Signal Transduction

Inhibition of NF-κB Signaling Ablates the Invasive Phenotype of Glioblastoma

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Abstract

Glioblastoma multiforme, the most common primary brain tumor, is highly refractory to therapy, mainly due to its ability to form micrometastases, which are small clusters or individual cells that rapidly transverse the brain and make full surgical resection impossible. Here, it is demonstrated that the invasive phenotype of glioblastoma multiforme is orchestrated by the transcription factor NF-κB, which, via metalloproteinases (MMP), regulates fibronectin processing. Both, cell lines and tumor stem cells from primary glioblastoma multiforme, secrete high levels of fibronectin which when cleaved by MMPs forms an extracellular substrate. Subsequently, forming and interacting with their own microenvironment, glioblastoma multiforme cells are licensed to invade their surroundings. Mechanistic study revealed that NF-κB inhibition, either genetically or pharmacologically, by treatment with Disulfiram, significantly abolished the invasive phenotype in the chick chorioallantoic membrane assay. Furthermore, having delineated the underlying molecular mechanism of glioblastoma multiforme invasion, the potential of a disulfiram-based therapy was revealed in a highly invasive orthotrophic glioblastoma multiforme mouse model.

Implications: This study defines a novel therapeutic approach that inhibits micrometastases invasion and reverts lethal glioblastoma into a less aggressive disease. Mol Cancer Res; 11(12); 1611–23. ©2013 AACR.

Introduction

Glioblastoma multiforme is the most common tumor of the central nervous system (1). The current standard of care consists of tumor resection followed by radiotherapy and a course of temozolomide (2), but despite intense efforts, glioblastoma multiforme remains one of the most lethal tumors with a mean patient survival of 14 months (3). The high mortality of patients is partially due to the particular growth pattern of this malignancy. Indeed, the presence of micrometastases in the absence of a distinct tumor mass is sufficient to cause progressive neurologic dysfunctions and even death (4).

Glioblastoma multiforme grow diffusely and are highly invasive, infiltrating the surrounding brain tissue, thus making localized treatment, e.g., surgery, particularly ineffective (1). Therapeutic interventions to ablate the invasive phenotype of glioblastoma multiforme have so far proved to be insufficient, as systemic chemotherapy or whole brain irradiation have failed to eradicate invasive cells and micrometastases (4). It is therefore imperative for any new therapeutic intervention to consider this highly invasive nature of glioblastoma multiforme and ablate this aggressive phenotype as efficiently as possible.

Although the NF-κB pathway is frequently found aberrantly activated in glioblastoma multiforme (5), its role in glioblastoma multiforme biology remains elusive. While some findings point to a role in mediating DNA-damage repair (6) and modulating sensitivity to death receptor-mediated apoptosis (7), it has also been shown that in glioblastoma multiforme NF-κB does not play a role in proliferation and resistance toward therapeutic intervention based on chemotherapy (8). Recent evidence points to a possible role of this transcription factor in the tumor-invasion potential of glioblastoma multiforme cells (9, 10). However, so far, neither the precise molecular mechanisms of this role nor the potential therapeutic benefits of these findings have been systematically addressed.

Materials and Methods

Cell culture

U87MG cells from American Type Culture Collection (ATCC) were maintained in Dulbecco’s Modified Eagle Medium (DMEM; Gibco, Life Technologies, Inc.),...
supplemented with 10% fetal calf serum (FCS; Biochrom), 1 mmol/L glutamine (Biochrom), and 1% penicillin/streptomycin (Biochrom). Control and 1kbΔ superrepressor (SR) cells were created as previously described (6).

Patient-derived glioblastoma spheres were grown from surgical specimens, after patients’ consent was obtained. Specimens were minced and taken up in ice-cold PBS (Biochrom), followed by 5-minute centrifugation at 345 ×g/room temperature. After discharging the liquid, the tumor pellet was taken up in 5 mL TrypLE Express (Gibco, Life Technologies), supplemented with 1-glutamine, EGF (Biochrom, Life Technologies), and incubated for 5 minutes. Tumors were then filtered through a sieve (pore size, 70 μm) and taken up in DMEM/F-12 (HAM) medium (Gibco, Life Technologies), supplemented with 1-glutamine, EGF (Biochrom, Life Technologies), basic fibroblast growth factor (bFGF; Miltenyi Biotec GmbH), and B27 (Gibco, Life Technologies).

