ADAM8 affects glioblastoma progression by regulating osteopontin-mediated angiogenesis

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Abstract: Glioblastoma multiforme (GBM) is the most aggressive type of brain cancer with a median survival of only 15 months. To complement standard treatments including surgery, radiation and chemotherapy, it is essential to understand the contribution of the GBM tumor microenvironment. Brain macrophages and microglia particularly contribute to tumor angiogenesis, a major hallmark of GBM. ADAM8, a metalloprotease-disintegrin strongly expressed in tumor cells and associated immune cells of GBMs, is related to angiogenesis and correlates with poor clinical prognosis. However, the specific contribution of ADAM8 to GBM tumorigenesis remains elusive. Knockdown of ADAM8 in U87 glioma cells led to significantly decreased angiogenesis and tumor volumes of these cells after stereotactic injection into striate body of mice. We found that the angiogenic potential of ADAM8 in GBM cells and in primary macrophages is mediated by the regulation of osteopontin (OPN), an important inducer of tumor angiogenesis. By in vitro cell signaling analyses, we demonstrate that ADAM8 regulates OPN via JAK/STAT3 pathway in U87 cells and in primary macrophages. As ADAM8 is a dispensable protease for physiological homeostasis, we conclude that ADAM8 could be a tractable target to modulate angiogenesis in GBM with minor side-effects.

Keywords: ADAM8; angiogenesis; GBM cell lines; glioblastoma; macrophages; osteopontin; tumor microenvironment.

Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive type of brain cancer of middle-aged patients with a grim prognosis (Koshy et al. 2012). Until now, the standard clinical treatment of GBM is limited to a combination of surgery, radiation and chemotherapy. Despite continued efforts over several decades to improve patient outcome, GBM remains an incurable disease with a median survival of only 15 months. In 2009, the FDA approved bevacizumab, a drug targeting neovascular formation, for patients with recurrent glioblastomas (Davis 2016). Neo-angiogenesis is critical for tissue growth and repair, and forms the basis for tumor progression (Javan et al. 2019; Viallard and Larrivee 2017). Thus, anti-angiogenic drugs have been widely suggested for clinical cancer treatment. This type of treatment is
considered to be beneficial as it can not only reduce the blood and nutrient supply of tumor cells in the center of a tumor, but also alleviate edema formation and subsequently neurological symptoms (Berghoff et al. 2014; Takano et al. 2013). However, for bevacizumab, fast mutating tumor cells can rapidly develop resistance by circumventing Vascular Endothelial Growth Factor (VEGF) signaling (Iwamoto et al. 2009), so that alternative routes of blocking angiogenesis are required.

As a large non-tumor cell population, macrophages/microglias is a prominent cell type of the GBM tumor microenvironment and is a mediator of inflammatory processes. Macrophages can build networks in the tumor that form vascular-like structures to provide oxygen and nutrients for tumor growth, thereby promoting tumor growth (Barnett et al. 2016).

As shown in numerous studies, macrophages can polarize into two extreme types, either M1-like or M2-like. M1-like macrophages are characterized by a pro-inflammatory and anti-tumor phenotype, while M2-like macrophages are considered as anti-inflammatory and tumor-supporting macrophages (Atri et al. 2018). According to a previous study performed in GBM patients, ADAM8 expression is highly expressed in macrophages/microglias and equally correlated with M1- and M2-like markers, CXCL10 (M1) and CCL13 (M2) (Gjorgjevski et al. 2019).

A disintegrin and metalloproteases (ADAM) constitute a family of multi-domain enzymes with important roles in cell adhesion, migration, proteolysis and signaling. As transmembrane proteins, ADAMS are associated with the process of ectodomain shedding that releases membrane-associated cytokines and receptors into the tumor microenvironment (Kataoka 2009; Murphy 2008). The metalloprotease-disintegrin 8 (ADAM8), is a proteolytically active member of the ADAM family (Blobel 2005; Murphy 2008) and was initially identified in macrophages (Yoshida et al. 1990). High expression levels of ADAM8 in tumor cells have been shown to be associated with invasiveness and metastasis of carcinomas, such as breast and pancreatic tumors (Conrad et al. 2017; Romagnoli et al. 2014; Schломann et al. 2015). For these cancers as well as for glioma, high ADAM8 expression levels correlate with poor patient prognosis (Conrad et al. 2019).

