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Role of Adamts-1 in Pleomorphic Xanthoastrocytoma Tumor Cells Progression

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ABSTRACT

AIM: To analyze the expression of ADAMTS-1, NF-κB, and STAT3 in human pleomorphic xanthoastrocytoma specimens, and their correlation with glioma advancement.

MATERIAL and METHODS: Pleomorphic Xanthoastrocytoma tumor cell lines were treated with low and high doses of cytokines at 24 and 48 hours to replicate the inflammatory environment. The effects of IL-1 were assessed with the scratch wound-healing assay, and the expression levels of ADAMTS-1, NF-κB, and STAT3 of the groups were determined by western blot analysis.

RESULTS: Cytokine treatment significantly increased the migration of PXA glioma cells after scratching at 24h and 48h time points. Similarly, 10 and 30 ng/mL IL-1 induced 1.86 and 1.94 fold increases, respectively, in ADAMTS-1 expression after 24h, and 3 and 3.27 fold increases, respectively, after 48h, compared with the non-treatment control group.10 and 30 ng/mL IL-1 doses caused 2.5 and 2.6 fold increase, in NF-κB protein levels after 24h, and 3.16 and 3.41 fold increases after 48h, compared with the non-treatment group. The protein levels of STAT3 after 24h were 2.62 and 2.43 fold higher, and 3.78 and 3.84 fold higher after 48 hours, with 10 and 30 ng/mL IL-1, compared with the non-treatment group.

CONCLUSION: The proliferation and progression of glioma cells were proportional to the increased expression levels of ADAMTS-1, NF-κB, and STAT3. Our findings indicate that the proteolytic function of ADAMTS-1 may be associated with the malignant transformation of low-grade gliomas.

KEYWORDS: Pleomorphic Xanthoastrocytoma, Matrix Metalloproteases, NF-kappa B, STAT3 transcription factor, Glioma

ABBREVIATIONS: ADAMTS: A disintegrin and metalloproteinase with thrombospondin motif, **ECM:** Extracellular matrix, **GBM:** Glioblastome multiforme, **IGFBP-2:** Insulin-like Growth Factor Binding Protein 2, **IL-1:** interleukin-1, **NF-κB:** Nuclear Factor-kappa B, **PXA:** Pleomorphic xanthoastrocytoma, **STAT3:** Signal transducer and activator of transcription 3

INTRODUCTION

Glioma cells attain migratory and invasive properties early in tumorigenesis due to uncontrolled atypical processes, including cell proliferation, cell invasion, extracellular matrix (ECM) remodeling, migration, and the establishment of vascular supply (10). Among these processes, post-translational modifications to the ECM structure complex caused by alterations in protease expression have been strongly associated with tumor progression (57).

PXA is a relatively rare low-grade brain tumour, with a 30% recurrence and 75–80% overall survival rates following surgical removal (39). PXA has lack of genetic properties which are characteristics of infiltrative gliomas (19). PXA has a favorable prognosis, but cases showing mitoses more

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than five per 10 high-power field with or without necrosis are termed 'anaplastic PXA'. Cases with signs of anaplasia have poor prognosis, and the tumors' clinical behavior can be more aggressive. A previous study reported that a high mitotic index is associated with increase in recurrence and decrease in overall survival (9). The mitogen-activated protein kinase/extracellular signal-regulated kinase signaling pathway may lead to the promotion of cell proliferation and differentiation, and thus may increase the invasion and migration of PXA. However, its rarity and the lack of knowledge about the natural clinical course of PXA tumor cells hinder advances in defining the factors that predispose these cells to the acquisition of anaplastic features.