**Adhesion assay**
The trypsin-based adhesion assay was performed as previously described (11).

**Time-lapse photography and wind-rose analysis**
Time-lapse photography was performed as previously described (11). Final analysis and wind-rose depiction were performed with ImageJ (Rasband, W.S., ImageJ; http://imagej.nih.gov/ij/, 1997–2011).

**Migration assay**
Cellular migration was determined after 12 hours by analyzing cells migrating through either a collagen-coated (BD Biosciences) or a fibronectin-coated (Sigma-Aldrich) membrane in an 8-μm pore Costar Transwell insert (Corning Inc.). Medium containing 20% FCS served as chemotactant, while cells were seeded in medium containing 10% FCS.

**Biologic invasion assay**
The invasion of cells into a biologic structure was assayed via the chorioallantoic membrane (CAM) assay (12). Briefly, 1 × 10⁶ cells were seeded in a 1:1 mixture of serum-free medium and Matrigel (BD Biosciences) onto the CAM of 1-week-old fertilized eggs. Four days after seeding, the tumor and surrounding CAM were extracted, embedded in paraffin, and cut.

**Tissue immunohistology**
Avian, murine, and human tissue samples were stained for hematoxylin and eosin. In addition, staining for Vimentin (Abcam plc) and Fibronectin (Dako Deutschland GmbH) was performed, according to the previously published protocol (12).

**Western blot analysis and integrin surface expression**
Western blot analysis and integrin surface expression were performed as previously described (11), using the following antibodies: mouse monoclonal anti-Vinculin (Sigma-Aldrich), mouse monoclonal anti-Src (Cell Applications Inc.), rabbit polyclonal anti-FAK (Millipore), mouse monoclonal anti-uPAR (American Diagnostica), mouse monoclonal anti-α-tubulin (Millipore), mouse monoclonal anti-β-actin (Sigma-Aldrich), anti-mouse and anti-rabbit IgG-HRP (Santa Cruz Biotechnology) and APC-labeled anti-CD29 (BD Biosciences) for integrin β₁.

**Fluorescence microscopy**
Fluorescence microscopy was performed as previously described (11), using the following antibodies: rabbit polyclonal anti-p65 (Santa Cruz Biotechnology) Texas Red anti-rabbit (Vector Laboratories), Texas Red antimouse (Vector Laboratories), and fluorescein isothiocyanate (FITC) anti-rabbit (Milipore), as well as 4',6-diamidino-2-phenylindole (DAPI; Roche Diagnostics) or Hoechst 33342 (Sigma-Aldrich) to counterstain the nuclei and phalloidin-TRITC (Sigma-Aldrich) to visualize the actin cytoskeleton. For fibronectin staining, rabbit anti-fibronectin (Siemens Healthcare Diagnostics GmbH) was used in conjuncture with anti-rabbit immunoglobulin G (IgG) Biotin (Dako Deutschland GmbH) and SA-Alexa Fluor R568 (Molecular Probes, Life Technologies). Pictures were taken with an AX70 "Provis" microscope (Olympus).

To present pictures more clearly, the depiction of the actin cytoskeleton in Fig. 2C was performed similarly as described earlier, but using a laser scanning confocal microscope (Leica DM IRB).

**Analysis of the actin cytoskeleton**
The amount of intact cytoskeleton was determined by analyzing the ratio of G-actin to F-actin using the G-actin/F-actin In Vivo Assay Kit (Cytoskeleton, Co) according to the manufacturer’s instructions. The densitometric analysis was performed with ImageJ.

**Quantitative determination of Fibronectin synthesis**
The amount of secreted Fibronectin was determined as previously described (11).

