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Collagen XVI expression is upregulated in glioblastomas and promotes tumor cell adhesion

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Abstract **The poor prognosis of glioblastoma patients is related to diffuse brain invasion and interaction of tumor cells with extracellular matrices (ECM). We describe expression and function of the FACIT-collagen XVI in glioblastomas. We found upregulation of collagen XVI mRNA as well as protein in glioblastomas as compared to normal cortex. SiRNA knockdown resulted in decreased cell adhesion whereas increased adhesion was observed on surfaces coated with collagen XVI. The migration of glioblastoma cells on this substrate remained unchanged. Our results demonstrate de-novo expression of collagen XVI in glioblastomas as part of the tumor specific remodeling of the ECM.**

Structured summary:
MINT-6743179:
Collagen IV (uniprotkb:P02462-1) and *Collagen XVI* (uniprotkb:Q07092) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)
MINT-6743170:
GFAP (uniprotkb:P14136) and *Collagen XVI* (uniprotkb:Q07092) *colocalize* (MI:0403) by *fluorescence microscopy* (MI:0416)

Keywords: Collagen XVI; Glioma; Glioblastoma; Adhesion

1. Introduction

The fibril forming collagens I, III, V and VI are normally not found in the brain parenchyma, but are components of mesenchymal structures like cerebral vessels and choroid plexus stroma. Correspondingly, in gliomas only a minority of tumor cells deposit these interstitial collagens in the peritumoral ECM. Collagen IV is a major component of basement membranes beneath vascular endothelial cells and choroid plexus epithelial cells in normal brain. In glioblastomas collagen IV is present in virtually all tumor vessels, as well as in some giant glioma cells and in tumor cells around vascular proliferations. In addition, rare types of gliomas, such as pleomorphic xanthoastrocytoma exhibit a collagen network around individual tumor cells. Collagens VII and VIII are absent from normal brain, but may be expressed in glioma tissues. The expression pattern of collagens changes during growth of gliomas in cell culture. Glioma cell lines deposit large amounts of collagens I, III, IV and VI, a feature that is known as mesenchymal drift [1].

Remodeling of the tumor associated ECM is one mechanism by which glioma cells force their invasion into brain tissue [2]. Using differential gene expression microarray analysis to compare various glioma cells with increased versus decreased migratory phenotype, we detected upregulation of collagen XVI gene expression in the subpopulation of cells showing increased migration [3]. Collagen XVI is a member of the fibril associated collagens with interrupted triple helices (FACIT-collagens). The 213 kDa collagen XVI protein consists of 10 collagenous (Col) domains separated by 11 non-collagenous (NC) regions. It is encoded by a single gene located on chromosome 1p34-35 in humans and there is no evidence for different isoforms [4]. Collagen XVI belongs to the minor collagens which are components of connective tissues like skin or cartilage. In skin it is located near basement membranes of blood vessels and at the dermo-epithelial junction (DEJ) [5], with an increased expression level in fibrotic skin diseases [6]. Collagen XVI forms homotrimers, with a length of 240 nm, visualized as a rod-like structure with various kinks terminated by a large N-terminal domain (NC 11) as determined by atomic force microscopy and rotary shadowing [7]. Members of the FACIT-family were first described as being associated with the surface of fibrillar collagens, hypothesized to interconnect heterotypic collagen fibrils with each other to form large collagen networks and to link these fibrils to macromolecules of the extrafibrillar matrix. In skin, collagen XVI is integrated into particular fibrillin-rich microfibrils lacking an amorphous elastin core. In cartilage, collagen XVI is a component of small heterotypic D-banded fibrils, mainly occurring in the territorial matrix of chondrocytes [8]. In contrast to members of fibril forming or basement membrane collagens, the FACIT-collagen XVI has not yet been described in brain or tumor tissue. Here, we study the expression of collagen XVI in glioblastomas and investigate its impact on migration and adhesion of glioblastoma cells.

2. Materials and methods

2.1. Cell culture

Established human glioblastoma cell lines U87MG, U343MG, U373MG and 86HG39, as well as primary glioblastoma cells (TB288) were cultured as described [3]. For detection of collagen XVI in cell culture supernatant, 1×10^6 cells were incubated in a 10 cm dish for 24 h in serum free medium.