ADAMTS family of 19 identified metalloproteases have been widely involved in cancer development and tumor progression through the degradation and polymerization of structural ECM proteins. ADAMTS-1 is the member of these matelloproteases with a molecular structure characterized by domains, modules, and motifs. The signal peptide, prodomain, metalloproteinase, and disintegrin domains constitute the amino half of ADAMTS proteases (N-terminal), whereas the carboxyl region (C-terminal) consists of the cysteine-rich domain, spacer region, and thrombospondin type 1 motif. The metalloprotease and disintegrin-like domains of ADAMTS-1 catalyze the breakdown of versican and aggrecan, which are known to be the components of ECM (45,46). The binding ability of ADAMTS-1 to the ECM is attributed to its C-terminal thrombospondin motifs (21). The lectican family forms a lattice structure in normal adult brains by linking to hyaluronan and tenascin-R. Aggrecan and brevican are proteoglycans of the lectican family, which are essential for maintaining the integrity of the brain ECM (59). The proteases remodel the host tissue environment, precipitate new blood vessels formation, and facilitate glioma progression through proteolytic ECM degradation. In addition to the remodeling of the ECM, inflammation is another important process to be involved in tumor development, from initiation to metastatic progression (12). Initially, ADAMTS-1 was identified as an intermediator of inflammation in an experimental model of colon carcinoma (23). Several studies have reported increased expression levels of ADAMTS-1 after IL-1 administration, which suggests that ADAMTS-1 production could be up-regulated by inflammatory mediators and cytokines (14,20,51). The effect of ADAMTS-1 on tumor invasion and the development of inflammation related cancer could be directly due to its proteolytic activity (7,24,35).

Nuclear Factor-kappa B (NF-κB) and Signal Transducer and Activator of Transcription 3 (STAT3) are transcription factors linking inflammation to cancer development. Both are critical regulators that contribute to the process of tumorigenesis through interaction with immune and inflammatory functions (41). NF-κB is abnormally regulated in glioblastoma multiforme (GBM), in which translocation of the phosphorylated form of NF-κB into the nucleus plays a critical role in tumor cell proliferation (34). Furthermore, STAT3 is commonly activated in several cancers (4,11,60). The tyrosine and serine phosphorylation status of STAT3 has been determined to be constitutively active (27). Levels of activated STAT3 are elevated in high-grade gliomas and are reported to be positively associated with tumor grade (1,3). Moreover, McFarland et al. analyzed these transcription factors in primary human GBM xenografts and cultured GBM cell lines and reported that inhibiting the activity of NF-κB and STAT3 causes a decline in glioma size and aggressiveness (33).

The expression of ADAMTS-1 in pleomorphic xanthoastrocytoma (PXA) tumors has not yet been reported. It is therefore of interest to investigate whether ADAMTS-1 is involved in the cytokine-mediated invasion and progression of PXA. Herein, PXA cell lines were treated with different cytokine doses. Development and progression of PXA associated with the activity of ADAMTS-1, NF-κB and STAT3 were examined under the replicated inflammatory conditions in this *in vitro* model.

MATERIAL and METHODS

Cell Cultures and IL-1 Cytokine Treatment

The human PXA tumor primary cell line was kindly supplied by Ankara University. The cell lines were originally isolated from astrocytic tumors that were diagnosed as PXA. Dulbecco's Modified Eagle Medium (DMEM) and 4.5 g/L Glucose (Lonza), 10% fetal bovine serum, %1 L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin were used to culture the cells in a 5% CO_2-humidified atmosphere at 37°C. Incubation of the 1.0×10^5/mL cell density were supplied with low-dose (10 ng/mL), and high-dose (30 ng/mL) IL-1 for 24 and 48 hours. Afterwards, cells harvest and isolation of proteins were achieved.

In Vitro Wounding Migration Assay

PXA tumor primary cells were seeded in 6-well culture plates until confluent and left overnight. The next day culture medium was removed, and a pipette with a tip of 200 µL was used to draw across the diameter of each well. To wash the cells, PBS was added in an incubator at 37 °C and 5% CO_2. After scratching, cells in medium with %1 FBS were cultured and treated with doses of 0 ng/mL, 10 ng/mL, and 30 ng/mL IL 1. Wound closure was observed, and cells in the scratched area were counted and photographed under an optical microscope at 40× magnification after 24 and 48 hours .

Cell-free areas of the representative images from the wound-healing assay were evaluated using the Wimscratch platform. Data represent three replications, and were analyzed using Student's t-test.

Cell Protein Extraction

After the different treatments, phosphate-buffered saline was used to wash the cells which then tyrpsinized from the plates. Cells (3×10^6) were solubilized in 1485 µL of CelLytic MT Mammalian (Sigma) with a protease-phosphatase inhibitor cocktail. Then the cells were cleared by centrifugation at 13,000 × g at 4°C for 15 min, and protein concentration of the collected supernatant was analyzed with the ThermoScientific Bradford Assay protein kit.

Cell protein lysate samples were boiled to denaturate at 95°C in Laemmli Sample buffer with β-mercaptoethanol for 7 min. A

total of 15-µg protein was used for Western blot analysis was performed with a total of 15-µg protein.