**Electrophoretic mobility shift assay**
Nuclear extracts were prepared according to a previously established protocol (8) using following γ-[32P]-ATP-labeled oligomer: 5′-AGTTGAGGGGGACCTTTTCCCACGGC-3′.

**Proteolytic cleavage of fibronectin by metalloproteinases**
The recombinant catalytic domains of metalloproteinase (MMP)-2 and MMP-9 (ProSpec) were added upon seeding at a 1:10 dilution to 100 μL medium containing 6.0 × 10⁶ cells. Cells were allowed to settle for approximately 1 hour before fixation.

**MMP quantification**
MMPs were quantified by Fluorokine Multianalyte Profiling (MAP) kits following the manufacturer’s instructions (R&D Systems), using following kits: Human Fluorokine MAP Base Kit, MMP Panel, Human MMP-2 Fluorokine MAP, and Human MMP-9 Fluorokine MAP.
Gelatin zymography

Zymography was performed as previously described (13).

Apoptosis measurement

The apoptosis readout was DNA fragmentation as assessed by fluorescence-activated cell-sorting (FACSscan; Becton Dickinson) analysis of propidium iodide–stained nuclei as previously described (11).

Changes in cell number

Cells were seeded and allowed to proliferate for indicated times. This was followed by prolonged treatment with a trypsin/EDTA solution (Biochrom) until all cells were in suspension. The cell suspension was diluted 1:100 in CASYton solution (Innovatis) and cell numbers were then determined using CASY1 DT (Innovatis).

Chemical and pharmacologic inhibitors

Disulfiram was obtained from Sigma-Aldrich, whereas Antabuse (Actavis Group PTC) was used for the orthotopic mouse experiments. Cells were seeded at a concentration of 1 μmol/L GM1489 (Santa Cruz Biotechnology), 1 mmol/L RGD peptide (Enzo Life Science), and 200 pmol/L VPLCK (D-Val-Phe-Lys Chloromethyl Ketone; Merck Chemicals).

Orthotrophic mouse model

Human glioblastoma multiforme cells were transplanted into mouse brains as previously described (14). Briefly, 1.0 × 105 cells were stereotactically implanted into the right striatum of NOD.Cg-PrkdcscidIl2rgtm1Wji/SzJ mice. Animal experiments were approved by the Regierungspräsidium Tübingen, Germany.

Statistical analysis

Statistical analysis was carried out by a two-sided Student t test, unless stated otherwise.

Results

NF-κB inhibition reduces of glioblastoma multiforme cell/microenvironment interaction

After recent suggestions (9) that in glioblastoma multiforme cells, NF-κB might contribute to cell invasion, we further investigated this intriguing possibility using U87MG cells stably transfected with a previously described IkBα superrepressor (SR) construct that inhibits NF-κB activity (refs. 6, 8; Supplementary Fig. S1A). Although proliferation and spontaneous apoptosis seemed unaffected (Supplementary Fig. S1B and S1C), the SR cells displayed a striking retardation in cell spreading (Fig. 1A) and detached more readily after trypsin treatment (Fig. 1B). This diminished adhesion also affects cell locomotion, i.e., undirected movement (Supplementary Fig. S1D): control cells travel in a roughly linear fashion, SR cells move more randomly, frequently altering direction, in particular after reattachment upon transient loss of cell-substrate interaction (Supplementary Fig. S1D, arrowheads).

Although a scratch assay suggested that SR cells also behave differently from control cells in migration, i.e., directed movement control cells (Supplementary Fig. S1E), we further confirmed this by ascertaining the migratory capacity of the cells through a collagen-coated membrane. As shown in Fig. 1C, control cells migrate significantly faster through the membrane, suggesting that their increased interaction with their substrate leads to increased motility, despite potentially reduced locomotion. To verify that these findings are of physiologic relevance, we used the so-called chick CAM assay (15). Intriguingly, although both cells remained viable and appeared to proliferate on the CAM, only the control cells exhibited an invasive phenotype, whereas the SR cells did not grow into the CAM (Fig. 1D). Taken together, these data strongly argue for a critical role of NF-κB–mediated signaling in glioblastoma multiforme invasion.

