

ARTICLE

The Distribution of Extracellular Matrix Proteins and CD44s Expression in Human Astrocytomas*

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Aims of the study were: 1. to establish the prevalence of CD44 protein expression in human astrocytomas; 2. to compare the distribution of the extracellular matrix in these tumors; 3. to investigate the relation between CD 44, the extracellular matrix proteins and the histological grade of the tumor. CD44, Type IV Collagen (Col IV), Laminin (LN), Fibronectin (FN), and Tenascin (TN) expression were detected by immunohistochemistry in formalin fixed paraffin embedded tissue samples of 52 astrocytic tumors: 35 glioblastomas (GB), 7 Anaplastic astrocytomas (AA) and 10 astrocytomas (A). The localization of Col IV was observed in the basement membrane of the vessel walls in most of the astrocytomas (88.4%) with a similar pattern obtained with LN staining. 7 of 10 A (70%), 2 of 7 AA (28%) and 9 of 35 GB (25.7%) showed LN positivity. There was a negative correlation between LN expression and tumor grade ($p=0.03$). FN was either localized in the basement membrane or showed thick multi-layered immunoreactivity of the vessel walls. FN expression was seen in 6 A (60%), 4 AA (57%) and all of 35 GB (100%). The FN distribution was not uniform and its

staining intensity showed decrease in GB. 3A (30%), 3 AA (42%), 27 GB (77.1%) showed TN expression in the vessel walls and in some tumor cells of 19 GBs. TN expression was positively correlated with the degree of vascular endothelial proliferation in GB ($p<0.05$). The expression of CD44s was seen as plasma membrane positivity of glioma cells in 5 of 10A (50%), 3 of 7AA (42.3%) and 29 of 35 GB (82.8%). The intensity of immunoreaction was quite strong especially near the vessels. There was a good correlation between TN and CD44s expression in human astrocytic tumors ($p=0.005$). No relationship was observed between GFAP, ECM proteins and CD44s expression. Both CD44s and TN expression showed increase with malignancy in astrocytomas. These findings indicated that the histological malignancy of the astrocytomas was correlated with expression of TN and CD44s. It was suggested that in astrocytomas there was a biological relationship only between CD44 and TN, but none with the other ECM proteins. TN may play a role in angiogenesis in human astrocytic tumors. (Pathology Oncology Research Vol 6, No 2, 118–124, 2000)

Keywords: astrocytic tumors, extracellular matrix proteins, CD44, tenascin, immunohistochemistry, anaplastic astrocytoma, glioblastoma

Introduction

Human malignant gliomas are characterized by uncontrolled cell proliferation and infiltrative growth within the brain. Their complete surgical resection is difficult due to

dissemination throughout the brain. Extracranial metastases of astrocytomas and glioblastomas are extremely rare (0.1–0.5 %).^{6,27,30,34}

Various hypotheses have attempted to explain the lack of dissemination of gliomas outside the brain. However none of them have yet identified the underlying molecular mechanism of this disability. Some observers say that differences in the extracellular environment between the brain and other tissues may play a role in this biological phenomenon.^{27,31,36} It may be due to specific interactions between tumor cell surface receptors and specific extracellular matrix (ECM) components.³⁰

Normal and neoplastic cells in central nervous system (CNS) have shown to produce *in vitro* and *in vivo* ECM proteins such as fibronectin (FN), laminin (LN), type IV

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collagen (Col IV), tenascin (TN) which all are found within the framework of basement membranes of vessels.^{3,30,31,36}

Tenascin (TN) appears to be particularly important. Its distribution is much more restricted than the tissue distribution of FN, LN, and Col IV. During embryogenesis it exists transiently in several developing organs including teeth, kidney, cartilage, CNS, bone. Its expression is believed to correlate well with cell proliferation and migration.^{4,5,8,10,17,23,28,32} During mammary carcinogenesis, it has been found to be re-expressed in the stroma of malignant and some benign tumors.⁷ Specific roles suggested for TN include interference with FN activity and immunomodulation.^{7,17,23} It may also facilitate new blood vessel formation. In this study we formed the hypothesis that tenascin could be increased in more malignant astrocytomas compared to other ECM proteins.