Western Blot Analysis and Antibodies

15 µL cell protein lysate from each sample, including protein markers, were subjected to western blot BioRad Miniprotean Stain-Free TGX protein Gels, 4–15%, 12-well comb, 20 µL, #456-8085 within BioRad 1X Tris/Glycerine/SDS under a voltage of 250 for 20 min for electrophoresis, then immediately transferred to PVDF membranes which were kept in a 2.5% dry non-fat milk for one hour in TBS-T and co-incubated with the primary antibodies including anti-ADAMTS-1 (Abcam, Cambridge, UK. #28284), anti-STAT3 [Santa Cruz Biotechnology, Inc. #sc-482), anti-NF-κB (Santa Cruz Biotechnology, Inc. #sc-372) and anti-β-actin (Santa Cruz Biotechnology, Inc. #sc-69879) under 23 °C for 16 hours. Membranes were washed with TBS-T three times for 10 min each at 23 °C, and then incubated with the secondary antibodies for one hour (Santa Cruz Biotechnology, Inc.) using HRP conjugation (1:2500) kit for one and a half hour at 23 °C room temperature. Membranes were then washed for three times with TBS-T. Antibodies labeled with HRP were visualized with Immun-StarTM WesterCTM kits (BioRad, Australia) and BioRad ChemiDoc MP Imaging System. Image J software was used to analyze densitometry. The protein expression was quantified by normalized to the expression of β-actin.

Statistical Analysis

Data from the western blot assays were analyzed with the use of SPSS 21.0 software. All western blot bands were compared to the control group. Statistically significance of the density values was calculated using paired sample t-test. p-<0.05 was regarded as results statistically significance. All the data are expressed as mean ± SEM.

RESULTS

Wound Migration Assay

To estimate the migratory ability of PXA tumor cells, a wounding migration assay was conducted. The tumor cells were observed at different time points (24 and 48 hours after scratching) and treated with different IL-1 doses (10 and 30 ng/mL). As shown in Figure 1A, PXA cells in the control group had not invaded the scratched area at the 24 and 48 hour timepoints. However, glioma cells were observed in the scratched area after 24 hours in both the 10 and 30 ng/mL IL-1 conditions. Migration increased significantly after 48 hours with both IL doses. The images were also evaluated with the WimScratch platform for proper quantification to provide unbiased results (Figure 1B). Twenty-four hours after scratching, the amount of PXA cells in the scratch significantly increased with the 10 ng/mL IL-1 dose compared to that of the no treatment control (63.46% ± 0.72 wound closure versus 79.7% ± 0.7, respectively, p=0.001). Furthermore, 24 hours after scratching, the amount of PXA cells in the scratch significantly increased with the 30 ng/mL IL-1 dose compared to that of the control group, and no statistically significant difference was found in wound closure between the 10 ng/mL and 30 ng/mL IL-1 treatments at the 24 hour timepoint (80.3% ±1.00 versus 79.7% ± 0.7, respectively, p=0.184). When we observed the PXA cells in the scratched area at the 48 hour timepoint, the amount of cells in the scratch significantly increased, as compared to that at the 24 hour timepoint, with both IL-1 doses (97.8% ±0.47 versus 79.7% ± 0.7, respectively, in 10 ng/mL IL-1; 99.6% ± 0.15 versus 80.3% ± 1.00, respectively, in 30 ng/mL IL-1; p=0.003, p=0.002, respectively). No significant difference was found between 10 and 30 ng/mL IL-1 treatment doses at the 48 hour timepoint (p=0.054) (Figure 2). From this, it can be concluded that IL-1 treatment provoked faster closure of the wound and migration increased over time.

Western Blot

The expression levels of ADAMTS-1, NF-κB, and STAT3 were detected by western blot analysis. ADAMTS-1 was as a single 75 kDa band, comparable to the expected size of the mature enzyme. NF-κB was seen as a single 65 kDa band, and STAT3 as a single 86 kDa band in lysate from PXA cells that received different treatments and at different timepoints. Fragments of ADAMTS-1 were not detected (visualized), and expression levels of ADAMTS-1, NF-κB , and STAT3 were expressed as the fold change.