IkBα superrepressor–expressing cells do not efficiently process Fibronectin

Before investigating the underlying molecular mechanisms that link NF-κB signaling to migration/invasion, we independently confirmed the role of NF-κB in a second glioblastoma multiforme cell line, T98G, expressing the control vector or the IkBα superrepressor (Supplementary Fig. S2).

Next, we concentrated on integrin-mediated adhesion, which we have previously shown to play a role in glioblastoma multiforme (11). Although the protein expression of several focal adhesion proteins, as well as the surface expression of the β1 integrin subunit did not differ between control and SR cells (Fig. 2A and B, respectively), when visualizing the actin cytoskeleton, which links focal adhesions to cell morphology and motility (16), we found that SR cells almost completely lacked organized stress fibers (Fig. 2C). To further verify this, we separated filamentous F-actin from globular G-actin and compared the ratio of these two (Fig. 2D). To assess whether focal adhesions formed and were unable to connect to the cytoskeleton, or whether cells cannot form focal adhesions and thus do not provide a tethering point for the actin stress fibers, we visualized the cellular localization of the focal adhesion. Interestingly, the typical punctuate staining, which is indicative of focal adhesion (Fig. 2E, first column), is almost completely lacking in SR cells (Fig. 2E, second column). However, this difference does not seem to be due to the SR cells’ intrinsic inability to form focal adhesions, because when provided with an external fibronectin matrix, they are fully capable of forming focal adhesions (Fig. 2E, third column). Importantly, the failure to interact with their substratum seems to be a specific effect and not a general deficit of the SR cells to interact with their environment, as the SR cells’ ability to form adherens junctions is unaffected (Supplementary Fig. S3A).

To verify that the differences in migration are due to altered focal adhesion formation/actin organization in the absence of fibronectin, we repeated the Transwell assay shown in Fig. 1D with a fibronectin-coated membrane (Fig. 3A). When
provided with an external source of fibronectin matrix, both control and SR cells migrate equally well through the membrane (Fig. 3A), suggesting that, indeed, the differences in environmental interaction of those cells are due to the SR cells’ inability to provide their own fibronectin matrix. As a putative NF-κB response element was found in the fibronectin gene (17), we initially speculated that SR cells might produce and thus secrete less fibronectin; this, however, is clearly not the case (Fig. 3B). As the distinct SR phenotype is lost upon providing the cells with an artificial fibronectin matrix, SR cells might be incapable of processing intrinsic fibronectin efficiently and are thus unable to incorporate it into a matrix. To analyze this hypothesis, we stained cells for fibronectin (Fig. 3C). Whereas control cells produce long strands of fibronectin (Fig. 3C, top, gray arrowheads) and also lay down the beginning of an extracellular fibronectin matrix (Fig. 3C, top, white arrowheads), these features seem to be absent in the SR cells (Fig. 3C, bottom).

As MMPs have been implicated in the invasive phenotype of glioblastoma multiforme (10) and are also regulated by NF-κB (18–20), we next investigated whether they are involved in NF-κB–dependent processing of fibronectin. Therefore, we added a recombinant catalytic MMP domain to the cells before seeding and analyzed fibronectin-processing the next day (Fig. 3D). Upon addition of the recombinant catalytic MMP domain, SR cells appear more spread out and a reduction in fibronectin nodules is visible (Fig. 3D). To further confirm a direct connection between MMP activity and fibronectin processing, we again repeated the Transwell assay with a collagen-coated membrane and in the presence of either the RGD peptide, that blocks the interaction between fibronectin and integrins, or the pharmacologic inhibitor GM1489, which is highly specific for MMPs, or both (Fig. 3E). Each substance reduced migration by 40% to 50%, but, importantly, combining both had no additive effect (Fig. 3E), suggesting that there is a linear relationship...
between both targets. As predicted by this emerging model, combining those two inhibitors has no additional effect on SR cells (Supplementary Fig. S3B).