Osteopontin (OPN, gene name SPP1) is a secreted sialoprotein found in all body fluids and expressed in many tissues and cells. OPN is a chemoattractant for many immune cell types including macrophages, dendritic cells, and T cells. Furthermore, OPN enhances B lymphocyte immunoglobulin production and proliferation. When secreted by tumors, for example by glioma cells, OPN has been shown to promote proliferation, migration and angiogenesis via integrin αvβ3/P13K/AKT signaling (Ramchandani and Weber 2015; Wang et al. 2011).

In our study, we initially found in GBM tissue that ADAM8 is widely expressed both in neoplastic glioblastoma cells as well as in tumor-associated cells in the tumor microenvironment. We investigated the impact of ADAM8 on GBM progression in vivo by stereotactic injection of ADAM8 deficient glioma cells in the brain of nude mice. Our results suggest a significant contribution of ADAM8 during tumor angiogenesis, as demonstrated in vivo and in vitro by functional analysis of ADAM8 deficient U87 glioma cells and by analyzing primary macrophages isolated from ADAM8 knockout mice. Notably, mice lacking ADAM8 have no evident developmental or pathological defects as confirmed by phenotyping report of ADAM8 knockout mouse (Kelly et al. 2005), which suggests ADAM8 as a potential candidate target protein for GBM therapy with minor expected side effects.

**Results**

**ADAM8 expression is increased in glioblastoma multiforme and is localized to tumor cells and tumor-associated macrophage/microglia**

A total of 50 GBM tissues obtained from hospitalized patients were examined for ADAM8 expression by qPCR, indicating that ADAM8 expression is significantly higher in GBM compared to normal brain tissue (Figure 1a). Western Blot analyses confirmed higher expression levels of ADAM8 (Figure 1b). Furthermore, ADAM8 localization in GBM tissue was assessed by immunohistochemistry (Figure 1c). In normal brain tissue, ADAM8 expression is hardly detectable and restricted to some neuronal cells. In GBM tissue, ADAM8 expression is significantly higher (approx. 10-fold, \( p < 0.05 \)) and can be detected in tumor cells, neoplastic endothelial cell with proliferations, and in tumor-associated cells. In order to characterize these cells further, we performed double staining with immune cell markers and found that ADAM8 co-localizes with CD68, a marker for macrophages/microglia in the brain (Figure 1c, image g). Thus, we conclude that ADAM8 might be functionally important for tumor cells and for tumor-associated macrophages.

To analyze the function of ADAM8 in GBM, we performed loss-of-function experiments in U87 MG cells. Among all human GBM cell lines, U87 MG cells showed the highest level of ADAM8 expression. Briefly, endogenous ADAM8 was knocked down by small hairpin loop RNAs (shRNA) and a number of cell clones \((n = 8)\) were obtained with significantly reduced ADAM8 expression levels...
(>50-fold). The cell clone with the highest ADAM8 reduction assessed by qPCR and western blot (Figure 2a) was used for further functional analyses in vitro and in vivo (Figure 2). The proliferation behavior of cells showed no difference (Figure 2b), while cell migration was significantly reduced in U87_shA8 cells (Figure 2c).