ADAMTS-1, NF-κB, and STAT3 were found to be constitutively expressed in the treatment and non-treatment groups by western blotting of PXA cell lysates. Comparing the PXA cell lines treated with either 10 or 30 ng/mL IL-1, after 24 hours, the 10 ng/mL IL-1 treatment significantly increased the ADAMTS-1 expression levels to 1.86 fold higher and the 30 ng/mL IL-1 treatment induced 1.94 fold increase compared to that of the non-treated control (p=0.004 and p=0.005, respectively) (Figure 3A). There were no significant differences in ADAMTS-1 expression levels between the 10 and 30 ng/mL IL-1 treatment groups (p=0.067).

Moreover, the 10 ng/mL IL-1 induced an additional 1.6-fold upregulation in ADAMTS-1 gene expression in PXA cell lines after 48 hours as compared to the 24 hour timepoint. The 30 ng/mL IL-1 treatment induced an additional 1.68-fold upregulation in ADAMTS-1 gene expression in PXA cell lines after 48 hours as compared to the 24 hour timepoint. ADAMTS-1 expression levels after 48 hours, for both 10 and 30 ng/mL IL-1 treatments, were significantly higher than those after 24 hours (p=0.001 and p=0.004, respectively) (Figure 3A). Significant difference in ADAMTS-1 expression was not detected between the 10 and 30 ng/mL IL-1 treatment groups after 48 hours (p=0.047).

The 10 ng/mL and 30 ng/mL IL-1 treatments caused a 2.5 fold and 2.6 fold increase, respectively, in NF-κB protein levels in PXA cells after 24 hours when compared with the non-treatment group (p=0.025, p=0.005; respectively). The levels of NF-κB were 1.52 and 1.57 fold higher after 48 hours than those at the 24 hour timepoint under IL-1 treatment of 10 and 30 ng/mL, respectively, and were significantly different (p=0.009 and p=0.005, respectively) (Figure 3B). Significant difference was not found between the IL-1 10 and 30 ng/mL treatment groups at the 24 and 48 hour timepoints (p=0.834 and p=0.092, respectively).