Next, we investigated whether MMP expression (Fig. 3F) or activity (Fig. 3G) differed between control and SR cells, focusing on MMP-2, together with MMP-9, the dominant MMP in glioblastoma (10, 21). As we saw clear difference in MMP activity, but not expression level, we also looked at further molecules through which NF-κB might regulate MMP activation. Although MT1-MMP (22) and tissue inhibitor of metalloproteinases (TIMP; ref. 23) were not dynamically regulated when comparing control with SR cells (Supplementary Fig. S3C and S3D, respectively), we found clear differences in uPAR expression (Fig. 3H). In particular, we found that SR cells expressed two high molecular weight bands. Although additional bands are often suggested to be due to glycosylation, it has also been reported that uPAR exists in different isoforms, which can be either membrane-bound or secreted (24). This is of particular interest, as NF-κB binds to the uPAR promoter (25) and MMP-2 and -9 can be activated by plasmin-activated uPAR-bound uPA (26), which has also been shown to play a role in glioblastoma invasion (27). To confirm the involvement of uPAR in NF-κB–mediated MMP-2 activation, we used the highly specific plasmin inhibitor VPLCK (Fig. 3I). VPLCK strongly inhibited transmigration through a collagen-coated membrane, but combining VPLCK and GM1489 had no additional effect (Fig. 3I), again suggesting a linear relationship.

Taken together, these data show that glioblastoma multiforme cells activate MMPs via NF-κB–dependent regulation of the uPA/uPAR complex and thereby process secreted fibronectin. This allows the cells to form an extracellular...
matrix to which the cells then adhere strongly via focal adhesions. These focal adhesions also form tethering points for the actin cytoskeleton, allowing the cells to spread rapidly. Our data suggest that glioblastoma multiforme cells activate NF-κB upon encountering an unfavorable microenvironment, experimentally simulated by keeping cells in suspension (Supplementary Fig. S3E).

**Therapeutic potential of NF-κB inhibition in glioblastoma multiforme**

To assess whether NF-κB inhibition might be of therapeutic benefit in glioblastoma multiforme treatment, we used disulfiram, the active component of Antabuse, which was originally described as a well-tolerated inhibitor of acetaldehyde metabolism (28–30). It can cross the blood-brain-barrier (30–33) and has been shown to inhibit invasion via modulation of MMPs (34, 35), although this effect has so far not been linked to NF-κB signaling and fibronectin, and is already in clinical use.

First, we assessed whether disulfiram inhibits NF-κB signaling in glioblastoma multiforme cells. Treatment with Disulfiram leads to inhibition of both basal and TNF-α-induced NF-κB signaling, as shown by localization of the p65 subunit (Fig. 4A) and the transcription factor’s binding to DNA (Fig. 4B). Disulfiram has a significant effect on cell numbers (Fig. 4C), presumably due to its strong intrinsic capacity to induce apoptosis (Fig. 4D). Apoptosis is likely to be initiated by anokis, as cells detach (Supplementary Fig. S4A). This effect of disulfiram is specifically due to NF-κB inhibition, as it is much less marked in SR cells (Supplementary Fig. S4B) and transient, as cells that have been seeded for longer and thus, presumably, formed their own extracellular matrix do not detach as readily as freshly seeded cells (Supplementary Fig. S4C). In addition, the distinct punctuate staining of the focal adhesion and the fibrous actin cytoskeleton are also lost upon disulfiram treatment (Supplementary Fig. S4D). This set of data indicates that the SR construct and disulfiram have similar, but not identical mechanisms of action upon cellular behavior. This notion is further confirmed by antibody-based proteomic screening (Supplementary Fig. S5).

