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ORIGINAL ARTICLE

Overexpression of TIMP-1 and Sensitivity to Topoisomerase Inhibitors in Glioblastoma Cell Lines

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Abstract The multifunctional protein - tissue inhibitor of metalloproteinases-1 (TIMP-1) - has been associated with a poor prognosis in several types of cancers including glioblastomas. In addition, TIMP-1 has been associated with decreased response to chemotherapy, and especially the efficacy of the family of topoisomerase (TOP) inhibitors has been related to TIMP-1. As a second line treatment of glioblastomas, the vascular endothelial growth factor (VEGF) antibody bevacizumab is administered in combination with the TOP1 inhibitor irinotecan and glioblastoma cell levels of TIMP-1 could therefore potentially influence the efficacy of such treatment. In the present study, we aimed to investigate whether a high TIMP-1 expression in glioblastoma cell lines would affect the sensitivity to TOP inhibitors, and whether TIMP-1 overexpressing cells would have alterered growth and invasion. We established TIMP-1 overexpressing subclones from two human glioblastoma cell lines. TIMP-1 overexpressing U87MG cells were significantly more resistant than low TIMP-1 expressing clones and parental cells when exposed to SN-38 (TOP1 inhibitor) or epirubicin (TOP2 inhibitor). No significant differences were observed for the TIMP-1 transfected A172 cells. Implantation of both U87MG and A172 spheroids into organotypic brain slice cultures revealed a reduced growth of TIMP-1 overexpressing U87MG spheroids, however, no significant differences in invasion were observed. The present study suggests that TIMP-1 overexpression reduces the effect of TOP inhibitors in glioblastoma. TIMP-1 also appeared to reduce spheroid growth, but did not influence invasion. Whether TIMP-1 plays a role in irinotecan resistance and has a predictive potential in glioblastoma patients remains to be elucidated.

Keywords Glioblastoma · TIMP-1 · TOP inhibitors · Chemosensitivity

Introduction

The most frequent primary brain tumor, the glioblastoma, is known for being highly malignant and invasive, making it practically impossible to remove surgically. Moreover, glioblastomas are known to be highly resistant towards chemotherapy and thereby remain incurable [1]. Several mechanisms, in addition to the existence of the supposed tumor stem-like cells, have been suggested to be involved in glioblastoma drug resistance [2–8]. Though, the more exact mechanisms behind chemoresistance appear complex and not fully understood.

The family of tissue inhibitors of metalloproteinases (TIMPs) is known to regulate the proteolytic activity of the extracellular matrix degrading matrix metalloproteinases (MMPs), making TIMPs interesting in cancer biology in regard to cell motility and invasion [9–12]. However, in contrast to an inhibitory effect of TIMP-1 on MMPs in cancer progression, TIMP-1 has been

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associated with cancer promoting properties such as increased tumor cell proliferation, increased angiogenesis and decreased sensitivity to chemotherapy [10, 13–19]. In clinical cancer studies, high TIMP-1 tumor cell or plasma levels have consistently been associated with a poor outcome for patients with colorectal and breast cancer among others [20–26]. We have previously reported that high tumor TIMP-1 protein immunoreactivity in glioblastomas predicted shorter overall survival [27]. In addition to this, a number of preclinical and clinical studies have shown an association between high tumor cell or plasma TIMP-1 protein levels and increased resistance to chemotherapy, especially in regard to treatment with topoisomerase (TOP) inhibitors [28–32].

More recently, the TOP1 inhibitor irinotecan has been introduced in combination with the vascular endothelial growth factor (VEGF) antibody bevazicumab as second line treatment of glioblastomas [33], making a possible association between TIMP-1 and TOP inhibitors increasingly interesting. Topoisomerases are protein complexes formed to solve the topological challenges associated with DNA unwinding during replication or transcription by introducing temporary breaks in the DNA. Irinotecan exerts its cytotoxic effect through its active metabolite SN-38, which stabilizes the DNA-TOP1 cleavable complexes preventing TOP1 to ligate the DNA strands after cleavage. These DNA damages lead to arrest of the DNA replication and apoptosis [34, 35].

Based on these data, we raised the hypothesis that TIMP-1 affects the cytotoxicity of TOP inhibitors in glioblastomas. In the present study, the aim was to investigate whether high levels of TIMP-1, in the two glioblastoma cell lines U87MG and A172, have an impact on the effect of TOP inhibitors. We transfected U87MG and A172 with a construct, conferring high TIMP-1 expression and protein production, and low and high TIMP-1 expressing subclones were isolated. Selected clones were exposed to a TOP1 inhibitor (SN-38, the biologically active irinotecan derivate) or a TOP2 inhibitor (epirubicin), and cell viability and cell death was evaluated. As mentioned above, invasive behaviour is one of the characteristic hallmarks in glioblastomas [1]. In the process of tumor cell invasion, degradation of the extracellular matrix is crucial and MMPs, and thereby the TIMPs, could play important roles in this process [9, 12]. As a second aim, we therefore investigated whether high TIMP-1 levels affected tumor cell tumor growth and invasion in an in vivo-like in vitro glioblastoma brain slice culture model.

Materials and Methods

Cell Line Culturing

The two adherent glioblastoma cell lines U87MG and A172 (obtained from ECACC, UK) were included in the present study. The cells were cultured in a standard medium consisting of Dulbecco Modified Eagle Medium (Sigma-Aldrich), 10% fetal calf serum (Sigma-Aldrich), 2% glutamine (LifeTechnologies), 4% nonessential amino acids (LifeTechnologies), 2% penicillin/streptomycin (LifeTechnologies), and 2 mM sodium pyruvate (Sigma-Aldrich). To obtain cell line-derived spheroids, the cells were cultured in 0.75% agar-coated culturing flasks under the same conditions as described above.

