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Expression of tenascin in human gliomas: its relation to histological malignancy, tumor dedifferentiation and angiogenesis

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Summary. The immunohistochemical distribution of tenascin (TN), fibronectin (FN), and laminin (LN) was investigated in 56 human gliomas (8 astrocytomas, 15 anaplastic astrocytomas, and 33 glioblastomas) with regards to the histological degree of malignancy and the degree of tumor cell differentiation evaluated by the staining of glial fibrillary acidic protein (GFAP). In 8 anaplastic astrocytomas and 28 glioblastomas, TN was predominantly immunolocalized in the basement membrane zone of the proliferating tumor vessels; sections of all astrocytomas were negative for TN staining. FN was localized in the basement membrane zone of the vessels in all astrocytomas, 12 anaplastic astrocytomas, and 22 glioblastomas. In 7 anaplastic astrocytomas and 19 glioblastomas, both TN and FN were expressed to various degrees in the tumor vessels. However, most of the TN-positive vessels did not express FN, and most of the FN-positive vessels were negative for TN staining. Furthermore, in 6 anaplastic astrocytomas and 12 glioblastomas, either TN or FN, but not both, were expressed in any area on serial sections. Most of the tumor cells around TN-positive, FN-negative tumor vessels did not express GFAP. On the other hand, GFAP was present in most tumor cells around TN-negative, FN-positive vessels. LN was detected in all vascular and pial-glial basement membrane zone of the tissues examined. These findings indicate that the degree of histological malignancy and the degree of cell dedifferentiation of human gliomas correlate well with the expression of TN, but are inversely correlated with the expression of FN. We postulate that the expression of TN, but not of FN, plays a role in the promotion of angiogenesis in malignant gliomas.

Key words: Glioma – Tenascin – Fibronectin – Cell differentiation – Tumor angiogenesis

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The tissue distribution of tenascin (TN), one of the extracellular matrix (ECM) glycoproteins, is much more restricted than the tissue distribution of other ECM glycoproteins such as fibronectin (FN) and laminin (LN) [8, 10, 11, 26]. During embryogenesis, TN is transiently present in the dense mesenchyme surrounding several developing organs such as the mammary gland [8], tooth [38], kidney [3], gut [2], cartilage, bone [25], or the central and peripheral nervous system [7, 10, 15] where the expression of TN is believed to correlate with cell proliferation and migration, as well as remodeling of the ECM [7, 11]. In normal postnatal tissue, only a faint expression of TN is detectable in the kidney [3, 20], skin, mammary gland, lung, liver [20], perichondrium and periosteum, ligaments, tendons, smooth muscle, and myotendinous junctions [10, 20, 25], while extensive accumulation of TN can be observed in the granulation tissue both of healing wounds [26] and in reparative and hyperplastic process [20]. These data suggest that the presence of TN in adult tissue is not always indicative of malignancy. On the other hand, TN is expressed intensely in the capillary basement membranes and/or stromal elements of several highly anaplastic tumors such as melanoma, fibrosarcoma [6, 20], squamous cell carcinoma of the skin [1, 20, 22], mammary carcinoma [20, 24], and Wilms' tumor [3, 20], while this glycoprotein is present in trace amounts, if at all, in differentiated tumors [1, 22, 24]. Bourdon et al. [5] demonstrated that glioblastomas expressed a glioma-mesenchymal extracellular matrix (GMEM) protein, which was later proved to be identical with TN by the use of monoclonal antibody 81C6 [6]. They also demonstrated that human glioma cell line U251MG can secrete a large amount of TN in vitro [6].

More than half of the TN molecule is homologous to the amino acid sequence of FN [11, 18, 37]. Furthermore, these two glycoproteins have opposite effects on several cell functions, both in vitro and in vivo. For example, soluble TN inhibits the attachment and spreading of cells on FN substrate [5, 9, 23]. Furthermore, TN promotes chondrogenic and osteogenic differentiation

in vivo and chondrogenesis in vitro, while FN has inhibitory effects [25].

In the central nervous system, FN and LN are localized chiefly in the basement membrane of vessels of both normal and pathological tissues [4, 16, 27, 28, 33]. Recently, it was demonstrated that TN was found within reactive astrocytes in case of cerebellar degeneration [29] and injured brain [21]. We have demonstrated that human malignant glioma cells can migrate in response to in vitro stimulation by FN [30]. Tumor cells were found to form clusters around fine networks of FN in the extracellular space of human glioma tissues, and to express FN receptors [16]. These data suggest that FN plays an important role in glioma cell invasion. On the other hand, the functional role of TN in human malignant gliomas remains unknown.