We are also interested in CD44, a cell surface glycoprotein, implicated in a number of cell to cell and cell to matrix interactions.^{12,13} The standard form of CD44 is a lymphocyte homing receptor.² In addition to the standard isoform, CD44 has several larger variants, that are generated by alternative splicing of more than 10 exons.^{12,13} Their functions include serving as the principle receptor for hyaluronic acid (hyaluran), modulating cell to matrix interreactions, and activating lymphocytes and monocytes.^{12,13,33}

It has been shown that CD44 and its variants are expressed in several neoplastic and non-neoplastic tissues.^{2,9,16,19,20,25,33} The variant forms of CD44 are considered to be implicated in tumor progression and metastatic development. Standard CD44 is present only in primary tumors.^{1,25,27}

In this study we investigated relationship between the distribution of ECM proteins (FN, LN, Coll IV, TN) and the expression of CD44s in astrocytic tumors by using immunohistochemical techniques.

Materials and Methods

Tissue specimens

Surgical specimens from 52 human gliomas were chosen for study. The tissues were retrieved from the archives of the Department of Pathology of Istanbul University, Cerrahpaşa Medical Faculty.

The specimens had been routinely fixed in 10% formalin and embedded in paraffin. HE stains of every case

were reviewed by the two pathologists of this study together and graded according to 1993 WHO CNS Tumor Classification.²² Our study concerns 35 Glioblastomas (GB), 7 Anaplastic Astrocytomas (AA), and 10 Astrocytomas (A). After reviewing all available routinely stained slides, each slide and the corresponding paraffin block were chosen for immunohistochemical study. The degree of Vascular Endothelial Proliferation (VEP) in Glioblastomas was scored semiquantitatively; as mild (+), moderate (++) and intensive (+++) according to the intensity of vessels in the high power field of the microscope.

Immunohistochemical staining

Sections were cut 5 µm thick, deparaffinized then pre-treated twice with citrate buffer for 5 minutes in a microwave oven. The endogenous peroxidase activity was blocked using 3% H₂O₂ and the slides were washed twice in Tris-Buffer Saline (TBS).

For anti-Tenascin staining, the slides were treated with Swine Serum (DAKO Patts, X-901) for 30 minutes, followed by incubation in the primary antibody for TN (Monoclonal mouse anti-human TN, DAKO, M636, diluted 1/25) at room temperature and inside a humidified chamber for another 30 minutes. This was followed by peroxidase conjugated avidin-biotin complex technique (PAP-DAKO). The sections were rinsed three times with TBS and subjected to a 20-minute reaction 3-amino 9-ethylcarbazole (AEC solution-DAKO Patts).

In order to stain with FN, LN, Col IV and GFAP performed by labelled streptavidine biotin (LSAB) immunostaining method, the same pretreatment was performed (as cited above) except of incubation within the swine serum. Then the primary antibody was treated with FN (monoclonal rabbit anti-human FN, DAKO, A245, diluted 1/200), LN (monoclonal mouse-anti-human LN DAKO; M638, dilution 1/20), Col IV (Monoclonal mouse anti-human Col IV DAKO, N1536, prediluted) and GFAP (monoclonal rabbit anti-human GFAP, DAKO N 1506, prediluted) each for 30 minutes. After staining, the sections were rinsed three times in TBS, then reacted with AEC solution for 20 minutes.

Table 1. The results of ECM proteins and CD44s staining (+ and ++) in astrocytic tumors

	LN	%	Col IV	%	FN	%	TN	%	CD44s	%
GB	9/35	25.7	30/35	85.7	35/35	100	27/35	77.1	29/35	82.8
AA	2/7	28	7/7	100	4/7	57	3/7	42.8	3/7	42.8
A	7/10	70	9/10	90	6/10	60	3/10	30	5/10	50
n=52	18/52	34.6	46/52	88.4	45/52	86.5	35/52	63.4	37/2	71.1 I

Spearman Rank correlation was used to compare all the results of immunoreactivity; p=0.03

Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP) method was used for CD44s, with the primary antibody of monoclonal mouse anti-human CD44 (DAKO, M7082, diluted 1/40). The slides were coated with Fast Red for 10 minutes and lightly counterstained with hematoxylin and mounted with gelatin (DAKO).