**ADAM8 reduced angiogenesis and tumor volume in a GBM model**

Using U87 control (scramble, U87_shCtrl) and U87 ADAM8 knockdown (U87_shA8) cell clones, intracranial tumor cell injections into striatum of nude mice were carried out to assess tumorigenesis. To detect tumors, MRI scanning was started three weeks after implantation in weekly intervals and tumor volumes were calculated (n = 14 for each group, Figure 2d). All mice injected with U87_shA8 cells survived over an observation time of nine weeks. In contrast, all mice injected with U87_shCtrl cells reached the endpoint criteria or died spontaneously within the observation time (Supplementary Figure 1). Compared to the U87 control group, tumor volumes of U87_A8kd cells were significantly smaller and, in some cases (6 out of 14), tumor cells were completely absent three weeks after implantation (Figure 2a). To assess a possible tumor graft rejection, injection sites were analyzed one week after injection (Figure 2e). Whereas tumors derived from U87_shCtrl cells grew as a compact cell mass after one week, U87_A8kd cells showed a patchy growth characteristic. After staining for vascular structures using CD31 staining, we found significantly less (reduced to 46%, p < 0.01) vascular staining in U87_shA8 (8.7 × 10^4 ± 8) compared to the U87_shCtrl group (Figure 2e).

**ADAM8 regulates GBM cell angiogenesis via osteopontin**

Given the results of the in vivo experiments, we postulated that U87_shA8 cells could be deficient in angiogenesis induction. To test this hypothesis in vitro, we utilized conditioned medium from U87_shCtrl and U87_shA8 cells for angiogenesis induction of human umbilical vein endothelial cells (HUVEC) and quantified formation of tubes. After 6 h incubation with the respective cell culture supernatants, tube formation was quantified by counting the number of...
meshes formed (Figure 3a, b). ADAM8 containing supernatants from U87_shCtrl cells induced significantly higher numbers of tubes as compared to supernatants from ADAM8-deficient U87_shA8 cells ($p < 0.05$).

To identify the molecular mechanism by which ADAM8 affects angiogenesis, a differential analysis of cell supernatants from U87_shCtrl and U87_shA8 cells was conducted in an angiogenesis protein profiler array. Array membranes with spotted antibodies against proteins involved in angiogenesis were incubated with conditioned supernatants of U87_shCtrl and U87_shA8 cells and the proteins captured by antibodies on the array membrane were detected by chemiluminescence and quantified (Figure 3c). Several candidates were identified, most prominently IL-8 and Osteopontin (OPN, Figure 3c).

Differences in expression levels of OPN between U87_shCtrl and U87_shA8 cells were confirmed by qPCR, suggesting that OPN expression levels are dependent on the ADAM8 gene dosage (Figure 3d).

To provide a direct proof for our hypothesis, we analyzed whether exogenous OPN can be used to recover the deficiency of U87_shA8 cells in angiogenesis assays (Figure 3e, f). However, when adding IL-8, the reduced angiogenesis capacity of U87_shA8 cells was not restored (data not shown). In contrast, addition of...
recombinant OPN at different concentrations caused an increase in the number of meshes formed in the U87_shA group and reached similar values as in the U87_shCtrl group (Figure 3f). As the OPN concentration increases, the number of meshes increases in both groups, although to a smaller extent in U87_shCtrl cells due to saturation effects (Figure 3e).

**ADAM8 affects secretion of OPN via the JAK/STAT3 pathway in GBM cells**

From previous studies it is known that ADAM8 can directly affect intracellular signaling by either shedding of membrane proteins, binding to integrin β1, or by direct interaction with intracellular proteins interacting with the ADAM8 cytoplasmic domain. To explore the signaling pathway involved in OPN regulation by ADAM8, we used a panel of pathway inhibitors to target NF-kB, JAK/STAT3, p38, ERK1/2 and AKT/PI3K (Figure 4). U87_shCtrl and U87_shA8 cells were incubated with the respective inhibitors for 24 h and levels of secreted OPN in cell supernatants were determined by ELISA. While none of the inhibitors affects OPN secretion in U87_shA8 cells, OPN secretion was significantly decreased in U87_shCtrl cells treated with the JAK/STAT3 inhibitor only, whereas other inhibitors such as NF-kB and p38 inhibitor caused even slightly enhancing effects on OPN secretion (Figure 4).
clarify the role of the ADAM8 protease function in OPN regulation, U87_shA8 cells were transfected with either active or inactive (E to Q mutation at amino acid position 335) ADAM8. Both, on the mRNA and the protein level, ADAM8 upregulated OPN expression and secretion, however, independent of the protease activity (Supplementary Figure 2).