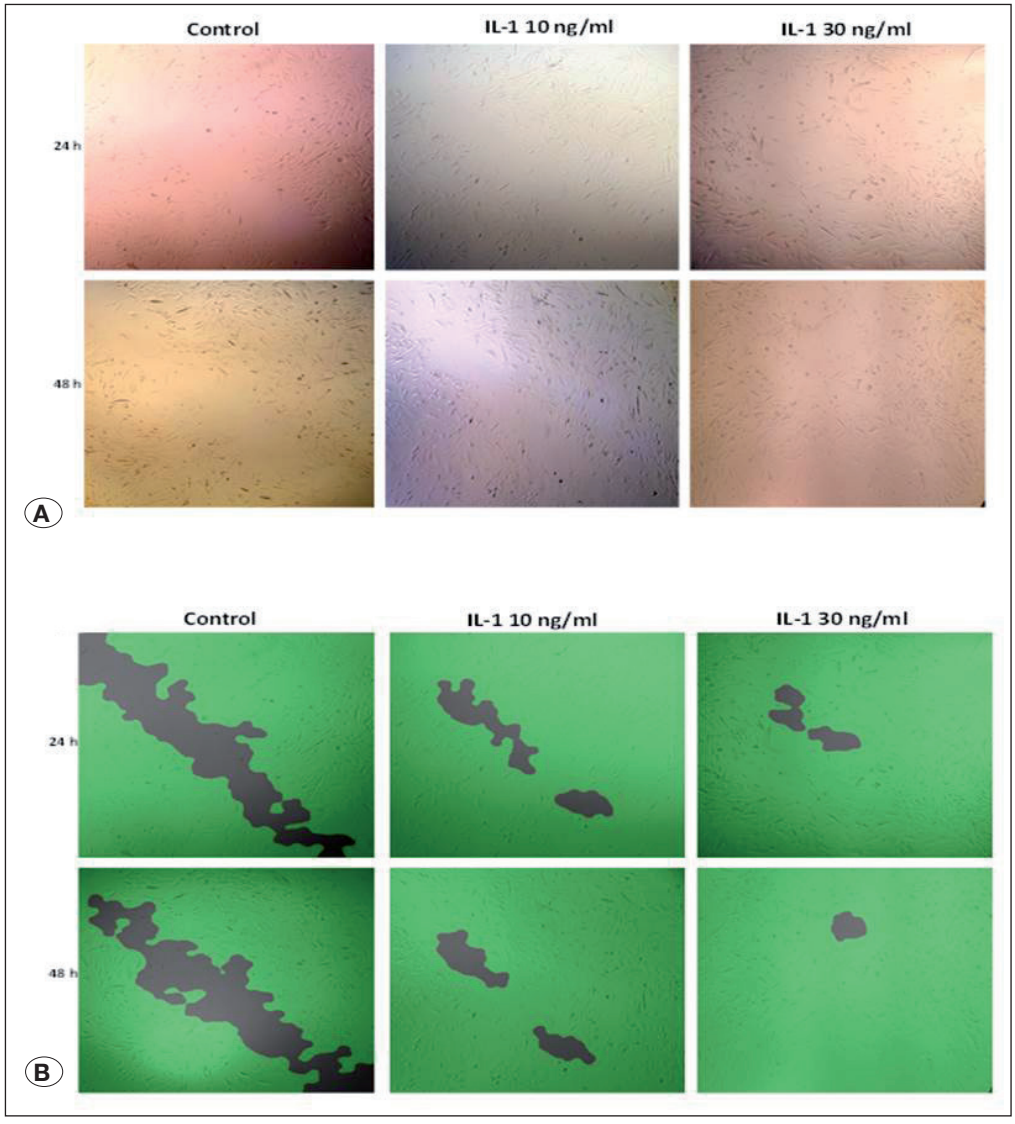

Figure 1: IL-1 effects on migration of pleomorphic xanthoastrocytoma. Cell migration was evaluated with the use of a wounding assay method. Pleomorphic xanthoastrocytoma cells were cultered with IL-1 (0, 10, 30 ng/ml) for the time indicated. **A)** Wound healing assay showing the migrated PXA cells at 24 and 48 h with and without treatment with IL-1 (10, 30 ng/ml). **B)** Pictures of the computerized processing of images are shown in green by the WimScratch platform.

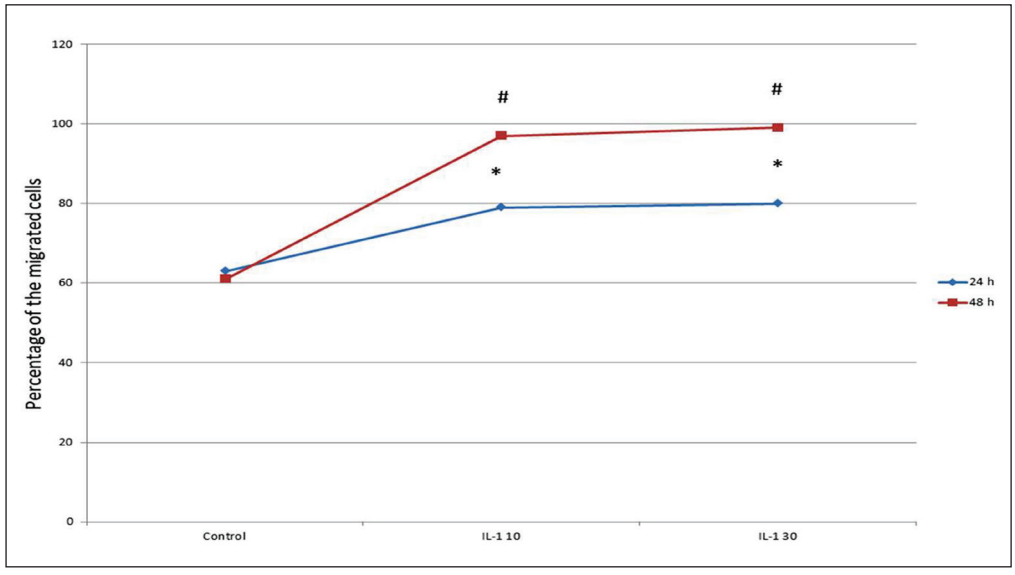

Figure 2: Graph show the precentage of the migrated cells resulted from the Wimscratch platform at 24 and 48 h. *Control versus 24 hours ($p<0.05$), # 24 hours versus 48 hours ($p<0.05$).

Similarly, 10 ng/mL and 30 ng/mL IL-1 treatment doses caused significant 2.62 fold and 2.43 fold increases, respectively, in STAT3 protein levels in PXA cells after 24 hours compared to that of the non-treatment control (p=0.002, p=0.001, respectively). The protein levels after 48 hours under 10 and 30 ng/mL IL-1 doses were 1.44 and 1.57 fold higher, respectively, than those after 24 hours, and were significantly different (p<0.001, p=0.001, respectively) (Figure 3C). No significant difference was found between the two treatment doses after 24 or 48 hours (p=0.074, p=0.286, respectively). All protein expression levels are outlined in Table I.