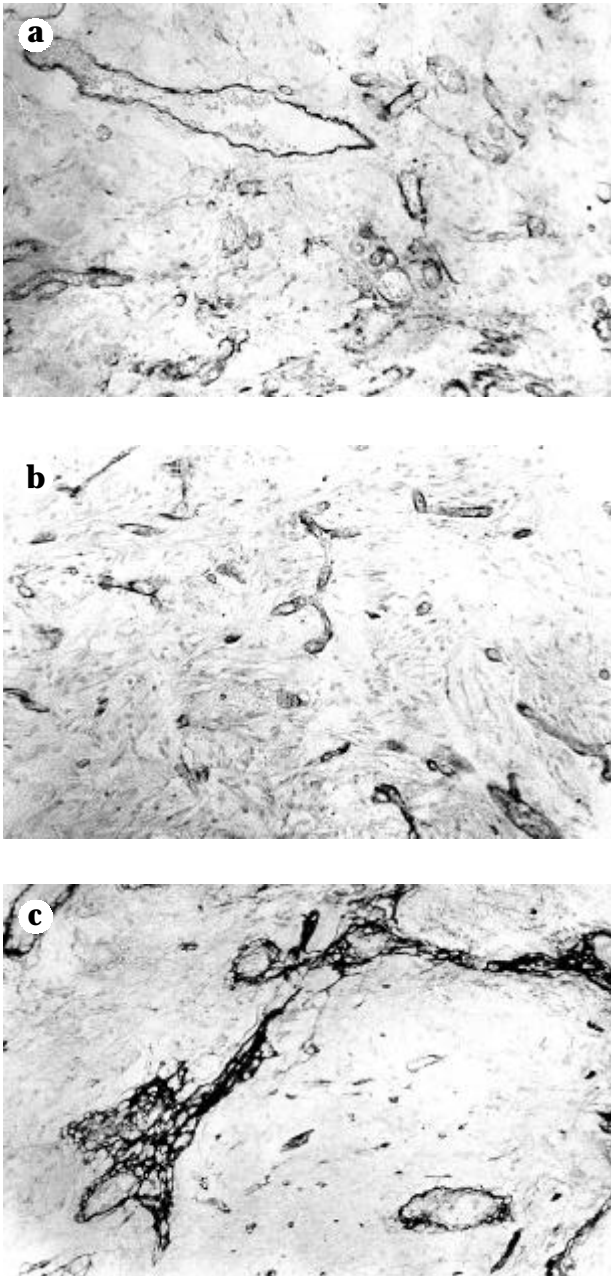


Figure 1. Immunostaining for Anti-Col IV. **a)** Anti-LN **b)** and Anti FN **a)** Note the linear, basal membrane type positive staining in vessel walls for Col IV (x100). **b)** Similar expression of LN; basal membrane type staining for LN. Lack of immunoreactivity in tumor cells (x 100). **c)** Immunostaining for FN in glioblastoma. Basal membrane type and thick, multi-layered staining in vessel walls (x 200).

Tonsillar squamous epithelium was used as a positive control for CD44s. The intensity of immunoreactivity for LN, FN, Col IV, TN, CD44s was scored semiquantitatively by two pathologists; as negative (0), mild (+) and moderate to strong (++) . For GFAP immunoreactivity, the tumors were scored as more than 50% of tumor cells (++) , or less than 50% of cell positivity (+).

Statistical evaluation

Spearman Rank correlation test was used to compare all of the immunoreactivity results. In addition to tumor grade, intensity of vascular endothelial proliferation (VEP), also was compared with immunostaining results by using the same statistical method.

Results

All the cases showed presence of the six antigens in blood vessel walls, in glioma cells and in extracellular space. The presentation of ECM proteins and CD44s were shown in *Table 1*.

Anti-Type IV Collagen (Col IV)

Col IV was detected in the vascular basement membrane of both the normal brain tissue and the tumoral component (*Figure 1a*). Positive staining was observed in 9 of 10 Astrocytomas (90%), all of the 7 Anaplastic Astrocytomas (100%) and 30 of 35 Glioblastomas (85.7%). Almost all gliomas showed extremely high reaction with Col IV (88.4%). The staining pattern was observed as an abundant network of thin-walled capillary vessels, of arterioles and venules. Vessel walls appeared occasionally thickened or bilayered. There was an increase in intensity of staining especially in AA and in GBs.

Anti-Laminin (LN)

A similar staining pattern with Type IV Collagen was observed using anti-LN primary antibody (*Figure 1b*). Anti-LN positivity was seen in 7 of 10 astrocytomas (70%), in 2 of 7 anaplastic astrocytomas (20%); and in 9 of 35 glioblastomas (25.4%).