Transfection of Cell Lines

The two glioblastoma cell lines A172 and U87MG were transfected with a pcDNA-3.1(Hyg)-TIMP-1 vector [36] or an empty vector as control. The cells were transfected using a Nucleofector™ 2b device (Lonza). Based on the manufacturer's instructions, the Cell Line Optimization Necleofector® Kit (Lonza) was used to create the optimal protocol for each cell line. In brief, seven different protocols were tested with two different transfection solutions. At first, the cells were transfected with pmaxGFP™-Green and cultured for 24 h. The following day, transfection efficiency and viability was determined using an Accuri C6 Flow Cytometer (BD Biosciences) for analysis. After optimization, 5×10^5 cells were transfected with 2 μg pcDNA-3.1/Hygro(−) with inserted TIMP-1 or an empty vector as a control.

Following the transfection, cells were selected using hygromycin (Sigma-Aldrich). As a control, untransfected cells were equally exposed to hygromycin. When the control cells were eliminated, the transfected cells were considered fully selected.

Dilution cloning was performed to isolate single-cell subclones expressing high and low levels of TIMP-1, respectively. The parental cell lines (U87MG and A172), controls transfected with an empty vector (U87MG_C and A172_C) and single cell clones expressing high levels of TIMP-1 (U87MG_TH1–3 and A172_TH1–3) or low levels of TIMP-1 (U87MG_TL and A172_TL) were included in the study.

The protein level of TIMP-1 in the cells as well as in the cell culturing medium was assessed for all cell lines i.e. controls and subclones. Cells were seeded in 6 cm petri dishes (Techno Plastic Products) in duplicates. After reaching a confluence level of 90% the culturing medium was collected and stored at −80 °C, and the cells were re-suspended in 120 μl lysis buffer (Mammalian protein extraction reagent, Thermo Scientific) supplemented with protease inhibitor cocktail set I (Calbiochem) and phosphatase inhibitor cocktail set II (Calbiochem), according to the manufacturer's instructions, and stored at −80 °C. The TIMP-1 protein levels in the medium and in the cells were estimated using a well-established TIMP-1 enzyme-linked immunosorbent assay (ELISA) [37, 38].

Finally, growth rates and doubling times of parental cell lines, subclones and controls were estimated. Cells were seeded in 6-well plates (45,000 cells/well) in 2 ml cell culturing medium. The cell number was estimated in triplicates using a Scepter™ cell counter (Millipore) at day 1–5, and the doubling times were calculated by t = ln2/k, where k is the cell growth rate estimated from the exponential regression model.

Chemotherapeutic Drugs

SN-38 (Tocris) and epirubicin (Sigma-Aldrich) were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and kept at −80 °C. At the day of cell exposure the stock solution was further diluted in cell culturing medium just prior to exposure. The final DMSO concentration in the wells with the highest chemoconcentration did not exceed 0.1%. A DMSO control was included in all experiments, and DMSO had no effect on the cells.

Cell Viability Assay (WST-1) and Cell Death Measured by LDH

The inhibition of proliferation was estimated using the WST-1 assay (Roche), based on the reduction of the tetrazolium salt WST-1 [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H–tetrazolium] by viable cells. Cell death was estimated using a lactate dehydrogenase (LDH) release assay (Cytotoxicity Detection Kit – LDH, Roche). Cells were seeded in a 96-well plate in cell culturing medium (described above) with 2500 cells/well for U87MG and 2000 cells/well for A172. For each cell line, two high TIMP-1 expressing clones (U87MG_TH1–2 and A172_TH1–2), one low TIMP-1 expressing clone (U87MG_TL and A172_TL) and the empty vector transfected control cell line (U87MG_C and A172_C) were included in each plate. After 24 h, SN-38 or epirubicin was added (15 nM – 400 nM). The A172 cells were exposed for 48 h, and U87MG cells were exposed for 72 h. Release of LDH was measured in accordance with manufacturer's instruction. In brief, the cell supernatant was transferred to a 96-well plate, and LDH reagent was added (1:1). After incubation for 30 min, the optical density at 490 nm (and 690 nm as reference) was measured using an ELISA reader (Buck & Holm).

The remaining culture medium was discarded and replaced by fresh medium and WST-1 (1:9) according to manufacturer's instructions. The plates were incubated for additionally three hours, and the optical density was measured at 450 nm (and 690 nm as reference). Finally, as a control all cell lines were exposed to the highest concentration of DMSO used in the study,. Each experiment was performed in triplicates and at least three times.

Tumor Invasion in Brain Slice Cultures

The effect of cellular TIMP-1 expression on glioblastoma tumor cell invasion was investigated using a well-established in vivo-like in vitro model where spheroids are implanted into organotypic rat brain slice cultures as described in a previous study [39]. The use of animals for organotypic brain slice cultures was approved by The Animal Experiments Inspectorate in Denmark (permission J. No. 2008/561–1572). The rats were decapitated and the brains were removed. In total 40 brains from new-born Wistar rat pups were cut into 400 μm thick slices and the corticostriatal slice cultures were placed on sterile porous membranes (Millipore) incubated in 6-well plates (TPP).