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2.2. Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA from native glioblastoma tissue ($n = 10$) obtained from neurosurgical resections and normal brain tissue ($n = 3$) obtained by autopsy from three adult subjects without neurological symptoms and immediately frozen in liquid nitrogen, was isolated with TRIZOL reagent (Invitrogen, Germany), while total RNA from cell lines was isolated with RNeasy Mini Kit (Qiagen, Germany). Total RNA (1 μg) was transcribed into cDNA with Omniscript RT (Qiagen). Conventional RT-PCR with subsequent ethidium bromide gel electrophoresis was performed using the following collagen XVI primers: 5′-ctggtgatcctgtacgagccagg-3′ (forward) and 5′-ccatcctgaaggttggacagggc-3′ (reverse). For quantitative RT-PCR TaqMan assays were performed with TaqMan Universal PCR Master Mix and the collagen XVI TaqMan Gene Expression Assay HS_00156876m1 using the GeneAmp 5700 Sequence Detection System (reagents and detection system from Applied Biosystems, USA). Primers and TaqMan-probe for GAPDH control and calculation of relative expression intensity (ΔΔCt-method) were described previously [3]. Quantitative RT-PCR for β1 integrin was performed with the primers: 5′-atc cca gag gct cca aag at-3′ (forward) and 5′-ccc ctg atc tta atc gca aa-3′ (reverse) using the SYBR-Green method, with GAPDH as internal loading control. Relative changes in gene expression were evaluated by the ΔΔCT-method. All steps were performed according to manufacturer's instructions.

2.3. Immunohistochemistry

For detection of collagen XVI in situ, 10 μm cryo sections from glioblastoma tissue were fixed in methanol:acetone 1:1 at −20 °C. After blocking with 0.5% BSA in PBS the sections were incubated with a polyclonal anti-collagen XVI antibody (raised in guinea pig; 1:100 dilution) [7] at 4 °C over night. Pre-immune serum obtained from the same animals served as negative control. Detection was performed with a biotin-conjugated mouse anti-guinea pig IgG secondary antibody (Vector Laboratories, USA) using the avidin–biotin-complex technique with peroxidase as enzyme and diaminobenzidine as substrate (Vector Laboratories).

For double-immunofluorescence deparaffinized and rehydrated sections were preincubated with protease XXIV (Sigma, Germany; 0.05% in PBS, 6 min, 37 °C) and hyaluronidase (Sigma; 0.1% in sodium acetate buffer pH 6.0, 60 min, 37 °C). After this treatment slides were washed several times in PBS and unspecific binding sites were blocked with PBS containing 1% bovine serum albumin (Biomol, Germany), 5% normal goat serum (DAKO, Denmark) and 20% protease inhibitor cocktail (Complete Mini, Roche, Germany) for 1 h at 37 °C. The blocking was followed by overnight incubation at 4 °C with primary antibody. Primary antibodies were diluted in blocking buffer, guinea pig anti-collagen XVI antibody 1:100, rabbit anti-collagen (α1)IV antibody 1:500 (kind gift from J.A. Eble, Muenster) and rabbit anti-GFAP 1:400 (DAKO). AlexaFluor555 (goat anti-guinea pig) and AlexaFluor488 (goat anti-rabbit) conjugated antibodies (5 μg/ml, Molecular Probes, USA) were added for 1 h at room temperature. Sections were counterstained with DAPI nucleic acid stain (300 nM in PBS, Molecular Probes) and mounted with DAKO fluorescent mounting medium (DAKO).

2.4. Western blotting

Cells and tissues were lysed with buffer containing 20 mM Tris, pH 7.6, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1% SDS. Cell culture supernatant was precipitated with trichloric acetic acid and the sediment was resuspended in lysis buffer. Equal amounts of proteins as determined using Lowry assay (BioRad, USA) were separated on a 7.5% polyacrylamide gel with subsequent transfer onto nitrocellulose membrane (Schleicher and Schüll, Germany) using the wet blot technique. Detection was performed with the polyclonal anti-collagen XVI guinea pig antibody and a peroxidase conjugated mouse anti-guinea pig IgG secondary antibody (Vector Laboratories) using the regular ECL plus detection kit (Amersham, Germany). To verify equal protein loading on each lane, blots were either stripped and reprobed for actin or checked by means of uniform PonceauS staining.

2.5. Migration assay

To determine migration of glioma cells on surfaces coated with recombinant collagen XVI [7], we used the monolayer migration assay as described elsewhere [3]. Surfaces were coated with 50 μg/ml recombinant collagen XVI or BSA as control and cells were observed over a period of 96 h. Increase of colony area was documented photographically and measurement was evaluated using the Sigma Scan Pro analysis software (SPSS Science, USA). Experiments were performed in duplicates with 3–6 colonies investigated.

2.6. Adhesion assay

For investigating adhesion on recombinant collagen XVI 96-well microtiter plates (Nunc, Germany) were coated by incubation with 100 μl per well of a recombinant collagen XVI solution (50 μg/ml) in serum free DMEM for 1 h at 37 °C. Wells were washed three times with PBS and incubated with 100 μl of a 1 mg/ml BSA/PBS solution for 30 min at room temperature. Adhesion depending on endogenous collagen XVI after knockdown experiments was tested on uncoated surfaces.