Laminin expression showed decrease in glioblastomas and anaplastic astrocytomas comparing to astrocytomas. Some of the GB slides, which also included the normal brain tissue adjacent to tumor, showed positive staining in the brain tissue but not in the tumoral counterpart. There was an inverse correlation between laminin expression and tumor grading in astrocytic tumors ($p=0.03$). Neither the cytoplasm nor the extracellular space showed positivity with LN and Col IV staining (*Figure 1a,b*).

Anti-Fibronectin (FN)

Anti-Fibronectin immunoreaction was positive in the vessel walls of both the normal brain and the tumoral tissue. The staining pattern was thicker than anti-LN and anti-Col IV. Rarely mild positive staining of perivascular matrix was observed in the surrounding connective tissue. A faint fuzzy staining was occasionally visible in between and also inside the glioma cells.

The endothelial glomeruloid proliferation of GBs, were stained in a multilayered fashion (*Figure 1c*), but at the same time the vessel walls showed a discontinuous and fragmented staining pattern not extending beyond the parietal cells. The intensity of immunopositivity was lower than low-grade astrocytomas with changes of immunoreaction in focal areas of the same slide. 6 of 10 astrocytomas (60%), 4 of 7 anaplastic astrocytomas (57%), and all of glioblastomas (100%) showed FN positivity. 45 of 52 astrocytic tumors expressed FN (86.5%). Although this was the highest ECM protein positivity that we observed, there was no difference between them statistically.

Anti-Tenascin (TN)

Tenascin expression was predominantly in the basement membrane of the vessel walls. The positivity with TN appeared as multilayered or thick linear staining within the endothelial-glomeruloid proliferation of GBs (*Figures 2 a,b*).

33 of 52 astrocytic tumor (63.4%) expressed TN positivity. 3 of 10 astrocytomas (30%), showed very thin and faint positivity of vessel walls in the tumor and in the brain tissue adjacent to tumor (*Figure 2 a*). In 3 of 7 Anaplastic Astrocytomas (42%) and in 27 of 35 glioblastomas (77%) TN positive immunoreaction was observed. Staining intensity changed from heavy to weak or absent within the same sections. 19 of 35 glioblastomas (54.3%) exhibited TN positive staining in the extracellular space, surrounding the tumor cells, which showed significant contrast

with the background staining. Intracytoplasmic staining was seen only in Glioblastomas as a cytoplasmic membrane type staining.

The positivity and intensity of staining for TN was correlated with the degree of vascular-endothelial proliferation in astrocytic tumors ($p < 0.05$) (*Table 2*). The expression of TN in glioblastomas and in anaplastic astrocytomas was compared with that of FNs. 27 of 35 glioblastomas (77.1%) and 2 of 7 anaplastic astrocytomas (29%) expressed both TN and FN. In these cases some of TN positive tumor vessels did not express FN or some of them showed weak FN positivity.

Anti-CD44 standard(s)

5 of 10 astrocytomas (50%), 3 of 7 anaplastic astrocytomas (42.8%) and 29 of 35 glioblastomas (82.8%) exhibited immunoreactivity with CD44s. 37 of 52 astrocytic tumors (71.1 %) showed positivity.

The immunostaining pattern of CD44s was observed as strong plasma membrane positivity within the tumor cells. The positivity increased especially around the vessels. But all the blood vessels, including very prominent endothelial proliferation were completely devoid of CD44s immunoreactivity. Mild positive immunostaining was observed within the white matter next to the tumoral counterpart. The pattern of staining was distinctly multipolar cell type with radiating process, characteristic of reactive fibrous astrocytes. When the CD44s positivity was compared with the other ECM proteins immunoreactivity, there was a positive correlation with TN staining ($p = 0.005$) (*Figure 4*).