Spheroids were derived from the TIMP-1 high expressing clones (U87MG_TH1–3 and A172_TH1–3), the two parental cell lines (U87MG and A172) as well as from the transfection controls (U87MG_C and A172_C). The day prior to implantation, all spheroids were labelled with the fluorescent dye DiI (1,1′ - Dioctadecyl - 3,3,3′,3′ - tetramethylindocarbocyanine iodide, Sigma-Aldrich) for 24 h. Spheroids (200–300 μm) were implanted into brain slice cultures between the cortex and striatum. After implantation, excess spheroids were fixed and paraffin-embedded, and the TIMP-1 expression was investigated immunohistochemically as a control. The co-cultures, each containing one spheroid per slice culture, were incubated using a standard tissue culture incubator (95% humidity, 95% air, and 5% CO_2) at 36 °C. The tumor cell invasion was followed over time using a Nikon inverted confocal microscope with perfect focus system. The invasion assay was performed twice for all cell lines. A Visiomorph classifier was developed to identify and calculate the area of the spheroid and the area of the invasive cells.

At the end of the experiment, the co-cultures were fixed, paraffin embedded, sectioned and investigated immunohistochemically using a human specific vimentin antibody visualizing the human cells invading the rat tissue.

Immunohistochemistry

Paraffin sections of the co-cultures were cut on a microtome and adjacent sections stained with TIMP-1 and vimentin immunohistochemical staining as previously described [27, 39].

Statistics

Data are expressed as mean + SEM. Mean values were compared using Kruskal-Wallis test with Dunn's post test (growth rates) and one-way analysis of variance (ANOVA) with Bonferroni correction (chemosensitivity, spheroid area and invasion area). Statistical analyses were performed using GraphPad Prism (GraphPad, San Diego, USA) or STATA

version 14 (StataCorp LP, USA). The statistical significance level was defined as $p < 0.05$.

Results

TIMP-1 Transfection

Following the TIMP-1 transfection, dilution cloning was performed generating 27 U87MG and 24 A172 single-cell subclones. TIMP-1 protein level in cell lysates and in cell culturing media was estimated for each clone using ELISA. Based on the TIMP-1 levels, high and low expressing clones were selected for the present study (Fig. 1a, b and e). The selected U87MG subclones expressed on average up to 8 and 13 times more TIMP-1, and the A172 subclones expressed up to 22 and 26 times more TIMP-1 than the parental cells (measured in collected cell lysates and culturing medium, respectively).

When comparing growth rates between TIMP-1 transfected clones and parental cell lines, we found that the U87MG growth rates were significantly different ($p = 0.021$). Comparing U87MG to the other clones, no significant

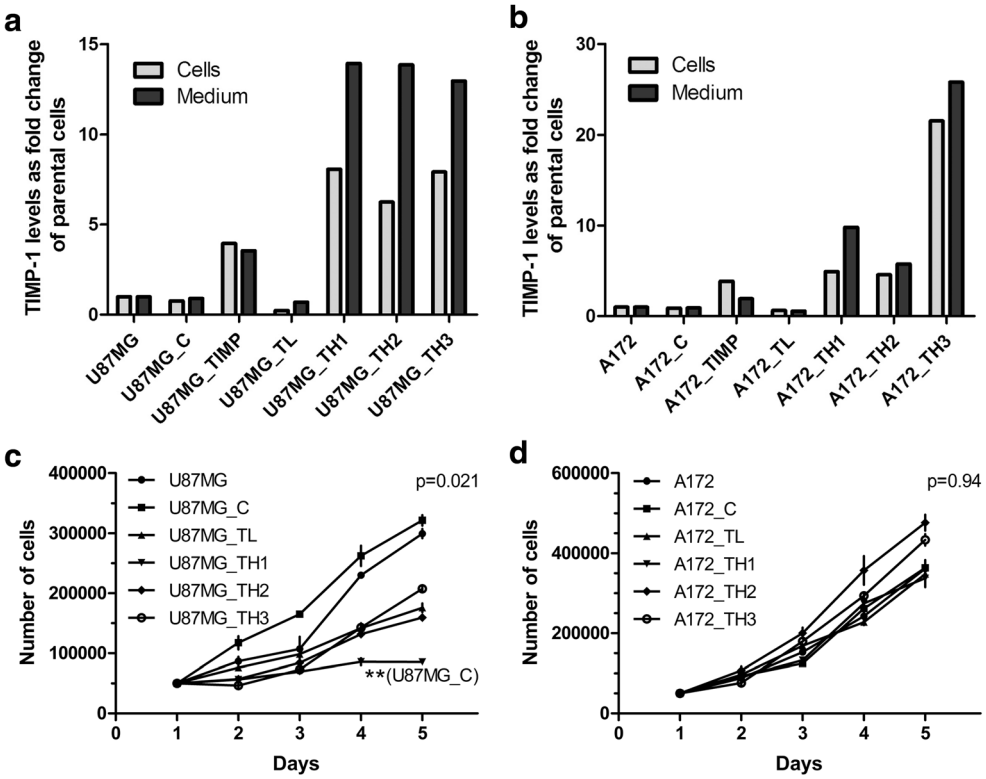

Fig. 1 The levels of TIMP-1 were measured by a well-established ELISA in the cell culturing medium and in cell lysates in the parental cell lines (U87MG and A172), the empty vector controls (U87MG_C and A172_C) and the TIMP-1 transfected cells (U87MG_TIMP-1 and A172_TIMP-1). Dilution cloning was performed and high TIMP-1 expressing subclones (U87MG_TH1–3 and A172_TH1–3) and low expressing subclones (U87MG_TL and A172_TL) were isolated. TIMP-1 fold changes were calculated (**a-b**) from the measured TIMP-1 levels (**e**). To characterize the cells, the growth rates (**c-d**) and doubling times (**f**) were estimated for all cell lines and subclones. ** indicates $p < 0.01$