1×10^5 cells per well were incubated for 1 h under standard conditions. Medium and non-adherent cells were aspirated and the wells were washed three times with PBS. Cells were fixed with 100 μl 3.7% formaldehyde/PBS for 15 min and stained with 100 μl 0.5% crystal violet in 3.7% formaldehyde/PBS overnight at 4 °C. After aspirating the dye solution, cells were washed four times with ddH_2O, air-dried and lysed in 100 μl 10% acetic acid on a shaker for 20 min. Absorption was measured at 570 nm using an ELISA reader. Results are presented as % absorption of controls using cells adherent on surfaces coated only with BSA.

2.7. siRNA transfection

Knockdown of endogenous collagen XVI was performed using siRNA oligonucleotides 5′-ugagcucauugagucaau-3′ (siRNA1) and 5′-ggacucaaauuggaacaca-3′ (siRNA2) with corresponding sequences as double strand (Eurogenetec, Belgium) and non-interfering siRNA (Qiagen) as control. Transfections were done in 6-well dishes with 3×10^5 cells each, using HiPerfect transfection reagent (Qiagen), following manufacturer's instructions. Efficiency of knockdown was analyzed by qRT-PCR 48 h after transfection. At the same time the cells were used for adhesion assays.

2.8. Statistical analysis

Differences in migration and adhesion were tested using two way ANOVA for each cell line, time point or substrate respectively, using LSD (least significant difference) as post hoc-test if necessary. $P < 0.05$ was considered as significant.

3. Results

3.1. Collagen XVI expression in glioma cells and tissues

Relative overexpression of collagen XVI in primary human glioblastoma cells was initially detected in our oligonucleotide microarray experiments [3]. To verify that these results obtained with primary glioma cells reflected in situ conditions and to exclude that collagen XVI expression represented a cell culture artifact, we prepared RNA from human glioblastoma specimens as well as from non-neoplastic cortex. In conventional RT-PCRs the collagen XVI amplicon (345 bp) could be exclusively detected in glioblastoma tissue, whereas in normal cortex tissue no transcription product was observed (Fig. 1A). Quantitative RT-PCR applied to determine altered expression, showed differences between tumor and normal tissues. Relative quantification of the data revealed 5–122-fold overexpression of collagen XVI mRNA in tumor tissues (Fig. 1B).

Using Western blotting collagen XVI protein was detected in lysates of glioblastoma tissue but not in extracts from normal cortex (Fig. 1C). In glioblastoma tissue, smaller truncated forms were detected beside the full length protein, indicating proteolytic digestion of collagen XVI in native tumor tissue. The sizes of the processed fragments, 180 and 130 kDa, are comparable to those seen with the recombinant forms [7] indi-