Anti-GFAP

GFAP positivity was observed in tumor cell cytoplasm. 7 of 10 astrocytomas (70%), 6 of 7 anaplastic astrocytomas (85.7%) and 24 of 35 glioblastomas (68.5%) showed more than 50% GFAP positivity (++) score). There was no

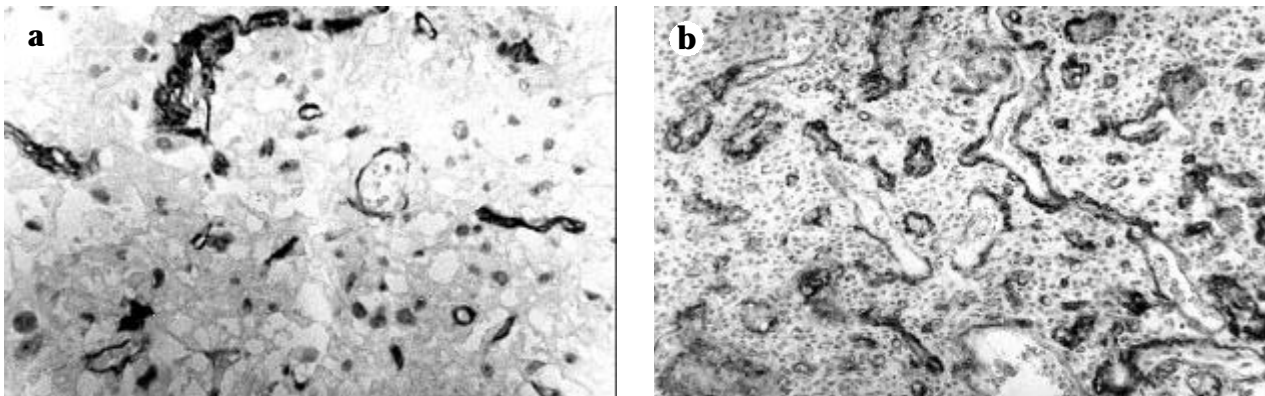


Figure 2. Immunostaining for TN in astrocytoma **a)** and glioblastoma **b)**. **a)** Faint and interrupted expression of TN in vessel walls (x400). **b)** Strong basement membrane type thick linear positivity in glioblastoma (x200).

immunoreactivity in the vessel walls. No relationship between ECM Proteins expression and GFAP Immunoreactivity score was found.

Discussion

The extracellular matrix is of a fundamental importance in directing development and differentiation of cells in an embryo and its organs. Fibronectin constitutes the major glycoprotein of the mesenchymal tissues whereas laminin is predominantly present in basement membrane. Both proteins are important for cell attachment, cell migration, differentiation and morphogenesis.

In normal and neoplastic conditions of central nervous system, ECM proteins such as LN, Col IV were found to be produced in-vivo within the framework of basement membranes. FN has been shown to be both in the intercellular matrix and the basement membrane of the vessel walls.³ To better understand the cellular mechanism of glioma cell invasion, many *in vitro* studies have been performed. They showed migratory responses and adhesiveness of human malignant glioma cells to ECM proteins; FN, LN, vitronectin and Col IV.^{15,30,31,35} Migration of astrocytomas was found to be variable and dependent on different ECM proteins.¹⁵ Our findings for Type IV Collagen, LN, and FN were similar to the literature.^{3,17} Col IV was found in basement membrane of vessel walls in most of the gliomas (88.4%). There was no correspondence between the intensity of immunoreactivity and the tumor grade.

Similar positive staining pattern was observed with anti-LN. But LN expression in glioblastomas (25.7%) and in anaplastic astrocytomas (28%) showed a decrease compared to astrocytomas (70%). ($p=0.03$). In literature laminin expression was generally observed in all grades of gliomas.^{3,17} But, our result was not an artifact since some of GB cases included both tumoral and non-tumoral areas in the same slides; and in these cases we observed the positive reaction in the vessel walls of the normal brain but not in the tumoral areas. Some observers showed that LN expression decreased in some malignant tumor such as in breast tumors, together with increasing of TN positivity.^{18,23}

In our study no extravascular immunoreactivity with Type IV collagen and laminin was observed in any of the gliomas. In most astrocytic tumors (86.5%), we found expression of FN in small and medium-sized vessel walls. In vascular endothelial proliferation FN positivity appeared as multilayered and thicker than the thin vessel walls. There was an extracellular immunoreactivity as a fine irregular network, which was confirmed in literature.^{3,17} According to these reports this feature might be the result of leakage of plasma fibronectin into the tumor.