e

Cell line	$Cell_{TIMP-1}$ (ng/ml)	$Medium_{TIMP-1}$ (ng/ml)
U87MG	168.9	79.7
U87MG_C	130.7	72.1
U87MG_TIMP-1	666.3	281.8
U87MG_TL	40.1	55.9
U87MG_TH1	1363.6	1110.4
U87MG_TH2	1055.3	1104.1
U87MG_TH3	1337.5	1032.1
A172	36.2	34.9
A172_C	32.0	31.7
A172_TIMP-1	137.9	67.4
A172_TL	23.2	19.8
A172_TH1	176.5	342.3
A172_TH2	163.9	200.6
A172_TH3	778.9	902.1

f

Cell line	Doubling time (days)
U87MG	1.4 ± 0.1
U87MG_C	1.9 ± 0.7
U87MG_TL	2.4 ± 0.7
U87MG_TH1	3.4 ± 0.4
U87MG_TH2	1.6 ± 0.4
U87MG_TH3	1.3 ± 0.2
A172	1.4 ± 0.1
A172_C	1.3 ± 0.1
A172_TL	1.6 ± 0.2
A172_TH1	1.2 ± 0.2
A172_TH2	1.2 ± 0.2
A172_TH3	1.0 ± 0.1

differences were found (p = 1.00 for all groups, except for U87MG_TH1 (p = 0.32)). Comparing U87MG_C to the other clones, U87MG_C was significantly different from U87MG_TH1 (p = 0.005), but not from the other clones (U87MG_TL: p = 0.81, U87MG_TH2: p = 0.20, and U87MG_TH3: p = 0.10) (Fig. 1c, f). For A172, we found no overall difference (p = 0.94), and no difference between the individual pairings (p = 1.00 for all comparisons) (Fig. 1d, f).

TIMP-1 and Chemosensitivity

For each cell line, low TIMP-1 expressing clones (U87MG_TL and A172_TL), high TIMP-1 expressing clones (U87MG_TH1–2 and A172_TH1–2) and the transfection controls (U87MG_ C and A172_C) were included (Fig. 2). When exposing U87MG cells to the two TOP inhibitors; SN-38 and epirubicin, significant differences in viability and thus chemosensivity were detected for both drugs (p < 0.001) (Fig. 2a, b). The two clones with high TIMP-1 expression were significantly less sensitive to SN-38 compared to TIMP-1 low expressing cells and controls at all concentrations (p < 0.001) (Fig. 2a). Similar was found for the cells exposed to epirubicin primarily at the low chemoconcentrations (15, 50 and 100 mM) (p < 0.01 or p < 0.001) (Fig. 2b). Further, the cell clone with low TIMP-1 expression showed less sensitivity to SN-38 compared to the control (p < 0.01 at 15, 50, 100, 250 mM and p < 0.001 at 150 mM). Overall, the viability decreased with increasing chemoconcentration being significantly lower for all four clones at 400 mM SN-38 and 400 mM epirubicin compared to 0 mM (p < 0.001). In the cytotoxicity assay, the control clone (U87MG_C) was significantly more sensitive to SN-38 compared to the low-expressing and high-expressing clones (p < 0.01 or p < 0.001) (Fig. 2c). No significant differences in chemosensitivity were detected when the