**In macrophages, ADAM8 is involved in the regulation of angiogenesis via OPN**

As shown above (Figure 1c), ADAM8 is additionally expressed in GBM associated macrophages/microglia (GAMMs) [9] which could contribute to tumor angiogenesis in GBM. To analyze if ADAM8 affects OPN expression in these cells similar to tumor cells, we used primary macrophages isolated from wild-type (WT) and ADAM8-deficient mice (Kelly et al. 2005) and no difference in macrophage development was observed (Bartsch et al., unpublished). Primary macrophages were isolated from bone marrow with a purity of >90% (Figure 5a, b). Supernatants from macrophages were collected and angiogenesis assays using HUVEC cells were performed. After 6 h incubation with supernatants from WT and ADAM8-deficient macrophages, HUVEC tube formation was significantly lower in supernatants from ADAM8-deficient macrophages compared to wild-type macrophages (Figure 5c–e). Similar to U87 cells, we analyzed differential proteins secreted from macrophages using a cytokine array. The strongest difference between WT and ADAM8-deficient macrophages was observed for osteopontin (Figure 5f).

**ADAM8 regulates OPN via JAK/STAT3 and NF-kB in macrophages**

Expression levels of OPN in WT and A8KO macrophages were confirmed by qPCR (Figure 6a) and ELISA assays (Figure 6b). We further analyzed if OPN added to A8KO macrophages restores angiogenesis; 100 ngl OPN restores the decrease of angiogenic ability caused by ADAM8 deletion but had little effect on WT macrophage supernatant (Figure 6c). Signaling pathway inhibitors were used in macrophages. Protein levels of OPN in cell supernatants were determined by ELISA. Inhibition of JAK/STAT3 and NF-kB caused a significant reduction of OPN expression in WT and A8KO macrophages (Figure 6d, e), suggesting that ADAM8 is one of the upstream proteins to regulate OPN expression through JAK/STAT3 and NF-kB.

**Discussion**

Expression of ADAM8 has been described to be involved in tumor cell invasion in gliomas (Wildeboer et al. 2006) and
Figure 5: ADAM8 regulates the angiogenic potential of macrophages.

(A, B) Enrichment of bone marrow derived macrophages (BMDM) from WT and ADAM8-deficient (A8KO) mice according to the protocol described in the MandM section. CD11b was used as macrophage marker to quantify purity of macrophage preparation. In WT bone marrow derived macrophages, ADAM8 (m-A8, green) and CD11b (red) stainings are positive. In bone marrow macrophages derived from ADAM8-deficient mice, only CD11b is positive. (C, D) With WT macrophage supernatants, HUVECs form tube-like structure after 6 h, whereas A8KO macrophage supernatants lead to smaller numbers of tubes after 6 h. (E) Number of meshes quantified in 20 viewing fields (n = 5). Angiogenesis induced from ADAM8-deficient macrophages is significantly decreased. (F, G) Differential analysis of cytokines and angiogenesis factors in supernatants of WT and A8KO macrophages. Note that osteopontin (OPN, blue box for WT, red box for A8ko) shows the biggest difference between the two experimental groups. White boxes, negative controls, asterisks mark the positive controls. Scale bar in (A) and (B) corresponds to 100 μm, (C) and (D) corresponds to 500 μm. Data from multiple replicates are presented as mean values and error bars indicate mean ± SEM.*p < 0.05, **p < 0.01, ***p < 0.001 by Student’s t-test.
high expression levels are unfavorable for glioma patients (He et al. 2012). More recently, ADAM8 expression was correlated to the occurrence of glioma-associated macrophages/microglia (GAMMs, Gjorgjevski et al. 2019), however the function of ADAM8 in tumor cells and in GAMMs remains elusive and tumor promoting effects in the glioma microenvironment can be postulated, since in all tumor entities described so far, ADAM8 is responsible for tumor invasion, migration, and chemoresistance, leading to poor clinical outcomes (Conrad et al. 2019).