Tenascin has much more restricted tissue distribution than the other ECM glycoproteins such as FN, LN, and Col IV. During embryogenesis TN is transiently present in

dense mesenchymal tissue, surrounding several developing organs, but a very faint amount is detectable in normal adult tissue.^{8,17} The major effect of Tenascin was on cell shape, which is believed to correlate with cell proliferation and migration, as well as remodeling of the ECM.¹⁷ Numerous studies have provided the re-expression of TN in different tumor tissue.^{5,8,23} The intact adult CNS

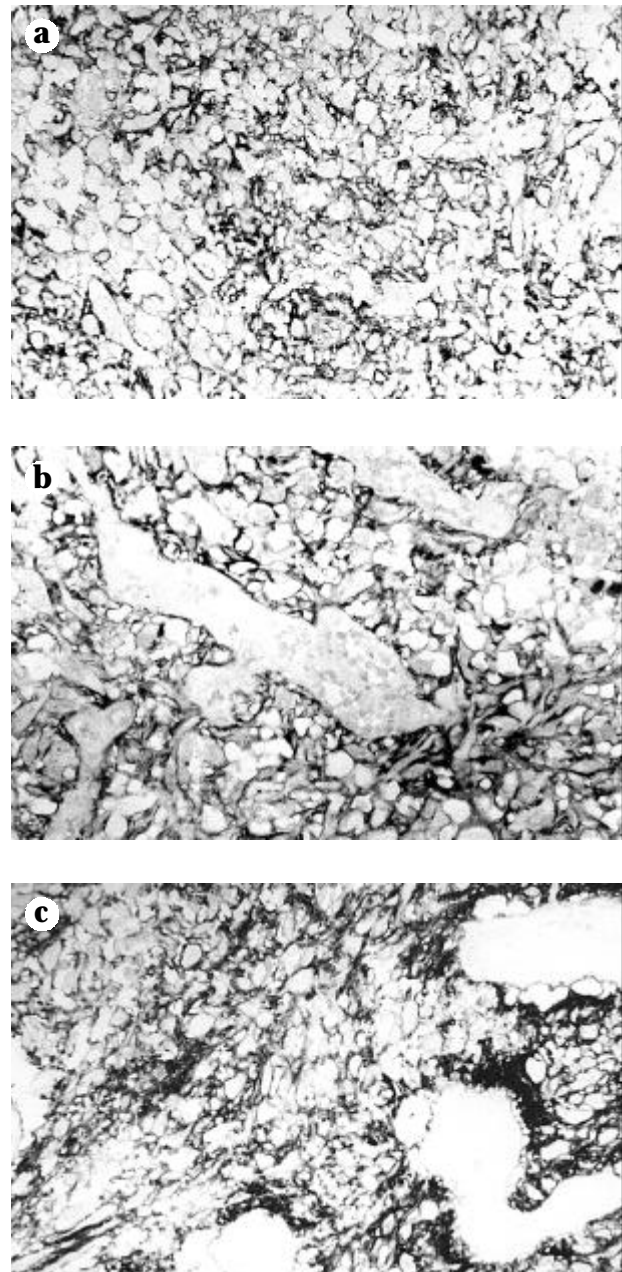


Figure 3. Immunostaining for CD44s. **a)** Membranous type staining in tumor cells in Anaplastic astrocytoma (x200). **b)** Strong membranous type in glioblastoma. Lack of immunoreactivity in vessel walls (x400). **c)** Note the increasing of CD44s expression around the vessel walls in glioblastoma (x200).

Table 2. The relation of Tenascin and CD44s expression with VEP in Glioblastomas

VEP in GB	Tenascin			CD44s		
	++	+	0	++	+	0
n=35						
+++	6	6	2	8	5	0
n=14						
++	6	1	4	7	2	0
n=11						
+	2	5	3	3	5	2
n=10						

Spearman Rank correlation test: $p < 0.05$

includes low levels of TN expression but it increases in wound healing and gliomas.^{4,10,28} Quantitation of TN in glioma cell lines revealed higher level than those in normal brain tissue extracts.^{29,35}

In our study TN expressed chiefly in the basement membrane of tumor vessel as reported previously.^{8,17,23} We demonstrated that the expression of TN was correlated well with the degree of histological malignancy of gliomas; TN was detected in 30% of astrocytomas, in 42.8% of anaplastic astrocytomas and in 77.1% of glioblastomas. This finding was consistent with the fact that the presence of vascular-endothelial proliferation is a reliable sign for a worse prognosis in malignant astrocytic tumors.^{22,34}

Distinctive reactions were seen around the hyperplastic vessels in 19 of 35 glioblastomas as previously reported.²³ This staining pattern was seen as linear cytoplasmic membrane type between the tumor cells.