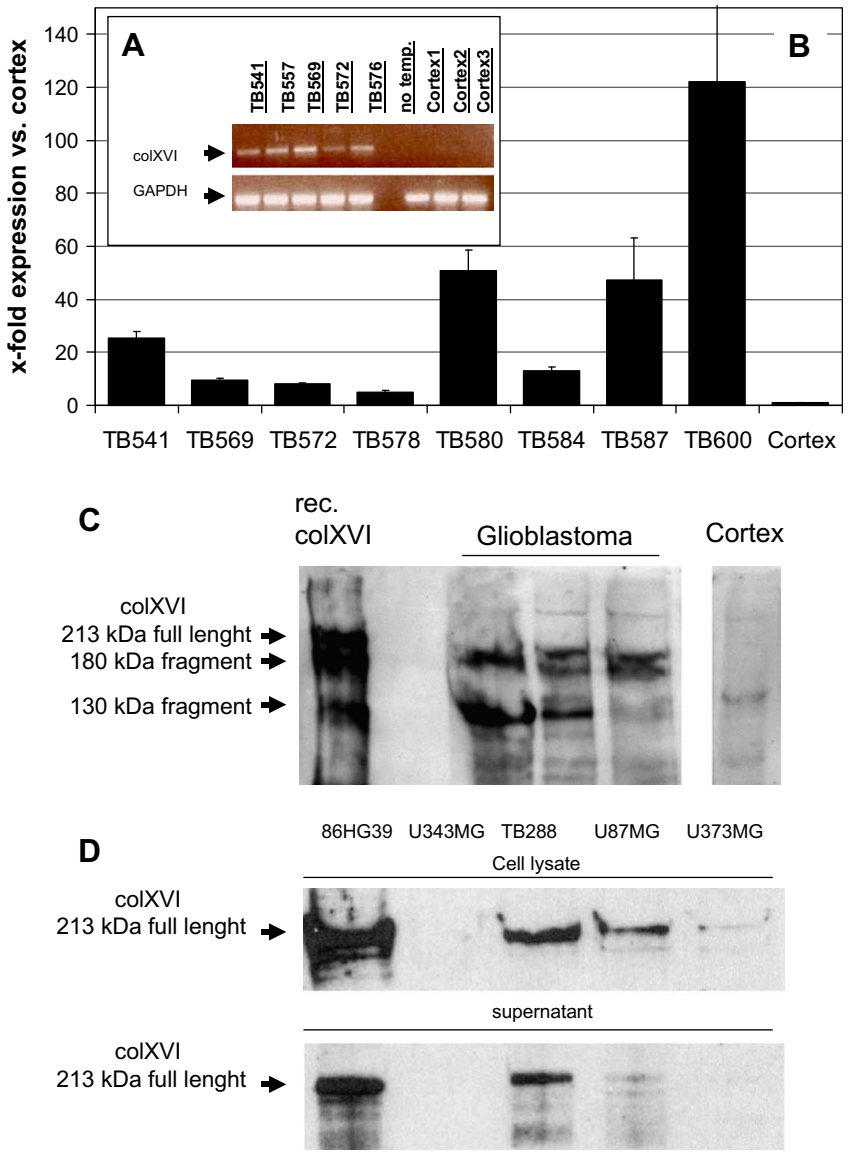

Fig. 1. Detection of collagen XVI transcripts and protein in glioblastoma specimens and cell lines. Conventional RT-PCR was performed with RNA from five glioblastoma specimens and with RNA from three non-neoplastic cortices. A 345 bp fragment specific for collagen XVI was only amplified in glioblastomas, control RT-PCR was performed with GAPDH primers (A). Quantitative RT-PCR resulted in 5–122-fold induced expression of collagen XVI mRNA in eight different glioblastoma specimens as compared to normal cortex. The mean expression of three non-neoplastic cortices is set to 1 and expression in glioma specimens is shown in *x*-fold relation to cortex (B). Full length collagen XVI with a molecular weight of 213 kDa was detected in tissue lysates of three glioblastoma specimen but not in normal cortex. Smaller bands (180 and 130 kDa) detected by an anti-collagen XVI antibody demonstrates the presence of processed fragments of collagen XVI, comparable to recombinant collagen XVI (rec.) (C). Collagen XVI protein is expressed in considerable amounts in primary cells of a glioblastoma (TB288) and the glioblastoma cell lines U87MG and 86HG39, whereas two other cell lines showed only very faint (U373MG) or no (U343MG) expression (D, upper panel). Secretion of collagen XVI was verified in supernatants of primary TB288 cells and the established glioblastoma cell lines U87MG and 86HG39, but not in U343MG and U373MG (D, lower panel).

cating N- and C-terminal processing of collagen XVI also in tumor cells. Full length collagen XVI (213 kDa) is found in Western blots of cell lysates from some established glioma cell lines or primary glioma cells. Western blotting of cell culture supernatants revealed collagen XVI secretion by glioma cells (Fig. 1D). Significant amounts of collagen XVI were detected in lysates from primary TB288 cells, as well as U87MG and 86HG39 cell lines. The other two cell lines revealed only a very faint (U373MG) or no (U343MG) signal at all. In addition, collagen XVI protein was detected in supernatants of two glioma cell lines (U87MG and 86HG39) and the primary TB288 cells, whereas two others (U373MG and U343MG) remained negative. These results were corroborated by quantitative measurements of mRNA levels from the respective cell lines (data not shown).

Immunohistochemistry showed expression of collagen XVI in glioblastoma specimens. We found strong staining of tumor cells (Fig. 2A) or tumor vessels (Fig. 2C) in all investigated glioblastomas. In tumors with cellular collagen XVI localization nearly all tumor cells were histologically positive, while normal cortex was virtually negative (Fig. 2E). Zones of the dermal–epidermal junctions in normal skin served as positive