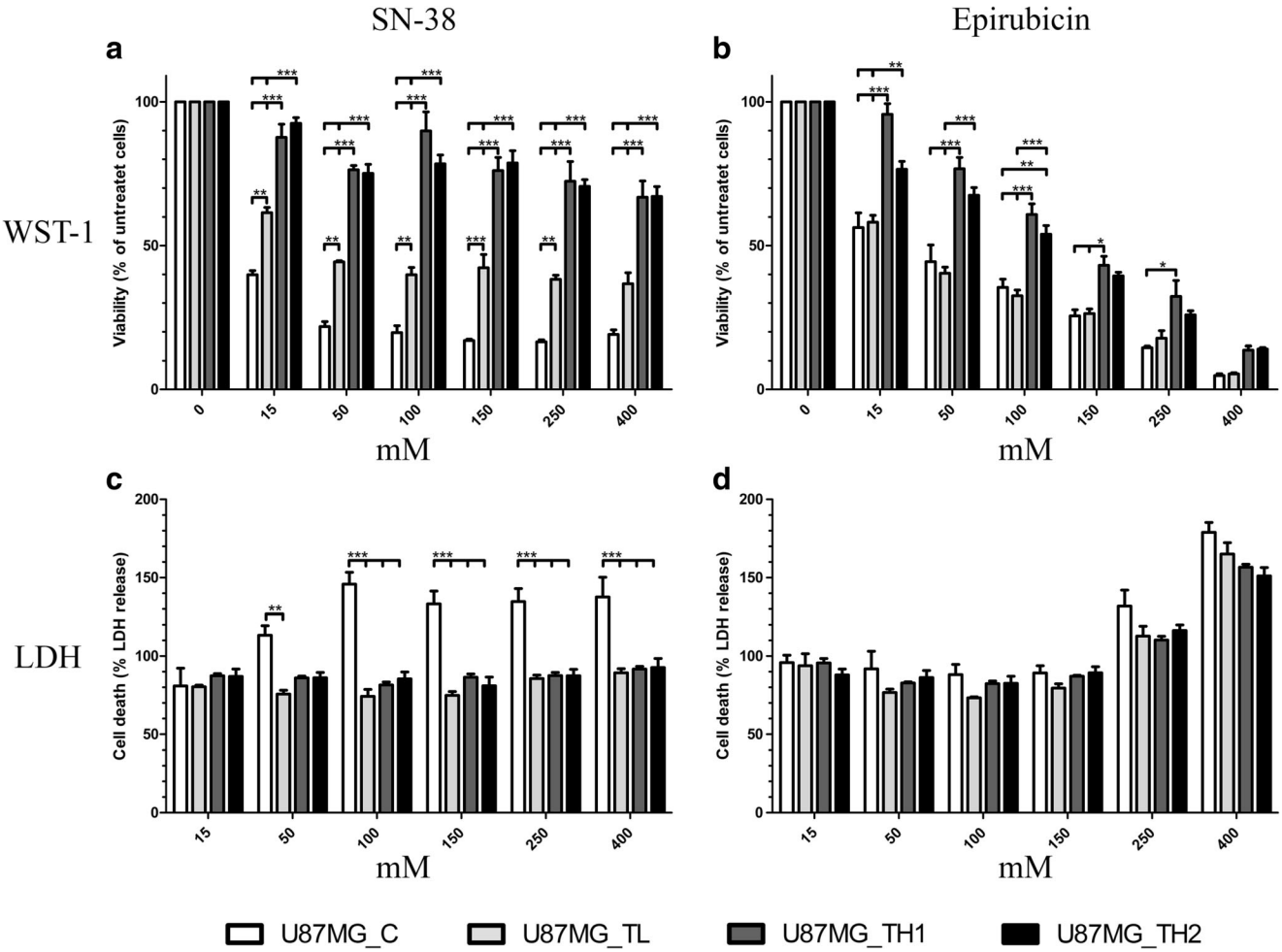

Fig. 2 The chemosensitivity of the TIMP-1 transfected U87MG subclones was assessed using a cell viability assay (**a-b**) and a cell death assay (**c-d**) following exposure to SN-38 (**a, c**) or epirubicin (**b, d**). The two high TIMP-1 expressing subclones (U87MG_TH1–2) were significantly less sensitive to drug exposure than the low TIMP-1 expressing subclone (U87MG_TL) and the control (U87MG_C) when exposed to SN-38 (**a**) or epirubicin (**b**). Using a cell death assay, significantly higher levels of cell death were observed for the control compared to the transfected cells when exposed to SN-38 (**c**), while no significant differences in cell death were observed when the cells were exposed to epirubicin (**d**). Viability and cell death is shown as percentage of untreated cells. * indicates p < 0.05; ** p < 0.01; and ***p < 0.001

four clones were exposed to epirubicin ($p > 0.05$), but the LDH release was significantly highest at 400 mM epirubicin for all four clone ($p < 0.01$ or $p < 0.001$) (Fig. 2d) The chemosensitivity did not significantly differ between the A172 subclones, for either SN-38 or epirubicin.

Brain Slice Cultures

Tumor cell invasion and the area of the implanted spheroids were estimated in the confocal Z-stacks (Fig. 3) for spheroids derived from U87MG ($n = 20$), U87MG_C ($n = 19$), U87MG_TH1 (n = 19), U87MG_TH2 ($n = 22$), U87MG_TH3 ($n = 35$), A172 ($n = 16$), A172_C (n = 16), A172_TH1 ($n = 24$), A172_TH2 ($n = 27$) and A172_TH3 (n = 27). Spheroid area increased over time for U87MG ($p < 0.001$) and U87MG_C ($p < 0.05$) (Figs. 3a-f and 4a), while the area was similar over time for the TIMP-1 transfected cells U87MG_TH1–3 (Fig. 4a). In the confocal images, the invasion varied between spheroids within each group ($p < 0.001$ or $p < 0.05$). The overall invasive patterns were generally similar for all U87MG cell lines (Fig. 4b).

For the spheroids derived from A172 control and transfected cells, the spheroid area tended to decrease over time (Figs. 3g-l and 4c). This was significant for A172_C and A172_TH2 ($p < 0.01$ or $p < 0.001$) which also had or tended to have the highest spheroid area (Fig. 4c). The decreased spheroid area was associated with high levels of invading tumor cells being similar for all A172 spheroids ($p < 0.01$ or $p < 0.001$) (Fig. 4d).

Immunohistochemical staining of the co-cultures at the end of the experiment confirmed elevated levels of TIMP-1 in the implanted spheroids derived from the single cell clones, although the TIMP-1 protein expression in A172 was rather low compared to U87MG (Fig. 5a-d). The sparse invasion of the U87MG spheroids and the pronounced invasion of A172 spheroids detected in the confocal images were confirmed using a human specific antibody against vimentin (Fig. 5e-h). No pyknotic nuclei were identified indicating that all spheroids and brain slices were viable.

Discussion

In the present study, we successfully performed TIMP-1 transfection of the two human glioblastoma cell lines U87MG and A172 and established subclones expressing high or low levels of TIMP-1. The U87MG TIMP-1 high expressing subclones U87MG_TH1 and U87MG_TH2 showed decreased sensitivity when exposed to each of the two TOP inhibitors, SN-38 and epirubicin, suggesting TIMP-1 to play a role in TOP-related sensitivity/resistance. However, for the A172 cell line, no significant differences were observed, suggesting that molecular mechanisms independent of TIMP-1 levels influence sensitivity to TOP inhibitors in glioblastoma cells. By determining growth rates of parental cell lines and their transfected subclones, we could to a great extent exclude that the observed changes in drug sensitivity were due to differences in growth rates. However, the high TIMP-1 expressing U87MG_TH1 subclone had a significantly slower growth rate than the control U87MG_C possibly explaining the reduced chemosensitivity of this subclone. The lower growth rate of U87MG_TH1 should also have resulted in lower viability. However, in the WST-1 cell viability assay we observed the opposite, indicating that we may actually underestimate the TIMP-1-induced resistance. In line with this the U87MG_TL and U87MG_TH2 subclones had identical growth rates but a pronounced and significant difference in viability.