Next, we confirmed whether NF-κB inhibition by disulfiram has the desired function effects on transmigration (Fig. 4E) and invasion (Fig. 4F). As our data so far were obtained from cell lines that might not fully reflect the genomic profile of glioblastoma multiforme in vivo (36), we turned to three primary-cultured tumor initiating cells (Supplementary Fig. S6A), which have been cultured as spheres, thus retaining the original tumor-expression profile (36). Two of these cell populations, G38 and G40, were obtained from patients with glioblastoma multiforme, whereas G55 has been identified as gliosarcoma. As G40 cells secreted the highest amount of fibronectin (Supplementary Fig. S6B), we investigated whether we could adapt the previously described orthotrophic mouse model (14) for G40 cells and treat the mice from day 16 postoperation, every second day with disulfiram. Interestingly, tumor bulk could not be found in brains of mice harboring G40 cells, although abnormal cells were clearly present (Supplementary Fig. S6C) throughout the whole murine brain, to no lesser extent than G38 cells, although G40 appear to micrometastasize in smaller groups or individual cells (Supplementary Fig. S6D). Disulfiram treatment led to an increase in cell–clusters’ size (Supplementary Fig. S6E and S6F), suggesting the possibility that it can efficiently inhibit further migration after cell division. It seems unlikely that disulfiram has increased proliferation of glioblastoma multiforme as mice appeared healthy when sacrificed. As cell morphology and growth rates of G38 and G40 cells are similar in vitro (Supplementary Fig. S6G–S6J), it is tempting to speculate that this marked difference in growth pattern in vivo is due to the striking difference in fibronectin secretion between those cells (Supplementary Fig. S6B).

Next, we repeated the in vivo experiment using the G38 cells (Fig. 5A). Twenty-seven days postoperation, the mice were sacrificed, as control mice began to show symptoms and the brain regions parietal of the tumor bulk were analyzed for micrometastasis (Fig. 5B). Number, size, and position of cell...
clusters were then compared between untreated and disulfiram-treated tumors (Fig. 5C and D), until cell clusters exceeded at least 100 cells, as these clusters cannot be safely referred to as micrometastasis. Over comparable brain areas, untreated tumors formed 448 clusters consisting of 2,051 cells, whereas disulfiram treatment reduced the amount of clusters to 19, consisting of 247 in total. Furthermore, tumor cells in untreated mice were found more parietal, i.e., invaded further, than in treated mice (Fig. 5D). Although tumor bulk (Fig. 5E), as well as range and amount of micrometastasis (Fig. 5D and F, respectively) were clearly reduced by disulfiram treatment, the size of individual cell clusters was actually significantly increased by disulfiram, from 4.6 to 13 cells per cluster (P value = 0.04, one-sided Mann–Whitney U test). These data suggested that disulfiram can successfully inhibit glioblastoma multiforme micrometastasis and tumor growth.

Finally, we analyzed human brain sections of 2 patients afflicted with glioblastoma multiforme (Fig. 6) to assess the relevance of our findings in a clinical setting. Importantly, the four key features necessary for veracity of our model were also present in these sections: (i) fibronectin expression is enriched at the leading/invasive edge of the tumor compared with tumor bulk (Fig. 6A). (ii) Cells of the vascular system expressing fibronectin, the only area in healthy brain that has been reported to express fibronectin (37), are clearly distinguishable from tumor cells (Fig. 6B). (iii) Fibronectin was not uniformly expressed/secreted in many of those cells, suggestive of directional incorporation of this matrix compound into the microenvironment (Fig. 6B and C). (iv) Several fibronectin-positive cells seemed to be associated with “trails” of this substance. These “trails” probably indicate the route taken by the invasive cells (Fig. 6B and C).
In this study, we delineate the molecular mechanisms that allow glioblastoma multiforme cells to alter their microenvironment so as to increase their invasive potential (Fig. 7). Furthermore, we suggest a possible therapeutic intervention that is able to prevent glioblastoma multiforme invasion/micrometastasis, the most difficult to treat and deadliest feature of this malignancy (3). In detail, we found that the suggested link between NF-κB activity and glioblastoma multiforme invasiveness is due to the processing of fibronectin by MMPs, which thus permits incorporation of this matrix component directly into the surrounding of the invading tumor cells. MMP-2 is processed and thus activated in an uPAR-dependent manner, which in turn is known to be regulated by NF-κB (38–40). Although the individual steps of this signaling cascade were previously proposed, they had so far not been demonstrated in glioblastoma multiforme. More importantly, however, the involvement of MMPs in fibronectin processing has so far only been viewed in the context of invasion facilitated by extracellular matrix destruction. Here, we present compelling evidence that the opposite can also occur, invasion mediated by fibronectin processing that leads to novel matrix formation.