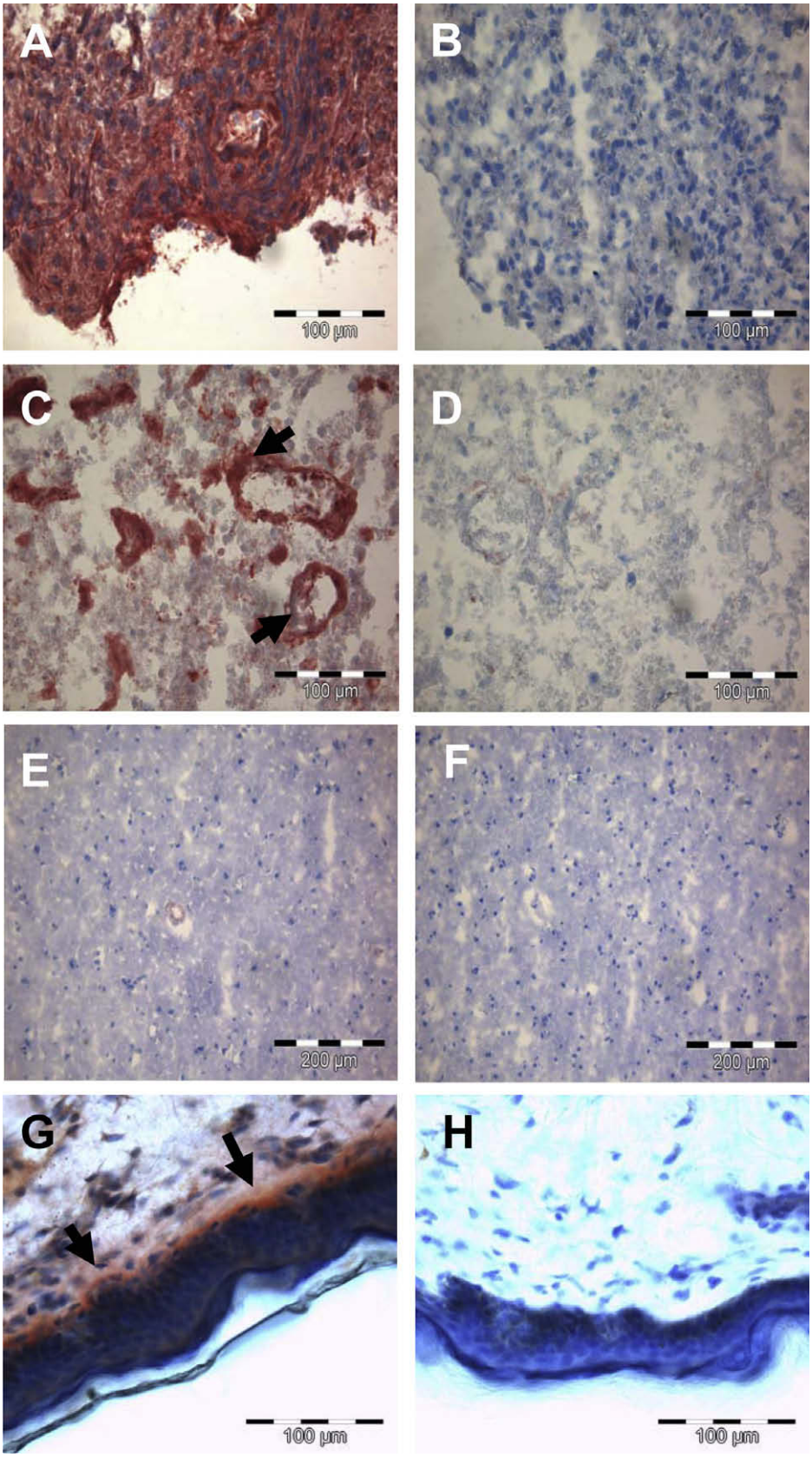

Fig. 2. Immunohistochemical localization of collagen XVI in glioblastoma tissues. Staining was found in tumor cells (A) and in tumor vessels (C, arrows). Virtually no staining was found in normal cortex (E). Normal human skin, where zones of the dermal–epidermal junctions are known to be positive for collagen XVI (G, arrows), served as positive control (see [15]). As negative controls tissues were incubated with pre-immune serum and showed no staining (B, D, F, H). Sections were counterstained with haematoxylin.