DISCUSSION

ADAMTS-1 and its potential role was evaluated in the invasiveness of PXA, which is a rare brain tumor with a

Table I: The Protein Levels of ADAMTS-1, NFκB, and Stat3

	Control 24 hours	Control 48 hours	IL-1 10 ng/mg 24 hours	IL-1 30 ng/ml 24 hours	IL-1 10 ng/ml 48 hours	IL-1 30 ng/ml 48 hours
ADAMTS-1	0.36 ± 0.01*,†	0.36 ± 0.02‡,§	0.68 ± 0.01*,¶	0.72 ± 0.01†,††	1.11 ± 0.007‡,¶	1.21 ± 0.01§,††
NF-κB	0.1 ± 0.02*,†	0.12 ± 0.005$^{\#,\S}$	0.25 ± 0.006*,¶	0.26 ± 0.008†,††	0.38 ± 0.005$^{\#,\P}$	0.41 ± 0.003§,††
Stat3	0.16 ± 0.006*,†	0.18 ± 0.009‡,§	0.41 ± 0.003*,**	0.39 ± 0.005†,††	0.71 ± 0.003‡,**	0.72 ± 0.009§,††

All data given as mean ± SEM. ***ADAMTS:*** *A Disintegrin and Metalloprotease with Trombospondin motifs,* ***NF-κB:*** *Nuclear factor –kappaB,* ***Stat:*** *Signal transducers and activators of transcription.* ****:*** *Control 24 h vs IL-1 10 ng/ml 24 h (p<0.05).* ***†:*** *Control 24 h vs IL-1 30 ng/ml 24 h (p<0.05).* ***‡:*** *Control 48 h vs IL-1 10 ng/ml 48 h (p<0.001).* ***#:*** *Control 48 h vs IL-1 10 ng/ml 48 h (p<0.05).* ***§:*** *Control 48 h vs IL-1 30 ng/ml 48 h (p<0.05).* ***¶:*** *IL-1 10 ng/ml 24 h vs IL-1 10 ng/ml 48 h (p<0.05).* *****:*** *IL-1 10 ng/ml 24 h vs IL-1 10 ng/ml 48 h (p<0.001).* ***††:*** *IL-1 30 ng/ml 24 h vs IL-1 30 ng/ml 48 h (p<0.05).*