We found that GBM tissues investigated from our tumor collection (n = 50 patients) show elevated expression of ADAM8. In GBM tissues, ADAM8, in addition to tumor cell expression, is co-localized with markers for macrophages (CD68), NK cells and neutrophils. By far, the most frequent cell types co-stained with ADAM8 are macrophages, suggesting that ADAM8 is important for tumor cell-macrophage communication in the tumor microenvironment. One of the most important functions of ADAM8 described so far, is the contribution to neovascularization and angiogenesis (Guaqil et al. 2010; Romagnoli et al. 2014). Thus, it is likely that the presence of ADAM8 in GBM is associated with increased angiogenesis. To analyze if angiogenesis induction is based on a systemic effect, we initially investigated tumor cells and macrophages separately in vitro using genetic means to reduce the ADAM8 gene dosage. Our results show that ADAM8 induced in GBM can either be expressed in tumor cells or in GAMMs. As demonstrated previously, there is no association of ADAM8 expression with a certain macrophage polarization state (M1/M2), so we can conclude that potentially both macrophage states could contribute to the observed angiogenesis effect (Dai et al. 2009). As an active metalloprotease, ADAM8 can cleave substrates relevant for angiogenesis, as shown in the context of retinal neovascularization (Guaqil et al. 2010). However, in this experimental setting, ADAM8 as a sheddase of angiogenesis factors such as Tie-2, VE-cadherin, and CD31 has an

Figure 6: In macrophages, ADAM8 regulates OPN secretion via JAK/STAT3 and NF-κB. (A,B) Relative OPN expression was determined by qPCR and OPN protein levels in supernatants by ELISA. A8KO macrophages express less OPN than WT macrophages. (C) By adding 100 ng/μl OPN, defective angiogenesis of HUVEC cells caused by ADAM8 deficiency can be restored. (D) Treatment of primary macrophages with signaling pathway inhibitors for p38, ERK1/2, PI3K/AKT, NF-κB, JAK/STAT3 followed by quantification of OPN using an ELISA. OPN expression of A8WT and A8KO is decreased after addition NF-κB and JAK/STAT3 inhibitors. (E) macrophages treated with NF-κB inhibitor and JAK/STAT3 inhibitor. Note that OPN expression is significantly lower than under control conditions (set to 1). Data from multiple replicates are presented as mean and error bars indicate mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001, Student’s t-test and two-way ANOVA.
opposite effect on angiogenesis i.e., blocks angiogenesis by inactivating angiogenesis-related proteins. By investigating the release of angiogenesis related molecules, we noticed that osteopontin is regulated by \textit{ADAM8} at the transcriptional level, leading to decreased levels of both, RNA and secreted OPN in tumor cells and macrophages as a result of reduced \textit{ADAM8} levels. Thus, \textit{ADAM8}, via a non-proteolytic function (Supplementary Figure 2), can modulate intracellular signaling in tumor cells and in GAMMs, thereby potentially affecting tumor angiogenesis in GBM patients. We admit that using HUVEC cell angiogenesis provides only a clue to the impact of \textit{ADAM8} in GBM angiogenesis, since we analyze a general angiogenic property and not particularly tumor angiogenesis as observed in GBM. However, OPN has been proven to be a potent effector in GBM angiogenesis and our \textit{in vivo} experiments prove this concept despite these limitations.