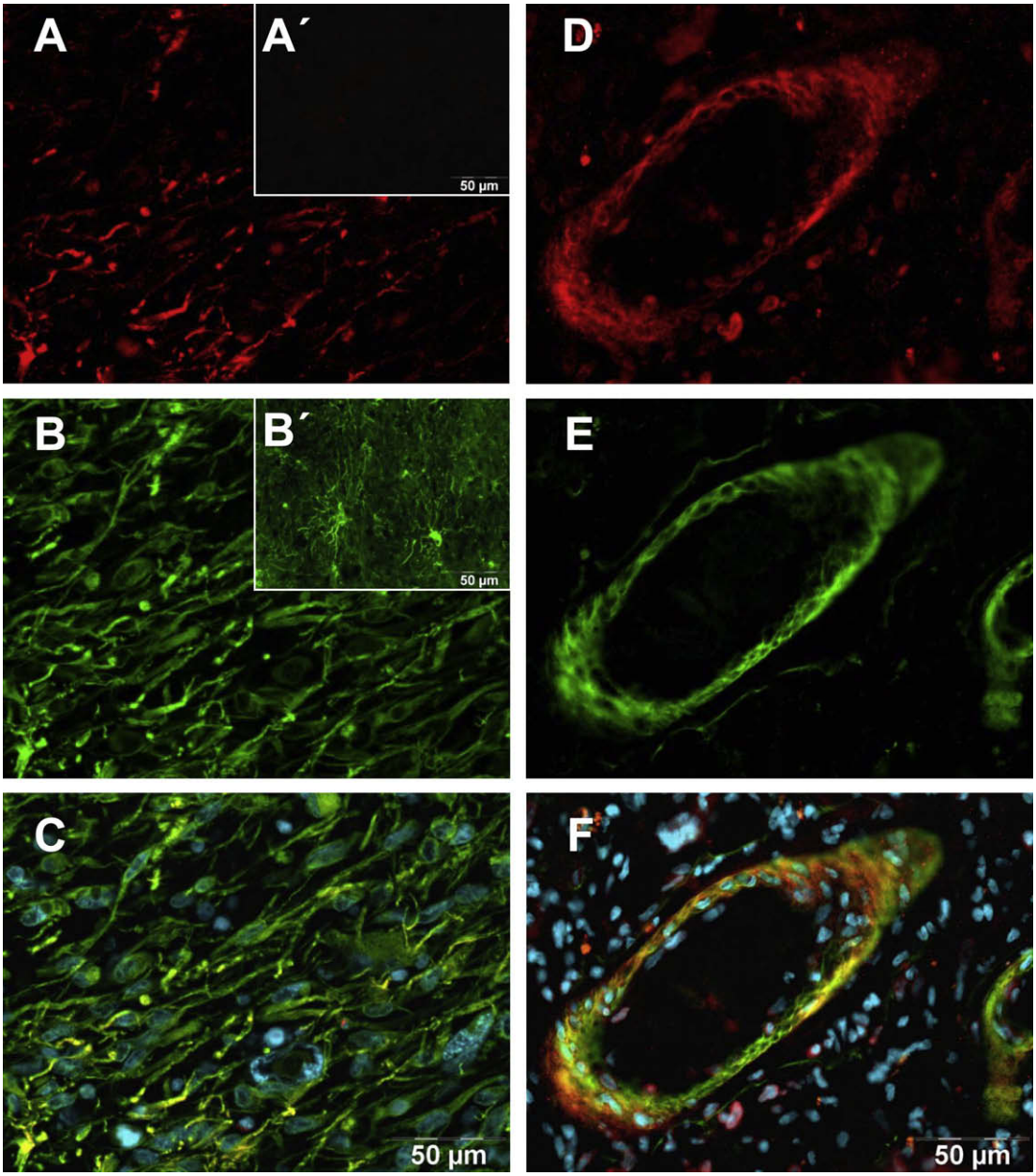

Fig. 3. Collagen XVI is expressed in GFAP positive cells and is underlying the basement membrane of tumor vessels. Double-immunofluorescence revealed collagen XVI expressing in GFAP positive cells, i.e. astrocytic tumor cells. Collagen XVI staining is shown (red in A), as well as staining with GFAP (green in B) resulting in yellow overlay (C) of both fluorescence staining and confirming coexpression of both proteins. Staining of normal cortex demonstrated only GFAP positive astrocytes (B′) but no collagen XVI fluorescence (A′). Furthermore collagen XVI is located around tumor vessels (red in D) together with collagen IV (green in E), overlay in (F). Nuclei are counterstained with DAPI.

control for collagen XVI staining (Fig. 2G). Double-immunofluorescence revealed staining of collagen XVI together with GFAP in the same cells, indicating collagen XVI is produced by the glioma cells (Fig. 3A–C). Collagen XVI was also found in tumor vessels underlying the collagen IV layer (Fig. 3D–F).

3.2. Migration and adhesion

Migration and adhesion assays were performed using the cell lines U87MG, 86HG39, U343MG and U373MG. Monolayer migration on collagen XVI (50 µg/ml) coated surfaces revealed no differences compared to BSA coated control surfaces in all four tested cell lines (Fig. 4). In contrast to the migration results, adhesion assays on surfaces coated with collagen XVI, demonstrated increased adhesion ($P < 0.05$) for all glioma cell lines at the highest coating concentration of 50 µg/ml compared to BSA control or lower concentrations of collagen XVI (Fig. 5A). There was also a significant difference in the strength of adhesion between the four cell lines at 50 µ/ml collagen XVI (U87MG > 86HG39 > U373MG > U343MG). Next, we studied effects of endogenous collagen XVI on adhesion rates, using the cell line U87MG, which expresses detectable amounts of protein in the supernatant and showed the strongest adhesion effect on the recombinant protein. Collagen XVI was knocked down with two different siRNA constructs;