Interestingly, these findings might also shed some light onto the more general mechanisms of fibronectin processing, about which only little is known. Soluble Fibronectin exists

Discussion

In this study, we delineate the molecular mechanisms that allow glioblastoma multiforme cells to alter their microenvironment so as to increase their invasive potential (Fig. 7). Furthermore, we suggest a possible therapeutic intervention that is able to prevent glioblastoma multiforme invasion/micrometastasis, the most difficult to treat and deadliest feature of this malignancy (3). In detail, we found that the suggested link between NF-κB activity and glioblastoma multiforme invasiveness is due to the processing of fibronectin by MMPs, which thus permits incorporation of this matrix component directly into the surrounding of the invading tumor cells. MMP-2 is processed and thus activated in an uPAR-dependent manner, which in turn is known to be regulated by NF-κB (38–40). Although the individual steps of this signaling cascade were previously proposed, they had so far not been demonstrated in glioblastoma multiforme. More importantly, however, the involvement of MMPs in fibronectin processing has so far only been viewed in the context of invasion facilitated by extracellular matrix destruction. Here, we present compelling evidence that the opposite can also occur, invasion mediated by fibronectin processing that leads to novel matrix formation.

Interestingly, these findings might also shed some light onto the more general mechanisms of fibronectin processing, about which only little is known. Soluble Fibronectin exists

Figure 5. Disulfiram also inhibits NF-κB in primary tumor-initiating glioblastoma multiforme cells. A, treatment scheme for therapeutic intervention in mouse harboring a G38-induced glioblastoma multiforme. A total of 0.5 × 10⁶ cells were orthotopically injected into mouse brains. After 16 days of tumor growth, 20 mg disulfiram are injected on day 16 and 17, then every second day until the experiment is terminated on day 27 (due to symptoms in the control mice). B, right lateral representation of a mouse brain, with the approximate position of the tumor depicted in blue. Ten 4 μm axial sections were analyzed, covering the area parietal of the main tumor bulk (shown in red). Individual sections were spaced at least 50 μm apart. C, frequency of cell cluster occurrences in ten comparable brain sections of either control mice or disulfiram-treated mice. Clusters were grouped into three categories: small (1–10 cells/cluster), intermediate (11–100 cells/cluster), and large (more than 100 cells/cluster). D, frequency and relative position of cell clusters (independent of size) in ten comparable brain sections of either control mouse or disulfiram-treated mice. E, maximal tumor bulk of either untreated mice (control) or mice treated with disulfiram, G38 cells are visualized with Vimentin (red). F, three sections of apical mouse brain 28 days after injection, untreated (control) or treated with disulfiram. Tissue was stained with hematoxylin (blue), G38 cells are visualized with Vimentin (red). In C and D, 10 consecutive and comparable brain sections of two mice per group were analyzed, whereas in E and F, exemplary results of two independent analyses are shown. Scale bar in E, 1 mm; in F, 0.2 mm.
as a compact, tightly packed protein, leaving many protein domains inaccessible for potential interactions (41). Activation of fibronectin is believed to occur via extending the protein, i.e., integrins on the cell surface mechanically stretch the fibronectin molecules into an open conformation that allows their incorporation into a mesh (42). Interestingly, fibronectin consists mainly of homologous type III repeats, which contain cryptic binding sites and are highly sensitive to proteolysis (43). Our data indicate that NF-kB-dependent activation of MMPs can mediate increased incorporation of fibronectin into a matrix.