Further we confirm that OPN could indeed promote the angiogenesis ability of HUVEC cells, we performed OPN "rescue" assays. When adding OPN to U87 cell supernatant of \textit{ADAM8} knockdown cells, OPN restores the ability of U87 cells to promote angiogenesis. In a study investigating the functional effect of OPN on HUVEC cells (Dai et al. 2009), a concentration of up to 5 μM (44 µg/ml) was used to activate PI3K/AKT and ERK1/2 signaling pathways. In our study, we used up to 200 ng of OPN corresponding to 0.2 μM, a concentration that is highly potent in inducing angiogenesis. For wild-type U87 supernatants, tube formation was only slightly increased compared to wild-type U87 supernatants without OPN. This indicates that at this point the ability of tube formation of HUVECs reached saturation. In contrast, when OPN was added to U87_shA8 cell supernatants, the ability of tube formation reached similar levels than the angiogenesis potential of U87_shCtrl cells. This prompted us to conclude that OPN is an \textit{ADAM8}-dependent factor essential for HUVEC tube formation and for tumor angiogenesis. In this regard, there are numerous studies supporting the notion that OPN promotes tumor angiogenesis. OPN was found along with VEGF in leukemia, involved in angiogenesis and chemoresistance (Mirzaei et al. 2018). In primitive connective tissue, OPN contributes as a key element to enhance osteogenesis and angiogenesis (Carvalho et al. 2020). In endothelial cells, OPN stimulates angiogenesis via phosphorylation and activation of the PI3K/AKT and ERK1/2 pathway (Ramchandani and Weber 2015). In conclusion, OPN has a general effect on angiogenesis promotion on different types of cells and expression of OPN in GAMMs is particularly essential for angiogenesis in GBM (Guaiquil et al. 2010). In addition, OPN is essential for infiltration of GAMMs in GBM (Wei et al. 2019). In GBM patients, elevated OPN levels were detected in serum samples, and their levels correlate with poor prognosis (Sreekanthreddy et al. 2010)

Our study provides novel evidence that \textit{ADAM8} regulates OPN expression and secretion. In order to further understand the mechanism of this regulation, several signal pathway inhibitors were used to block the specific pathways of NF-κB (Berbamine dihydrochloride), JAK/STAT3 (WP1066), P38 (SB203580), ERK1/2 (U0126), and PI3K/AKT (LY294002). We found that predominantly blocking JAK/STAT3 can significantly reduce expression levels of OPN, while other pathway inhibitors have an opposite (NF-κB) or no effect (p38, ERK1/2, PI3K/AKT) on OPN release in U87_shCtrl, but not in U87_shA8 cells. Based on these findings, we can conclude that \textit{ADAM8} derived from U87 can promote angiogenesis through OPN via the JAK/STAT3 pathway. STAT3 in particular was described as an important factor for glioma infiltration and growth (Priester et al. 2013) and the activation of this pathway by \textit{ADAM8} presents a novel finding.

Interestingly, a similar regulation of OPN by \textit{ADAM8} was also found in bone-marrow derived macrophages. To analyze this, we used primary macrophages from \textit{ADAM8}-deficient mice as the most suitable cell model. In these macrophages, not selected for a certain polarization type, expression of OPN decreased in the absence of \textit{ADAM8}, so that the ability to stimulate angiogenesis \textit{in vitro} decreased accordingly. Analogous to the experiments with U87 cells, exogenous addition of OPN restores the observed decrease of angiogenesis caused by \textit{ADAM8} deficiency, demonstrating that an \textit{ADAM8} to OPN signaling cascade is mode of action in these cell types with regard to angiogenesis. Considering macrophages as an eminent cell type in the microenvironment of GBM, elimination of \textit{ADAM8} in the tumor microenvironment could cause deprivation of OPN, thereby reducing neovascularization of the tumor blood supply. In contrast to U87 cells, OPN secretion in WT and in \textit{ADAM8}-deficient macrophages can be reduced by the JAK/STAT3 inhibitor. This suggests that there is an \textit{ADAM8}-dependent and -independent signaling for JAK/STAT3 activation in macrophages. However, the \textit{ADAM8}-independent OPN regulation occurs almost at a very basal level of expression. In macrophages, there is an additional effect of NF-κB inhibition on OPN expression.