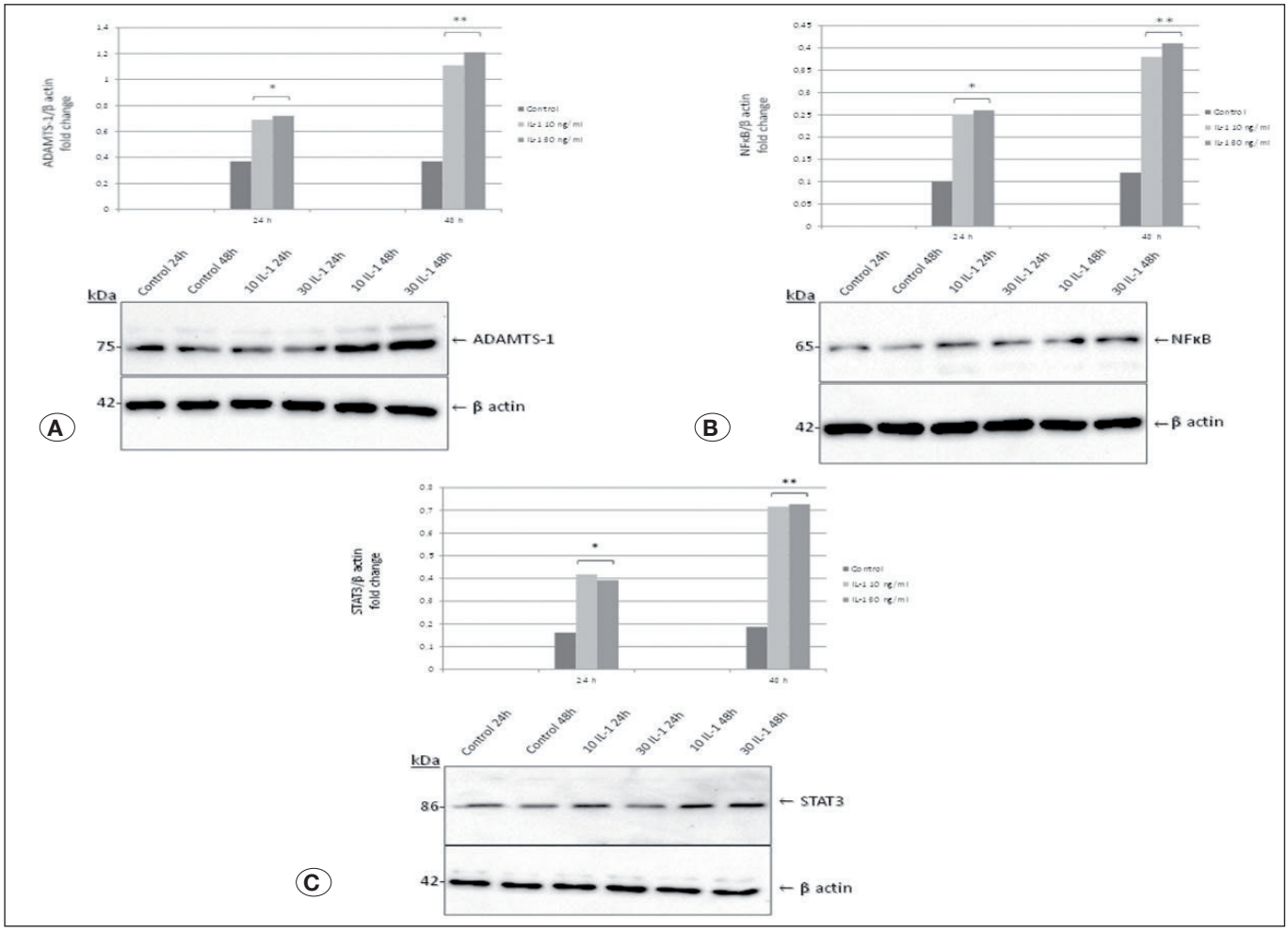

Figure 3: ADAMST-1 **(A)**, NF-κB **(B)**, and STAT3 **(C)** expression levels by Western blotting from 3 different experiments in PXA cell lines of control,10, and 30 ng/ml IL-1 treatment groups at 24 and 48 h time points. The expression levels were expressed as the fold change. *Control versus 24 hours (p<0.05), **24 h versus 48 hours (p<0.05).

favorable prognosis compared to that of diffusely infiltrative astrocytomas. Moreover, we analyzed the expression levels of NF-κB and STAT3 under treatment with the cytokine IL-1. PXA cell lines were incubated with low- and high-dose IL-1 for 24 hours or 48 hours. To determine migration of cells and invasion, we performed a wound-healing assay. The present study is the first report to examine ADAMTS-1 expression in PXA.

The migration, proliferation, and invasion of tumor cells increased under different doses of cytokine treatment, and aggressiveness increased significantly after 48 hours. Compatible with these findings, ADAMTS-1 was upregulated after cytokine treatment and significantly increased in activity after 48 hours. Similarly, the levels of NF-κB and STAT3 were concordant with the levels of ADAMTS-1.

Among the 19 identified ADAMTS proteases, the first to be discovered, ADAMTS-1, has been shown to have catalytic activity on proteoglycans, including aggrecan, versican, and brevican (22,23,37,45,47,58). Aggrecan is an ingredient of the brain ECM (59) and component of perineuronal nets (32). Brevican is also one of the main proteoglycans in adult brain (59). ADAMTS-1 (22), - 4 (18), and -5 (8) show the strongest aggrecanase activity. The degradation of proteoglycans by ADAMTS proteases has been shown to facilitate the invasion of surrounding normal brain tissues by glioma cells (36). In addition, the deregulation of ADAMTS-1 is involved in migration, invasion, and tumor growth (42,44). However, ADAMTS-1 has been known for its protumorigenic and antitumorigenic effects in different tumor types so the activities and contribution of ADAMTS-1 in tumorigenesis appears controversial (13,29,52). Altered ADAMTS-1 expression levels have been reported to be lower in various tumors, such as gastric, hepatocellular, and breast cancers (13,30,40,43). Several studies signify that the anti-tumoral activity of ADAMTS-1 is likely due to its anti-angiogenic effect through its thrombospondin motifs via the inhibition of endothelial cell proliferation, independent of its protease activity (38,54). However, higher ADAMTS-1 expression is often reported in primary gastric tumors with lymph node metastasis compared to that of primary gastric tumors (5). In addition, ADAMTS-1 is more highly expressed in breast and pancreatic cancers with the enhanced metastatic potential (17,30). An elegant commentary about the diversity in differential expression levels of ADAMTS-1 in various tumors has been provided by Liu et al., revealing that full-length form of ADAMTS-1 display pro-tumor activity whereas N- or C-terminal fragments generated by auto-proteolytic cleavage display potent anti-tumor activity (25). The pro-tumoral activity was shown to be regulated through the presence of a metalloproteinase domain (44).