We used a well-characterized pcDNA3.1hyg-TIMP-1 vector [36, 40] for TIMP-1 overexpression in the glioblastoma cell lines. This transfection system has been used previously in breast cancer models investigating the association between high TIMP-1 levels and chemosensitivity [36, 40]. The TIMP-1 ELISA used to determine TIMP-1 production in the different subclones is a validated assay capable of detecting uncomplexed and complexed forms of TIMP-1 [37, 38]. We performed single cell cloning to establish isogenic subclones representing low and high TIMP-1 expression and selected subclones were then used for the experiments.

The decreased chemosensitivity observed for the U87MG cell line is in line with previous findings in breast cancer cell lines where decreased chemosensitivity was observed when exposing TIMP-1 transfected cells to the TOP1 inhibitor SN-38 [40] or to TOP2 inhibitors such as epirubicin [40–42]. These in vitro data have successfully been validated in cancer patients where a significant association between high cancer cell TIMP-1 levels and decreased benefit from adjuvant epirubicin treatment has been shown for breast cancer patients [28, 31, 43], and a significant association was reported for irinotecan treatment and TIMP-1 plasma levels in patients with metastatic colorectal cancer [29, 32]. This association between TIMP-1 levels and irinotecan efficacy is becoming more interesting, since the VEGF inhibitor bevacizumab administered in combination with irinotecan is used as second line treatment of glioblastomas in the US and in Europe [33]. However, whether there is an association between high tumor TIMP-1 levels and the clinical response to irinotecan in glioblastoma patients, as has been reported for colorectal cancer, remains to be validated.

We did not observe differences in sensitivity to the TOP inhibitors between low and high TIMP-1 expressing A172 subclones. In general, the obtained levels of TIMP-1 protein expression in the transfected A172 subclones were lower than TIMP-1 levels obtained in the transfected U87MG subclones, although the fold changes, when compared to parental cells, were higher. TIMP-1 levels in the