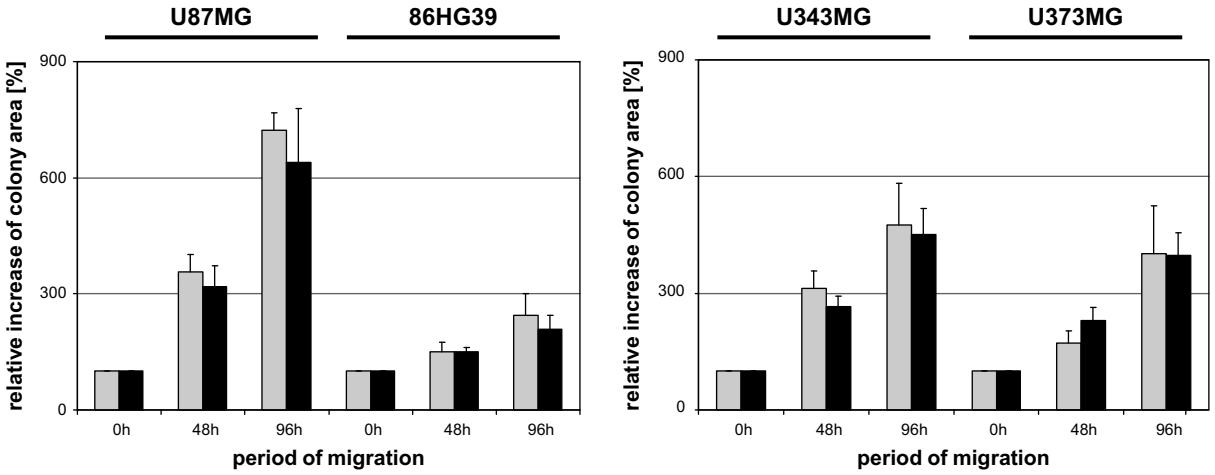

Fig. 4. Migration of glioblastoma cell lines on collagen XVI coated surfaces. On surfaces coated with 50 μg/ml collagen XVI (dark columns) migration rates remained unchanged compared to BSA (bright columns) coated surfaces. Results are the mean of two independent experiments with 3–6 replicates each.

efficiency was controlled with qRT-PCR and adhesion experiments were done on uncoated surfaces. In transient transfections with siRNA oligonucleotides the amount of endogenous collagen XVI mRNA decreased to 20% of controls transfected with non-interfering siRNA. Cells with reduced collagen XVI levels (using both siRNA oligonucleotides) exhibited significantly reduced adhesion rates ($P < 0.05$) compared with control transfected cells and with non-transfected cells (Fig. 5B). However, analysis of β1 integrin transcripts of siRNA transfected cells by quantitative RT-PCR, showed no changes in expression levels (data not shown). Thus, reduced adhesion is not a consequence of decreased β1 integrin mRNA levels.

4. Discussion

Collagen XVI represents the first FACIT-collagen detected in glioblastomas, as no other member of this protein family, like collagens IX, XII, XIV and XIX, has been described as being associated with this tumor so far. Immunohistochemically, collagen XVI is found in tumor vessels as well as in tumor cells. Compared with normal human cortex, collagen XVI expression is upregulated in glioma tissue and in a subset of glioma cell lines, where it is secreted into the supernatant. In cell culture assays with recombinant collagen XVI, there was no detectable effect on migration. However, glioma cell lines adhered strongly to collagen XVI coated surfaces, while adhesion was clearly reduced after blocking expression of endogenous collagen XVI.

In adult mice collagen XVI expression is low in the CNS which corroborates the absence of collagen XVI expression in normal human brain [9]. Interestingly, collagen XVI is expressed in neurite growth cones, following axotomy [10], connecting its expression to cell movement. It is important to note that collagen XVI expression in glioma cells is not a consequence of the mesenchymal drift of tumor cells during cell culture [11], since tumor cells express collagen XVI in situ. Furthermore, collagen XVI is an extracellular component of tumor vessels underlying the vascular basement membrane and it is well known that those membranes are preferred pathways for glioma invasion. Nevertheless, expression of collagen XVI is not a consistent feature of established glioma cell lines, because we have found cell lines with profoundly differing expression levels. This reflects the heterogeneity of glioma cells in situ, collagen XVI expression in a specific cell line presumably being a feature of the initial cell clone forming a permanent cell line. In fibroblasts collagen XVI expression is increased by transforming growth factor-β2 (TGF-β2) [12] which is upregulated in gliomas [13], suggesting that overexpression of collagen XVI in gliomas may be a downstream effect of this growth factor.

Because members of the FACIT protein family serve as molecular bridges between collagens and other components of the extracellular matrix, interconnection by collagen XVI may support the formation of migration pathways for glioma cells. Although we have not found a migration promoting effect of pure collagen XVI in our monolayer migration assays, collagen XVI may act as migration promoting substance only in combination with other factors, e.g. in allocation of three dimensional structures through interconnection to other structural proteins in brain specific ECM.