So far it was unknown how \textit{ADAM8} affects OPN expression and secretion. We have seen that the effect of \textit{ADAM8} on OPN is independent of its metalloprotease function. Rather, the disintegrin domain and signaling via the cytoplasmic domain seem to be involved in OPN regulation. A few clues came from previous research. In our previous studies of pancreatic cancer, we found that the expression of \textit{ADAM8} in pancreatic cancer cell lines Panc1
and AsPC-1 is related to migration and apoptosis. Invasiveness of pancreatic ductal carcinoma induced by activation of PI3K/AKT and ERK1/2 suggested that ADAM8 is highly correlated with PI3K/AKT and ERK1/2 signaling pathways (Schlomann et al. 2015). A previous study found that ADAM8 promotes migration and invasion of chondrosarcomas by activating NF-κB/MMP-13 (Liu et al. 2019). JAK/STAT3 is related to angiogenesis in some studies (Barry et al. 2007; Valdembri et al. 2002; Xue et al. 2017). By ELISA analysis, we found that the amount of secreted OPN in macrophages stimulated by JAK/STAT3 and NF-κB inhibitors decreased significantly, suggesting that OPN is regulated by these pathways. As the major target protein of ADAM8, we identified OPN regulated by ADAM8 via JAK/STAT3 signaling in GBM cells and in macrophages, thereby revealing a potential role of ADAM8 in angiogenesis.

In summary, our study identified ADAM8 as a relevant metalloprotease-disintegrin in GBM. Since ADAM8 is present in tumor cells and in GAMMs, we hypothesize that ADAM8 is an important modulator of the tumor microenvironment. In this respect, the extent of angiogenesis we have analyzed in vivo could be underestimated since we expect GAMM-derived OPN to additionally contribute to tumor angiogenesis in GBM. Considering that OPN derived from tumor cells is an important factor for recruitment of macrophages from the periphery (Wei et al. 2019), the additional OPN levels secreted from macrophages could form an angiogenesis-supporting microenvironment in GBM. Further studies will prove for these mechanisms in an animal model and further exploit these mechanisms in vivo with the goal to establish ADAM8 as a drug target in glioblastoma.

Materials and methods

Patient specimens

Approval from the local ethics committee (file number 185/11) was obtained to collect tumor tissue samples from patients who underwent surgical resection of GBM after providing written consent. Tumor tissue was either shock-frozen in liquid nitrogen (biochemical analyses) or embedded in paraffin for immunohistochemistry.

Cells

U87 MG cells were obtained from ATCC and their authenticity was checked by STR profiling and karyotype analysis with X- and Y-specific FISH probes. Cells were grown in DMEM with 10% FCS. HUVEC cells were purchased from Promo Cell (Heidelberg, Germany) and maintained according to the manufacturer’s instructions.

To generate U87 MG cells with an ADAM8 knockdown, cells were transfected with four shADAM8 constructs (TF314048, Origene, Rockville, MD, USA with the target sequence 5′-GCGGCACCTGCATGACAAGTACAGCTCA-3′) cloned into pRFP-C-RS vector using Lipofectamine LTX (Invitrogen, USA). Twenty-four hours after transfection, cells were treated with the respective selection antibiotic (1 mg/ml puromycin). Around 15 cell clones were selected and successful knockdown of ADAM8 was confirmed. For generation of control cell clones, a construct with a scramble shRNA (target sequence 5′-GCACTACACAGATGACTAGTACT-3′) sequence was used. ADAM8 expression in single U87 MG cell clones was analyzed by qRT-PCR, western blotting and ELISA.

Mice

ADAM8-deficient mice are kept on a C57Bl/6 background for at least 10 generations. For genotyping, diagnostic PCR assays were performed as described (Schlomann et al. 2015). For in vivo experiments, six-week-old athymic mice were obtained from Harlan (Indianapolis, USA). All animal experiments were performed in accordance with the German laws on the protection of animals (“Tierschutzgesetz”) and with permission by the local authorities (Regierungspräsidium Gießen V54-19c2015(1) MR20/18, and G60_2016).

Mouse bone marrow-derived macrophage isolation and culture

Mouse macrophages were isolated from mouse bone marrow. Mice were sacrificed by cervical dislocation, and femur and tibia were removed to flush bone marrow out with completed culture medium (10% FCS, 20 ng/ml M-CSF, DMEM) using a 2G needle. After separating the cells using a cell strainer (pore size 100 µm), cells were seeded in a petri dish in 10 ml complete medium. At day 4, 5 ml of the complete medium were replaced. After 7 days of incubation, macrophages were used for functional experiments.

