratios in 100 μL of medium. Spontaneous ⁵¹Cr release was determined by incubating the target cells with medium alone. To obtain the maximum ⁵¹Cr release, NP-40 (2%) was added. After incubation for 4 hours, 50 μL of the supernatant were transferred to a Luma-Plate 96 (Packard), dried, and measured. The percentage of ⁵¹Cr release was calculated as follows: 100 × (experimental release - spontaneous release) / [maximum release - spontaneous release]. The lytic activity of NK cell–depleted splenocytes was examined after 5 days of coculture with glioma cells. Irradiated glioma cells (5 × 10⁷) were seeded into 6-cm dishes. Splenocytes (5 × 10⁶) were added in 3 mL RPMI 1640 containing 10% FCS. Primed alloreactive splenocytes were removed at day 5 and used at different E:T ratios in a ⁵¹Cr release assay as described above.
Mice and animal experiments
VM/Dk mice are bred in our own laboratory. Mice of 6 to 12 weeks of age were used in all experiments. The experiments were done according to NIH guidelines, Guide for the Care and Use of Laboratory Animals. Groups of six mice were injected s.c. in the right flank with $10^6$ transfected SMA-560 tumor cells in 0.1 mL PBS as indicated. The mice were examined regularly over 30 days for tumor growth using a metric caliper. Before all intracranial procedures mice were anesthetized by an i.p. injection of 7% chloral hydrate. For intracranial implantation the mice were placed in a stereotactic fixation device (Stoelting) and a burr hole was drilled in the skull 2 mm lateral to the bregma. The needle of a Hamilton syringe (Hamilton) was introduced to a depth of 3 mm. SMA-560 glioma cells ($5 \times 10^3$) were injected in a volume of 2 μL PBS into the right striatum. The mice were observed daily and sacrificed when developing neurologic symptoms. For immunohistochemical stainings, brain cryosections were prepared from mice sacrificed on day 15 after glioma cell inoculation. The following antibodies were used: anti-CD3 (BD Bioscience), anti-CD11b (BD Bioscience), and anti-Ly49G2 (eBioscience). Subsequently, the slices were stained with secondary antibody and developed with DAB (Vectastain, Vector Laboratories).

Statistics
Where indicated, analysis of significance was done using the two-tailed Student's t-test ($^* P < 0.05$; $^{**} P < 0.01$). All experiments were done at least three times and representative experiments are shown.

Results
GDF-15 expression in malignant gliomas in vivo translates into elevated serum levels
To examine the expression of GDF-15 in vivo, placenta and gliomas of different WHO grades were stained by immunohistochemistry (Fig. 1A). In normal human white matter tissue specimens (top right), GDF-15 was detected on endothelial cells of small capillaries (arrow) but not on neuroepithelial cells. Diffuse astrocytomas (WHO grade II, middle left) and anaplastic astrocytomas (WHO grade III, middle right) showed an upregulation of GDF-15 on neoplastic glial cells beside the endothelial expression (arrows). Strongest GDF-15 expression was observed in glioblastomas (WHO grade IV, bottom left and right) both on neoplastic astrocytic cells and endothelial cells of vascular proliferations (asterisk). The distribution of GDF-15 was homogeneous within the five samples stained for each tumor entity.

We next aimed at testing whether glioma-derived GDF-15 was also detectable in the blood. To this end, we compared the GDF-15 levels in the sera of glioblastoma patients ($n = 13$) and healthy controls ($n = 20$). To avoid confounding effects of therapy-induced elevations of GDF-15, only samples from treatment-naive patients, that is, without prior radiation therapy and chemotherapy and without exposure to steroids, were measured by ELISA. This revealed a highly significant elevation of serum GDF-15 levels in the serum of glioblastoma patients ($P < 0.01$, unpaired two-sided Student's t-test; Fig. 1B).
Human malignant glioma cells express GDF-15 in vitro