We have demonstrated that several glioma cell lines can utilize collagen XVI as substrate for adhesion. The different strength of adhesion (U87MG > 86HG39 > U373MG- > U343MG) is consistent with former investigations, demonstrating different abilities of different cell lines to adhere to collagen IV [14]. Collagen XVI interacts with integrins α1β1 and α2β1, however, with a more avid binding to α1β1 [15]. Interaction of gliomas with collagens is controlled by specific integrin receptors provided by the cell. α1β1 and α2β1 integrins are known to be expressed on glioma cell surfaces and are involved in cell adhesion and migration [16,17].

Furthermore, knockdown of endogenous collagen XVI in U87MG reduced the adhesion capacity of this cell line. It is well known that glioma cells modulate their environment by segregation of different ECM components, with consequences for adhesion and migration [18] which is also conceivable for collagen XVI. For this FACIT-collagen the deposition into the pericellular ECM of fibroblasts and smooth muscle cells has already been reported [19].

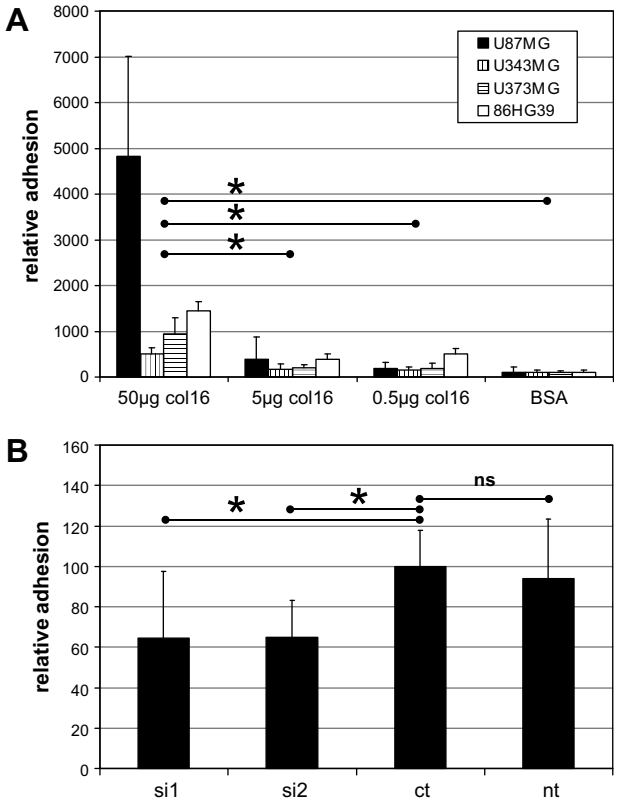

Fig. 5. Adhesion of glioblastoma cell lines on collagen XVI coated surfaces and after siRNA mediated inhibition of endogenous collagen XVI on uncoated surfaces. There was significantly higher adhesion of all four glioblastoma cell lines on surfaces coated with 50 µg/ml collagen XVI compared to lower collagen XVI concentrations and BSA coated controls. Results are the mean of two (U87MG, U343MG, U373MG) or three (86HG39) independent experiments with 6-fold replicates each. (* = $P < 0.05$) (A). SiRNA mediated knockdown of endogenous collagen XVI in U87MG cells was performed with transient transfection using two different siRNA oligonucleotides (si1 and si2). Both transfections resulted in collagen XVI inhibition of more than 80% compared to the non-interfering control transfections (ct). Transfection with two collagen XVI specific siRNA oligonucleotides resulted in decreased adhesion on uncoated surfaces, whereas adhesion was not significantly different for control transfected cells (ct) and non-transfected cells (nt). Results are the mean of three independent experiments with 6-fold replicates each. (* = $P < 0.05$, ns = not significant) (B).

We have detected full length collagen XVI and smaller fragments of lower molecular weight in glioblastoma tissue and TB288 cells, most likely being identical with the processed 180 kDa form lacking the N-terminal NC11 domain and the 130 kDa form which misses C-terminal regions [7]. This observation leads to the intriguing speculation that collagen XVI has functions other than mere matrix receptor interactions, perhaps including regulatory functions of the proteolytic 180 and 130 kDa fragments. Proteolytic fragments from collagen IV (tumstatin) and XVIII (endostatin) transfer anti-angiogenic effects, subsequently influencing cell growth and migration [20]. The C-terminal NC 1 domain of these collagens contains recognition sites for several matrix metalloproteinases, which are known to be hyperactive in gliomas [2]. It is intriguing to speculate that corresponding domains of collagen XVI fulfill regulatory functions in cell migration and adhesion. Notably, endorepellin, which represents the C-terminal domain of the heparansulfate proteoglycan perlecan [20], inhibits endothelial cell migration and binds to $\alpha 2\beta 1$ integrin, which has a binding site in the C-terminus of collagen XVI [15]. The cell line U87MG is therefore an interesting model for further investigations concerning collagen XVI functions in gliomas.

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