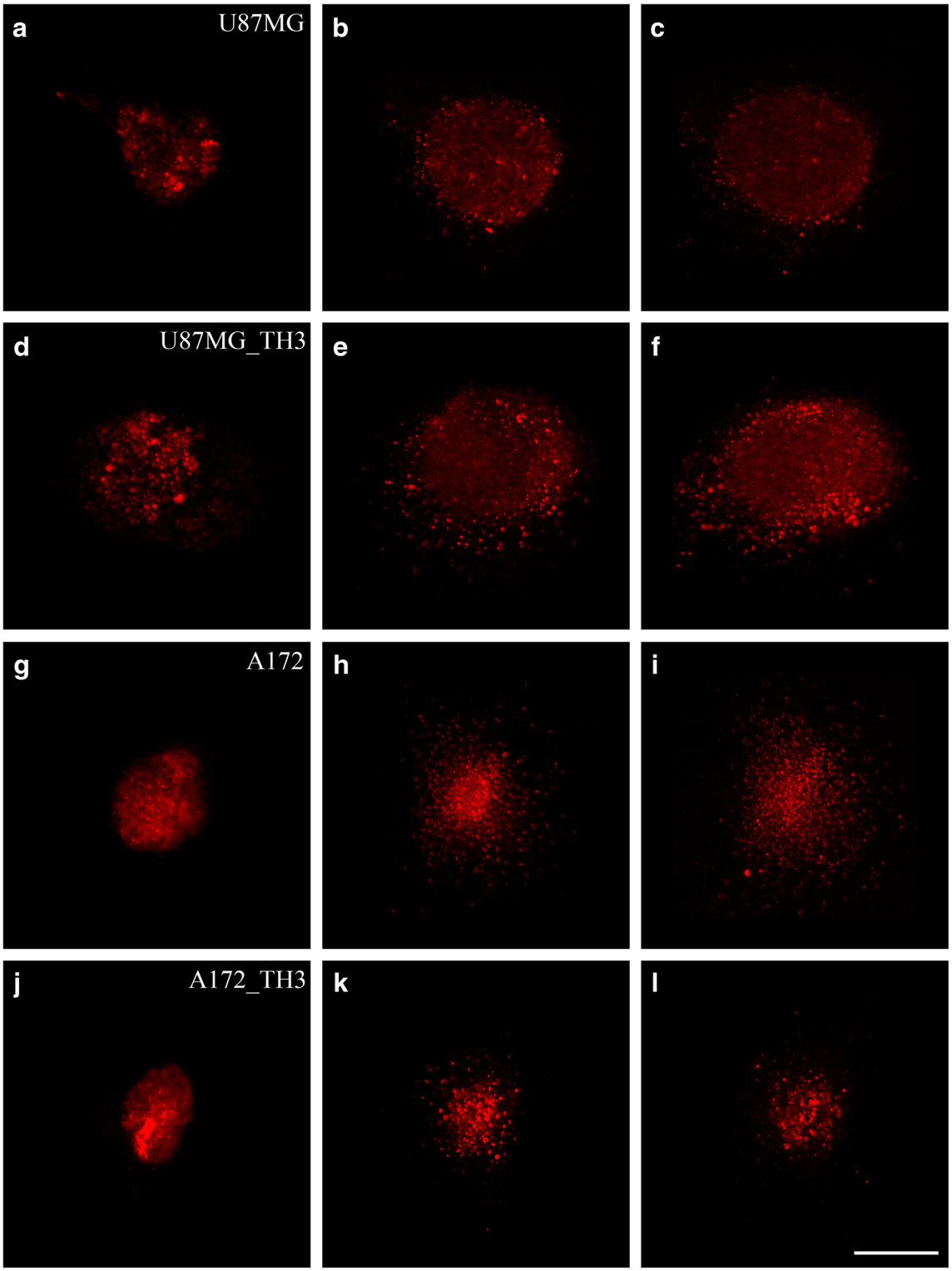

Fig. 3 Spheroids derived from U87MG and A172, the high TIMP-1 expressing subclones (U87MG_TH1–3 and A172_TH1–3) and controls (U87MG_C and A172_C) were implanted into organotypic brain slice cultures, and the invasion was followed for 6 days. Confocal Z-stacks were obtained at the day of implantation (**a, d, g, j**), day 3 (**b, e, h, k**) and day 6 (**c, f, i, l**) and the images in the Z-stacks were superimposed into one image. In general, the U87MG spheroid area increased over time (**a-c**), and only few invasive tumor cells were observed. The U87MG_TH1–3 subclones displayed different invasion patterns with most spheroids showing limited invasion, whereas few spheroids (U87MG_TH3) showed what appeared to be increased invasion compared to the parental cells (**d-f**). The area of the A172 spheroids decreased over time, and the cell line was highly invasive (**g-i**), whereas the invasion of the A172_TH1–3 A172 spheroids appeared to decrease (shown for A172_TH3) (**j-l**). Scalebar 250 μm

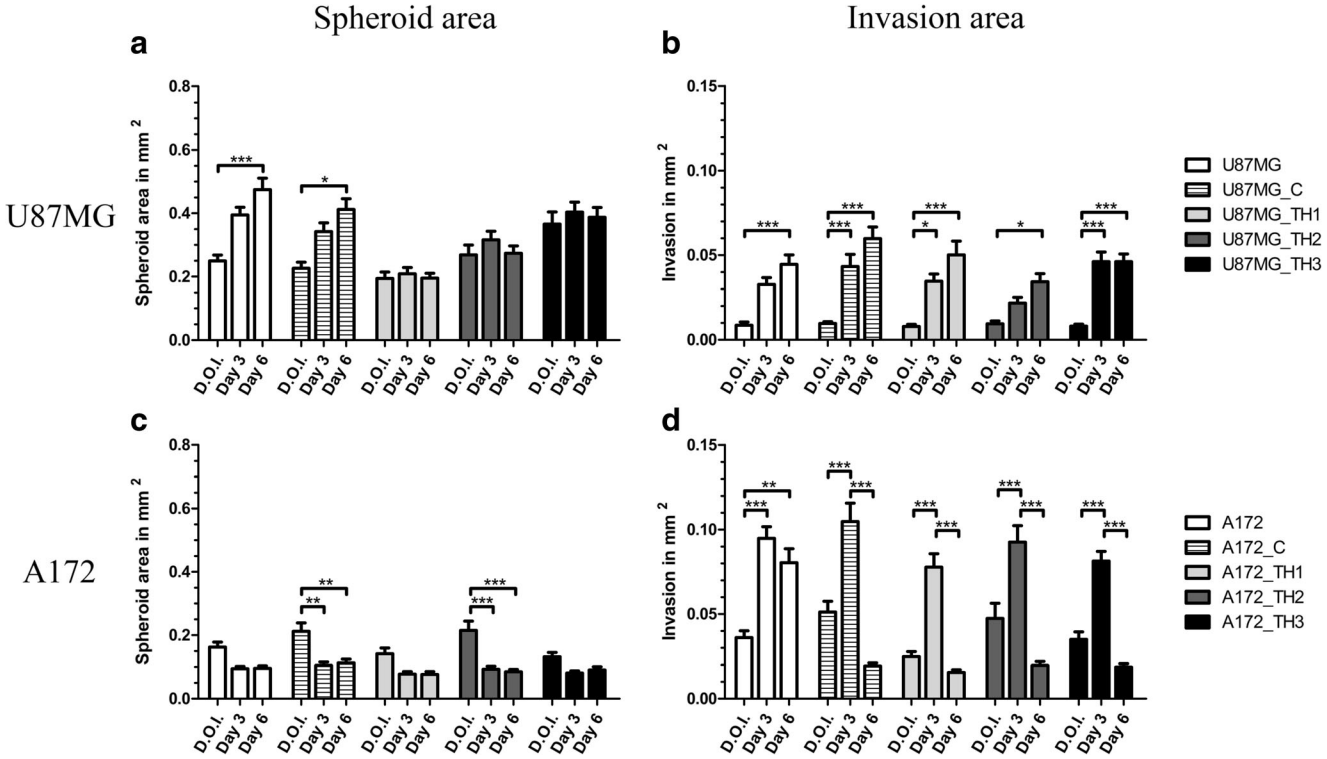

Fig. 4 Spheroids derived from the parental cell lines (U87MG and A172), controls (U87MG_C and A172_C) and high TIMP-1 expressing clones (U87MG_TH1–3 and A172_TH1–3) were implanted into brain slice cultures. The area of the implanted spheroids and the area of the invasive cells were estimated in the confocal images using Visiomorph software. For the U87MG-derived spheroids and controls, the area increased significantly over time, whereas the area was rather constant for the TIMP-1 transfected spheroids (**a**). In general, the level of invasion was similar for all subclones (**b**). The area of all the A172-derived spheroids decreased significantly or tended to decrease over time (**c**). All A172 subclones had the greatest invasion area at day 3 (**d**). * indicates $p < 0.05$; ** $p < 0.01$; and ***$p < 0.001$

A172 subclones may have been too low to affect the chemosensitivity in this cell line. Glioblastomas are very heterogeneous tumors and cell lines established from these tumors show different behaviour and properties, which may also explain the different results obtained with the two cell lines.