Proliferation assays

Cells were plated at 5000 cells/well in 96-well plates and cultured for 1, 2, 3, 4 and 5 days. On the indicated days, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent (AMRESCO, Solon, OH, USA) was added and incubated for 4 h at 37 °C, 5% CO2. The supernatant was discarded and replaced with 200 µl DMSO (Sigma-Aldrich Co., St. Louis, MO, USA) to dissolve the formazan product. Absorbance at 570 nm was determined in a 96-well-plate reader (BMG Labtech, Offenburg, Germany).

Scratch assays

Cell migration was determined by scratch assays in vitro in culture-insert microdishes (Ibidi GmbH, Planegg, Germany) according to the manufacturer’s instructions. Cells were seeded into each chamber at densities of 1 × 10^4 cells/ml in 100 µl DMEM medium with 10% FCS. After incubation at 37 °C, 5% CO2 for 24 h and serum starving for another 6 h, inserts were removed and 1 ml DMEM medium with 2% FCS was added into each dish. Images were taken immediately after insert removal and 18 h later. Rates of cell migration were determined.
by counting cells in the gaps (n = 3 viewing fields per plate in three independent experiments) using Image J software.

Immunofluorescence microscopy

1000 cells were seeded per well in an eight well chamber on glass (Lab-Tek, 4808), fixed and stained with antibodies (CD11b: Thermo Fisher, MAI-80091; mouse ADAM8: Biorbyt, orb4376; human ADAM8: R&D Systems, AF1031).

Angiogenesis tube formation assay

1 × 10^5 macrophages/U87 cells (ADAM8 deficient compared to respective control) were seeded in a six well in 1 ml of a 1:1 mix serum free DMEM and ECM MEDIUM (Promo Cell, C-22010). After 24 h, supernatant was collected and cleared by centrifugation at 1000 rpm for 5 min. 6 × 10^5 HUVECs per well were seeded on Angiogenesis Plate (IBIDI, 81506). After particular time points (Macrophages: 6 h; U87: 15 h), three pictures (center, upper left and lower right) were taken from each well and meshes formed were counted. For the OPN "rescue" assay, 2.5 ng/ml OPN (PeproTech, 120-35) was added to macrophage and 50 ng/ml OPN to U87 supernatants.

Cytokine array

A membrane-based antibody array [Proteome Profiler® Mouse XL Cytokine Array ARY028, RandD Systems, Abingdon, UK] was used to identify cytokine expression levels under different conditions, according to the manufacturer’s protocol. Briefly, conditioned supernatants of U87_shA8 and U87_shCtrl cells were incubated with a biotinylated antibody mix for detection on the nitrocellulose membrane by chemiluminescence. Quantification of dot optical intensities was performed using Image J software.

Real-time quantitative PCR

To determine gene expression, real-time quantitative PCR with SYBR Green (Precision® FAST master mix) on StepOnePlus Real-Time PCR System was performed using commercially available primers for m-ADAM8 (Qiagen, Hilden, Germany), m-SPP-1 (Qiagen), h-ADAM8 (Qiagen), h-SPP-1 (Apara Bioscience, Freiburg, Germany), RPLPO (Qiagen) was used as reference gene for normalization. Relative gene expression was calculated using comparative 2^-ΔΔCt method.

ELISA

Human and mouse osteopontin levels in cell culture supernatants were determined by ELISA kits for human OPN (R&D systems, DY1433, Southampton, UK) and mouse OPN (R&D Systems, DY441) according to the manufacturer’s instructions.

Statistical analysis

Unpaired two-tailed Student’s t-test was used to compare values from two independent groups. For multiple comparisons, Two-way ANOVA and multiple comparisons were used. Data from multiple replicates are presented as mean and error bars indicate mean ± SEM from at least three independent samples. Based on the obtained results, the data were considered not significant (p > 0.05), significant (*p < 0.05), highly significant (**p < 0.01) or very highly significant (***p < 0.001). Calculations were performed using the GraphPrism statistical analysis software.

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Supplementary Material
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