We went on to define the expression of GDF-15 in a panel of human glioma cell lines and primary polyclonal glioblastoma cell cultures in vitro. Real-time PCR revealed that GDF-15 mRNA expression levels were 10- to 600-fold increased in all of 12 examined glioma cell lines and 3 primary glioblastoma cell cultures when compared with normal human brain (Fig. 2A). Immunoblot analysis confirmed the presence of fully processed GDF-15 in 11 of 12 investigated glioma cell supernatants (Fig. 2B). There was a reasonably good correlation of mRNA and protein levels ($R^2 = 0.69$), with LN-428 and A172 showing the highest GDF-15 expression on both mRNA and protein levels, whereas the low mRNA expression found in U87MG, U251MG, and U373MG translated into weak, but still detectable protein levels. The apparent lack of GDF-15 in supernatant from U138MG cells might be due to poor processing, inadequate secretion, or to nonspecific adhesion of the cytokine to the cell surface (35). In line with previous studies (30), no correlation ($R^2 = 0.05$) was found between GDF-15 expression and p53 status of the cell lines (33).

GDF-15 promotes glioma cell proliferation and protects malignant glioma cells from NK and T cell-mediated cytotoxicity in vitro

To allow for studies in a syngeneic setting, we confirmed the expression of GDF-15 in supernatant of murine SMA-560 glioma cells. To perform loss-of-function experiments, we used RNA interference to stably silence the GDF-15 gene in these cells. Immunoblot revealed a downregulation of GDF-15 in SMA-560_shGDF-15 pool transfectants by >90% as quantified by densitometry (Fig. 3A). Under normal cell culture conditions, this had no apparent effect on growth or morphology of the cells. However, when we investigated the proliferation of these glioma cell sublines under serum-free conditions, SMA-560_shGDF-15 cells incorporated significantly less [methyl-3H]thymidine than did the corresponding control-transfected cells. This effect could be reverted by addition of exogenous GDF-15 (5 ng/mL) or FCS (10%) to the GDF-15-depleted cells (Fig. 3B and data not shown), whereas addition of GDF-15 to control-transfected cells did not further promote their growth. Thus, endogenously produced GDF-15 acts as an autocrine growth factor for glioma cells.

One of the most striking alterations caused by depletion of TGF-β in glioma cells is the increase in tumor cell immunogenicity (10). Therefore, we hypothesized that GDF-15 might also contribute to glioma-derived immunosuppression. Using syngeneic NK cells, we found that SMA-560_shGDF-15 cells were significantly more susceptible to immune cell lysis than were control cells (Fig. 3C). To investigate the effect of GDF-15 on immune cells other than NK cells, splenocytes were prepared from syngeneic VM/Dk mice and NK cells were depleted by magnetic-activated cell sorting. The cells were cocultured for 5 days with SMA-560_control or shGDF-15 cells and then used as effectors against fresh SMA-560_shGDF-15 targets. SMA-560_shGDF-15 cells were efficiently killed by splenocytes primed with GDF-15–deficient glioma cells, whereas priming with SMA-560_control cells induced significantly lower lytic activity (Fig. 3D). Of note, primed lymphocytes did not display cytotoxic activity against syngeneic splenocytes (lymphoblasts) under these conditions. Consistent with an increase of the lytic activity of immune effector cells, splenocytes cocultured with SMA-560_shGDF-15 cells generated higher levels of IL-2 and decreased IL-10 levels compared with splenocytes cocultured with wild-type cells ($P < 0.01$; Fig. 3E). In contrast, there was no difference in IFN-γ levels (data not shown). Control measurements without splenocytes confirmed that supernatant from glioma cells without splenocytes contained only negligible quantities of the respective cytokines: although IL-2 levels were below the detection limit, IL-10 concentrations were 16 pg/mL for control and 28 pg/mL for shGDF-15 cells.