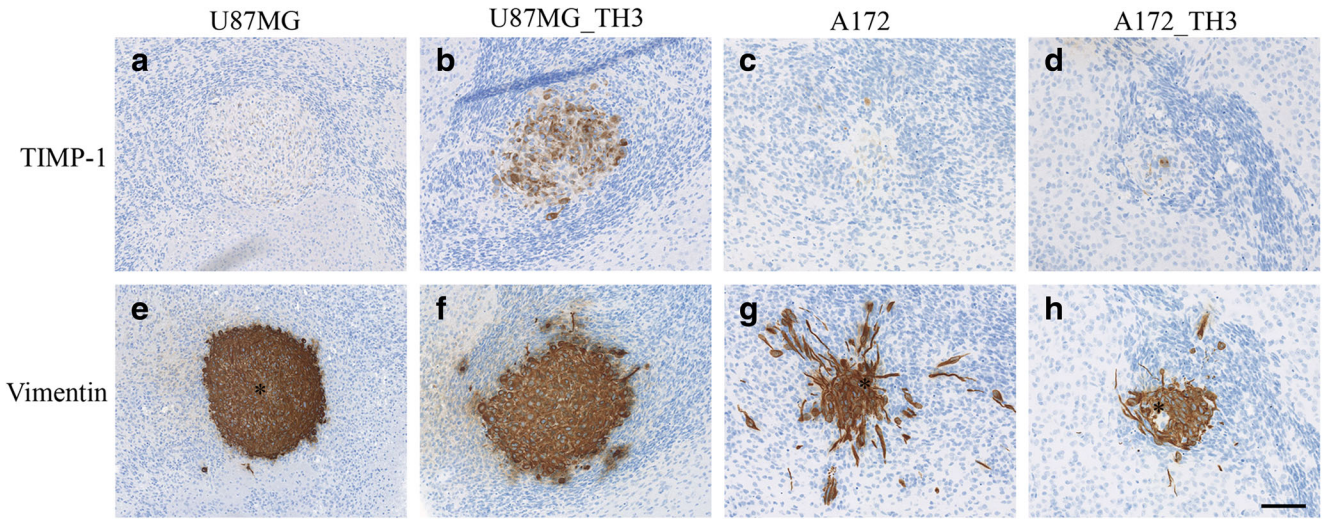

Fig. 5 Spheroids derived from U87MG and A172, controls (U87MG_C and A172_C) and high TIMP-1 expressing clones (U87MG_TH1–3 and A172_TH1–3) were implanted into brain slice cultures. Paraffin sections from cultures fixed at day 6 were stained immunohistochemically for TIMP-1 (**a-d**) and for vimentin (**e-h**). Each section represents one layer in the confocal Z-stack, and the invasion patterns observed in the confocal images were confirmed by immunohistochemistry (**e-h**). The levels of TIMP-1 in spheroids were in general low (**a-d**), although the levels in the TIMP-1 transfected spheroids (**b**, **d**) were increased compared to the parental cell line-derived spheroids (**a**, **c**). Scalebar 100 μm

Furthermore, we evaluated the effect of high TIMP-1 levels on tumor growth and invasion. Different growth and invasion patterns were seen for the two cell lines. When implanting the U87MG cell line-derived spheroids, only limited invasion was detected, which is in line with previous results obtained in vitro and in vivo [39, 44–46]. For both cell lines, the invasion of the TIMP-1 transfected cells was not significantly different compared to the controls. However, a tendency towards decreased invasion was observed when implanting TIMP-1 overexpressing spheroids into organotypic brain slice cultures. Although the invasion was limited, there appeared to be a decreased invasion for the U87MG_TH2 and U87MG_TH3 subclones compared to the control. This is in line with a study where glioblastoma cell lines, such as U87MG and A172, became less invasive in a dose dependent manner, when TIMP-1 was added to a chemotaxis chamber [47]. Furthermore, reduced growth rates and decreased invasion through matrigel-coated filters have been shown when comparing TIMP-1 transfected paediatric SF-188 glioblastoma cells to controls [48]. When evaluating the area of the implanted spheroids, the area of the TIMP-1 overexpressing U87MG spheroids was rather constant compared to the controls. In contrast to these findings, two independent breast cancer studies have shown that TIMP-1 overexpression stimulated tumor growth when TIMP-1-transfected breast cancer cells were implanted into nude mice [18, 49], suggesting that TIMP-1 may not exert the same effects in all cancers.

In conclusion, the present study showed that TIMP-1 overexpression reduces the cytotoxic effect of TOP inhibitors in the glioblastoma cell line U87MG. TIMP-1 overexpression also appeared to reduce spheroid growth but did not influence invasion. Whether TIMP-1, alone or in combination with other biomarkers, has a predictive potential in glioblastomas needs to be elucidated by tumor material obtained from appropriately designed prospective clinical studies.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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