Furthermore, our finding also shed light on the rather controversially discussed role of fibronectin in glioblastoma multiforme, which had been frequently dismissed as a cell culture phenomenon, as fibronectin is, vasculature aside, not generally expressed in brain tissue. Two of two human pathology sections of glioblastoma multiforme/human brain tissue intersection exhibited cells staining positive for fibronectin, which was previously predicted by gene expression profiling (44). This is in contrast to a previous study, which found fibronectin expression in the minority of samples investigated (37). However, it should be pointed out that whereas we focused on invading cells at the tumor periphery, the latter group investigated the glioblastoma multiforme tumor bulk. Because we propose a role for fibronectin in invasion/micrometastasis and have previously shown that homotypic cell–cell interactions, as found in the tumor bulk, can compensate for cell–substrate interactions in many respects (11), we would not necessarily expect high levels of fibronectin within the tumor. We found, as did others (37), an increase in fibronectin positivity in human glioblastoma multiforme cells grown as mouse xenographs, suggesting the possibility that fibronectin-expressing cells engraft better in a foreign environment, or that glioblastoma multiforme tumor stem cells themselves also produce high amounts of fibronectin (as shown here for G38 and G40) and these relative small tumors grown in mouse are enriched in stem cells. Obviously, these possibilities are not mutually exclusive. However, should additional research reinforce the suggestion that fibronectin production can also be a stem-cell characteristic; this would further strengthen the rationale for using disulfiram in glioblastoma multiforme therapy. Although disulfiram, a drug commonly associated with treatment of alcoholism (28, 29), is currently being clinically evaluated for treatment of brain tumors (44), it has so far only been studied as an inhibitor of aldehyde dehydrogenase and thus preventing cancer repopulation after chemotherapy (31). Our data suggest that disulfiram’s ability to prevent fibronectin’s incorporation into the matrix might also further inhibit stem-cell expansion, which has been shown to be facilitated by cell/fibronectin interaction (45). To establish a tool to use for investigating the potential role of disulfiram in future therapeutic regimens, we showed that G38 glioblastoma multiforme–initiating cells can form a highly invasive tumor when implanted orthotopically into mouse brains. Importantly, disulfiram seems to prevent both, micrometastatic spread and tumor bulk expansion, making it an ideal candidate for novel therapeutic approaches in glioblastoma multiforme.
Although this study focuses on inhibition of glioblastoma multiforme cell invasion, it also has further implications for other aspects of glioblastoma multiforme therapy, such as adhesion-mediated apoptosis resistance, or AMAR, a major obstacle in modern cancer therapy (46). Cell–fibronectin adhesion is not the only means by which glioblastoma multiforme cells interact with their microenvironment, as glioblastoma multiforme–astrocyte interaction (47) and homotypic glioblastoma multiforme–glioblastoma multiforme cell interaction (11) also play an important role in glioblastoma multiforme biology. Importantly, both these cell–cell interactions are mediated by gap junctions (47, 11), therefore, it is conceivable to envision a glioblastoma multiforme therapy that combines disulfiram with an inhibitor of gap junction, such as carbenoxolone (11). This could fully isolate glioblastoma multiforme cells from their surroundings and although it might not suffice to induce anoikis in glioblastoma multiforme, it would significantly reduce AMAR and thus sensitize glioblastoma multiforme cells for chemotherapy-induced apoptosis.

In summary, this work identifies a mechanism by which glioblastoma multiforme cells alter their microenvironment so as to enable their brain invasion and the establishment of micrometastasis, the most lethal hallmark of this particular malignancy. Furthermore, we have delineated the underlying molecular steps that facilitate this process, which also sheds new light on the processing of
fibronectin and thus the formation of the extracellular matrix in general. Our data and the resulting model (Fig. 7) offer a unifying platform for previously unconnected findings about glioblastoma multiforme invasion. For example, although the role of uPAR and MMPs in glioblastoma multiforme invasion has been known for some time (48) and a putative role for NF-κB has also been suggested (9, 10), so far, no one had been able to connect these individual findings. Furthermore, our model also predicts additional points of interest/targets for therapeutic intervention, for example as the interaction between glioblastoma multiforme cell and microenvironment is mediated via fibronectin and focal adhesion, we would expect that alterations in focal adhesion proteins would also affect invasion, which has been recently confirmed (49).

Finally, we also show how these findings might be translated into clinical use and strongly encourage further research into the incorporation of disulfiram, or related substances, into the current clinical standards of glioblastoma multiforme therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References


Inhibition of NF-κB Signaling Ablates the Invasive Phenotype of Glioblastoma


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Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-13-0435-T

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