There are several reports associating ADAMTS proteases are involved in progression of glioma mainly by the proteolytic activity on brevican and versican. (31,53,55). Recently, a report by Martino-Echarri et al. reported that high ADAMTS-1 regulation induces the proteolytic cleavage of Insulin-like Growth Factor Binding Protein 2 (IGFBP-2) in GBM to promote glioma invasion (28). Genomic studies by various groups assert that IGFBP-2 expression is a prognostic marker for the tumor grading of gliomas (6,56). One report revealed that high expression levels of ADAMTS-1 and IGFBP-2 induce shorter survival times in glioma patients. Furthermore, Lo Cicero et al. (26) confirmed that oligodendroglioma cells express ADAMTS-1, - 4, and -5, as has been previously shown in glioblastomas (15). Thus, overexpression of ADAMTS-1 might play an essential role in promoting tumor development and increasing the invasiveness of glioma cells.

We did not observe a persistent decrease in ADAMTS-1 levels proportional to the increase in PXA invasiveness compared with those of nonneoplastic tissue, as has been described in human breast carcinomas. IL-1 was found to increase ADAMTS-1 protein expression levels in PXA tumor cells until the termination of these studies after 48 hours. In agreement with previous findings, NF-κB and STAT3 expression levels were upregulated in the presence of IL-1 until the termination of these studies after 48 hours. NF-κB and STAT3 were chosen as potential transcription factors because they are known to be activated in glioma and collaborate to increase survival, proliferation, and tumor invasion and suppress anti tumor immunity via the activation of regulatory T cells (2,49,61). In addition, matrix metalloproteinase -2 and -9 expression is known to be directly upregulated by STAT-3. (16).

Although modulation of the *ADAMTS* genes is still not clear and not well understood, there is evidence that various factors, such as hormones, growth factors, and inflammatory cytokines, are involved in controlling their regulation (50). Rocks et al. reported that the transfection of human lung carcinoma BZR cells with full-length ADAMTS-1 accelerated *in vivo* tumor growth in severe combined immunodeficient mice, and increased ADAMTS-1 expression seem to be associated with higher content of IL-1 and transforming growth factor 1β (44). In addition, increased expression of ADAMTS-1 mRNA following hypoglossal nerve injury has been identified in N1E-115 rat cells treated with IL-1 (48). Cytokine expression in response to signals derived from glioma cells promote progression of glioma cells and modulate the recruitment and infiltration of immune cells (62). Our findings showed that increased ADAMTS-1 levels was related to IL-1 treatment of glioma cells.

CONCLUSION

Association between PXA and ADAMTS-1 proteases has never been postulated to date. In the present study, higher expression levels of ADAMTS-1 were detected in accordance with higher levels of NF-κB and STAT3 and increased invasiveness of PXA tumor cells. IL-1 stimulation may promote tumor progression, and ADAMTS-1, NF-κB, and STAT3 may be regulated in a way that favors the proliferation, invasiveness, and progression of tumor cells. Our findings about the role of ADAMTS-1 in PXA tumor cells may provide a basis for future work. Because these results associating high expression levels of ADAMTS-1 with the invasiveness of PXA is based on *in vitro* data, further studies, including *in vivo* experimental studies, are needed to verify the role of ADAMTS-1 in PXA. Inhibiting the proteolytic function of ADAMTS-1 mediated by IL-1 in gliomas may be a potential treatment to prevent malignant transformation of

astrocytomas. Furthermore, the impact of IL-1 on low-grade gliomas must be carefully observed to identify the pathways related to cytokine stimulation.

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