RNA interference–mediated GDF-15 depletion delays the growth of experimental gliomas in syngeneic mice

Taken together, the effects of GDF-15 on glioma cell proliferation and immune escape strongly suggest that endogenous GDF-15 expression might promote the tumorigenicity of glioma cells. Thus, we assessed the effect of GDF-15 depletion in vivo, using the syngeneic SMA-560 VM/Dk mouse model. In line with the in vitro data, tumor growth was significantly delayed when GDF-15–depleted rather than control-transfected glioma cells were s.c. inoculated in the flank (Fig. 4A; $P < 0.05$ from day 10, $P < 0.01$ from day 22 until the end of the experiment). Likewise, when SMA-560 cells were implanted stereotactically into...
the brains of VM/Dk mice, neurologic symptoms were first observed in animals carrying control cells. Accordingly, the median survival was prolonged from 19 days with control tumors to 24 days with shGDF-15 tumors (\[P < 0.01\] by two-sided, unpaired Student's t-test). Thus, although at day 21 none of the animals with a control tumor was still alive, 83% of the mice with shGDF-15 tumors were still viable (Fig. 4B). Two mice per group were sacrificed during the experiment for histologic analyses. Compared with mice with SMA-560 control tumors, experimental gliomas derived from shGDF-15 cells displayed an increased infiltration with T cells and macrophages (Fig. 4C). In contrast, the numbers of infiltrating NK cells were very low under both conditions (data not shown).

**Discussion**

The hallmarks of malignant glioma cells include their ability to deeply penetrate the surrounding healthy tissue and to inhibit antitumor immune responses. Together with their resistance to radiotherapy and chemotherapy, these properties translate into devastating tumor growth and a dismal prognosis. To gain a better understanding of the molecular mechanisms leading to the malignant phenotype of these cells, we characterized the role of GDF-15 for proliferation and immune escape of glioma cells. Immunohistochemistry confirmed that the majority of gliomas express GDF-15 \(\textit{in vivo}\) (Fig. 1). Compared with gliomas of WHO grades II and III, we noticed an increase of GDF-15 expression and more GDF-15–positive cells in WHO grade IV tumors. These findings apparently contradict the observation from Shnaper et al. (26) who found glioblastoma cells to be GDF-15 negative \(\textit{in vivo}\). However, the polyclonal rabbit serum provided by the Atlas consortium may be the first GDF-15 antibody to give reliable results in immunohistochemistry. Considering that previous studies also detected GDF-15 mRNA (36) and protein (37) in gliomas, we trust in the validity of the results.
our data. These immunohistochemical observations are further supported by GDF-15 expression analyses in established glioma cell lines. As shown in Fig. 2A, GDF-15 transcripts were upregulated in all of 12 permanent glioma cell lines and 3 primary polyclonal glioblastoma cell cultures compared with normal human brain. Further, GDF-15 protein was also detectable in the supernatant of most investigated glioma cell lines where its expression largely paralleled the mRNA data. Slight alterations might be due to posttranscriptional regulation and differences in secretion of the protein in different cell lines (35). In summary, our results clearly show that glioma cells express GDF-15. Nevertheless, tumor-associated microglial cells might also make a substantial contribution to the total GDF-15 levels observed in glioma patients, as proposed by others (26). A second important finding from our study which had not been observed in a previous publication were the increased serum GDF-15 levels of glioblastoma patients (Fig. 1B). The very clear (P < 0.01) difference noticed in our study might be due to the fact that we exclusively included treatment-naïve patients, thereby avoiding any confounding effects by steroids, chemotherapeutics, or irradiation.