In literature there were several reports that TN interferes with tissue fibronectin action,^{5,7,8,23} encouraging us to investigate the relationship between FN and TN in the same case. 27 GB (77.1 %) and 2 AA (29%) showed expression of both TN and FN. We tried to find the same vessel in the same slides, in order to compare the FN and TN positivity. Although some of TN positive vessels did not show FN expression or the inverse, there were some vessel, which presented with both TN and FN positivity.

Higuchi et al. suggested that tumor neovascularization may be related to TN expression in the high-grade gliomas. TN may suppress the expression of FN in the tumor vessels and then produce TN or induce the tumor vessels to express TN. Although there was no astrocytoma specific ECM proteins, it has been demonstrated that TN is a much less effective substrate for attachment of various cells than FN.^{8,15,17} If the TN is expressed instead of FN in the tumor vessel this may result in loosening of the adhesion of endothelial cells to the surrounding matrix.^{8,15}

As previously indicated,^{18,23} there was a negative correlation between the expression of TN and LN. We found that when the LN expression decreased or was absent in

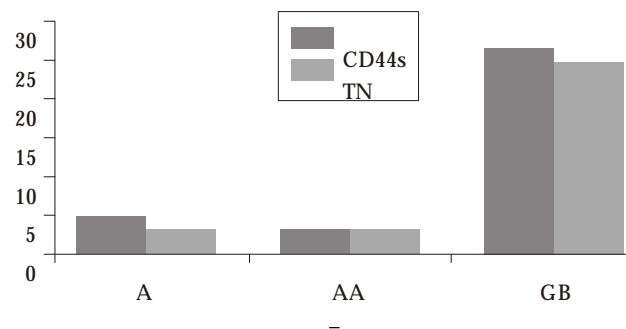
glioblastomas. Inversely the immunoreactivity of TN increased and was intense.

Although GB expressed high level TN more than low-grade astrocytomas,^{17,23,35} there was a marked variation in the intensity of immunostaining not only among different tumor samples but also in different regions on the same section of individual tumors. This suggested that the expression of TN might be due to the biological heterogeneity of malignant glioma.¹⁷ We found no relationship between positivity and intensity of GFAP and TN or other ECM protein expression. But Higuchi et al. reported that TN positive tumor matrix did not express GFAP.¹⁷

It is generally accepted that CD44 is a cell adhesion receptor and that hyaluronan is one of its ligands, which is a common component extracellular matrix and extracellular fluids.²⁶ The involvement of adhesion molecules in disseminating or metastasis was recently provided by the demonstration of standard CD44 (CD44s) and its variants. Clinical studies have confirmed that most carcinomas usually express a large spectrum of CD44 isoforms. However the metastatic potential of carcinoma has been related to expression of variants of CD44 especially CD44 v3.^{9,11,12,13,27}

Previous reports revealed that expression of variant CD44 isoform was observed in secondary brain tumors, but primary brain tumors such as glioblastomas expressed only CD44s.^{12,24,27} That's why, we decided to study only CD44s expression in astrocytic tumors, other than its variants.

Five of 10 A, 3 of 7 AA, and 29 of 35 GB showed CD44s immunoreactivity with the plasma membrane staining pattern within glioma cells, as was confirmed in literature.^{1,27} We did not see any immunostaining in vessel walls of the brain tissue or the gliomas. But there was a weak CD44s expression in normal white matter or cortex as was observed in literature.^{12,14} Reactive astrocytes near or inside the gliomas showed intense reaction expressing high levels of CD44s on their surfaces. These cells can be identified by positive binding to anti GFAP.¹⁴ Khoshyomn et al. reported that the CD44s expression was localized especially at the margin of the glial tumor.²¹ We observed that intensity of CD44s expression varied in focus areas being denser near the vessel walls. A number of experimental studies suggest-

**Figure 4. The correlation between tenascin and CD44s**

ed that the interaction of this receptor with ECM proteins might be partly mediating human glioma cells adhesion and invasion of brain tissue.^{21,26} We found that there was a strong correlation between TN and CD44s expression in astrocytic tumor ($p=0.005$) (Figure 4).

We can speculate that there may be a biologically strong relation between the expression of Cd44s and TN in malignant astrocytic tumor; and also with their increase of expression, LN and FN presentation decrease. TN may play a role in angiogenesis in malignant gliomas. TN and CD44s may act together as an autocrine growth factor for glioma cells.

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