Fig. 4. RNA interference targeting GDF-15 delays the growth of s.c. and intracerebral gliomas in syngeneic immunocompetent mice. A, VM/Dk mice were s.c. injected with 10^5 SMA-560 control (●) or shGDF-15 (□) cells. Tumor size was measured using a caliper every 3 days. B, SMA-560 control or shGDF-15 transfectants were inoculated intracerebrally in syngenic VM/Dk mice. Survival data for six animals per group are presented as Kaplan-Meier plot. C, at day 15 after tumor inoculation, two animals were sacrificed. Their brains were removed, shock-frozen, and assessed for the infiltration of T cells and macrophages. Representative stainings are shown.
To define the functional role of glioma-derived GDF-15, we silenced GDF-15 expression in murine SMA-560 glioma cells by RNA interference. This allowed for immunologic studies and a functional characterization of GDF-15 in a syngeneic setting (Fig. 3A). We noticed a reduced proliferation of glioma cells that were depleted of GDF-15 (Fig. 3B). However, this effect was not only reverted by the addition of recombinant GDF-15 but also by FCS, indicating that in complete medium the lack of GDF-15 is counterbalanced by a multitude of growth factors. In contrast, our immunologic studies clearly point out a novel and until now unrecognized role of GDF-15 as a glioma-derived immunosuppressive molecule. As shown in Fig. 3C, glioma cells depleted of GDF-15 are more susceptible to the lytic activity of NK cells. Likewise, splenocytes from syngeneic mice display improved cytotoxic capacity against SMA-560 targets when primed with shGDF-15 cells rather than with SMA-560 control transfectants. This improved immune cell activation in the absence of GDF-15 is further corroborated by an increase in lymphocyte-derived IL-2 and a decrease in IL-10 in coculture studies (Fig. 3E). These data show that anti-GDF-15 strategies may partly relieve glioma-induced immunosuppression and enable effective antitumor responses in vitro.

In syngeneic mice, s.c. injected SMA-560 control cells grew faster than SMA-560 shGDF15 transfectants (Fig. 4A). In line with these findings, we observed a survival advantage for mice that had intracranial tumors derived from shGDF-15 cells (Fig. 4B). These findings are in contrast to an earlier publication that reported that GDF-15−transfected LNZ-308 glioma cells lost tumorigenicity in nude mice (30). However, this model is not only unsuitable for the detection of immunologic tumor-host interactions. Ectopic expression of GDF-15 may further lead to unphysiologically high cytokine levels that could affect angiogenesis (38). Thus, downmodulation of endogenously expressed GDF-15 in an immune-competent orthotopic mouse model (as described here) is likely to reflect the actual role of GDF-15 in glioma more accurately. Further evidence for the presumed immunomodulatory role of GDF-15 comes from the observation that GDF-15−depleted tumors show stronger infiltration with T cells and macrophages (Fig. 4C). This might be a double-edged sword, however, because infiltration with regulatory T cells (39) or the presence of tumor-associated macrophages (40) have been linked to a poor outcome in other tumor entities. In brain tumors, however, the presence of regulatory T cells seems to be prognostically neutral within the entity of glioblastomas (41).

Likewise, no correlation was found between the total number of tumor-infiltrating macrophages and prognosis (42), whereas a (rarely observed) severe lymphocytic infiltration was linked to a significantly longer survival (43). The magnitude of T-cell infiltration may be inversely proportional to intratumoral TGF-β2 levels and correlates positively with clinical outcome (44), which bears some resemblance to our in vivo data on the divergent TGF-β superfamily member GDF-15. The fact that even mice with GDF-15−depleted tumors died suggests that the lack of GDF-15 might have been compensated by other immune-inhibitory mechanisms such as TGF-β. In addition, a selection process for glioma cell clones with a less substantial knockdown of GDF-15 expression might have occurred. Unfortunately, due to a lack of adequate reagents this hypothesis cannot currently be tested by immunohistochemical analysis of mouse tumors. Finally, GDF-15 may also have been provided by host cells, e.g., by microglia in the tumor microenvironment. However, the current lack of knowledge regarding the GDF-15 receptor and its associated signaling mediators do not only limit the mechanistic understanding of the involved processes, but they also preclude experimental pharmacologic approaches like the inhibition of signaling at the receptor level or the specific blockade of GDF-15−dependent signaling cascades. Nevertheless, the reasonably mild phenotype displayed by GDF-15 knockout mice (45) suggests that such strategies might be more safely directed towards GDF-15 than towards TGF-β, where the targeting of receptor kinases (46) has given rise to serious concerns regarding the safety of this approach. Overall, our study clearly shows that GDF-15 confers immune privilege to malignant gliomas, contributes to the malignant phenotype of these cells, and thus represents a novel and promising target for future therapies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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