The distribution of TN, FN, and LN in gliomas is associated with tumor vessels [6, 16, 27, 28, 33]; however, no comparative study has been published of TN and FN and/or LN regarding their expression in serial sections from human gliomas of varying degrees of malignancy.

In the present study, we investigated the immunohistochemical distribution of TN, FN, and LN in 56 human gliomas, and analyzed the relationship between the degree of expression of these glycoproteins and cellular differentiation as evaluated by GFAP staining. We also discuss the possible role of TN in tumor angiogenesis.

Materials and methods

Tissue specimens

Surgical specimens from 56 human gliomas were studied. The tissues were fixed in 10 % formalin and embedded in paraffin. The histological diagnosis of these tumors was confirmed by hematoxylin-eosin staining, and they were classified into three grades according to the New World Health Organization Classification of Brain Tumors [40]; 8 were astrocytomas (grade II), 15 anaplastic astrocytomas (grade III), and 33 glioblastomas (grade IV).

Antisera and monoclonal antibody

Rabbit antiserum against human TN purified from U251MG glioma cells was purchased from Telios Pharmaceuticals Inc. (San Diego, Calif.). Rabbit anti-human FN and rabbit anti-LN antisera were obtained from Biomedical Technologies Inc. (Stoughton, Mass.) and Chemicon International Inc. (Tenecuka, Calif.), respectively. A mouse anti-GFAP monoclonal antibody was obtained from Labsystems (Helsinki, Finland).

Immunohistochemical staining

Immunohistochemical staining for TN, FN, LN, and GFAP was performed by the labeled streptavidin biotin (LSAB, DAKO Corporation, Carpinteria, Calif.) staining method [36]. Formalin-fixed and paraffin-embedded tissue blocks were cut into 6-μm-thick serial sections on a microtome. These sections were deparaffinized by incubation in xylene, followed by serial rehydration through 100 %, 90 %, 80 %, and 70 % ethanol. For staining of FN and LN, they were pretreated with 0.1 % pepsin (Sigma Chemical Company, St. Louis, Mo.) in 0.01 M HCl for 15 min at 37 °C to unmask the antigenicity of these proteins [17, 27]. Sections immunostained for TN and GFAP were not pretreated with the proteolytic enzyme. All tissue sections were incubated for 5 min with 3 % hydrogen peroxide to block endogenous peroxidase. They were rinsed and treated with 10 % normal goat serum for 5 min at room temperature. Primary antisera (anti-TN, -FN, and -LN antibodies diluted 1:50) and anti-GFAP antibody (diluted 1:100) were incubated overnight at 4 °C in a humidified chamber. Replacement of the primary antisera or antibody with nonimmune rabbit or mouse serum provided the negative control. After washing with 0.05 M TRIS/HCl buffer, the sections were incubated with biotinylated anti-rabbit/mouse immunoglobulins for 10 min, and then washed with 0.05 M TRIS/HCl buffer. After incubation with the peroxidase-labeled streptavidin for 10 min, the sections were rinsed and subjected to a 10-min reaction with 0.07 % 3-amino-9-ethylcarbazole (AEC) and 0.007 % hydrogen peroxide in 0.1 M acetate buffer (pH 5.2). The sections were then lightly counterstained with hematoxylin and mounted with glycerol gelatin (DAKO).

Results

Immunoperoxidase staining of TN

In 8 of 15 anaplastic astrocytomas (53.3 %) and 28 of 33 glioblastomas (84.8 %), TN was predominantly immunolocalized in the basement membrane zone of the tumor vessels (Figs. 1, 2A). Staining was intense in some areas and weak or absent in others within the same section. The intensity of the stain for TN correlated well with the degree of endothelial proliferation of the tumor vessels. Of 28 glioblastomas presenting TN-positive tumor vessels, at least 12 exhibited TN-positive staining in the extracellular space around tumor cells, which were distinguishable from background staining (not shown). Intracytoplasmic staining of TN was seen in only a few tumor cells (not shown). None of the 8 astrocytomas, nor brain tissue adjacent to the tumor, showed any expression of TN.

Immunoperoxidase staining of FN

FN was localized in the basement membrane zone of the vessels in all 8 astrocytomas, 12 of 15 anaplastic astrocytomas (80 %), and 22 of 33 glioblastomas (66.7 %) (Fig. 3B). In both anaplastic astrocytomas and

Fig. 1A,B. Immunohistochemical staining of tenascin (TN) in glioblastoma tissue sections. TN was predominantly localized in the basement membrane zone of tumor vessels. **A,B** × 100

Fig. 2A–C. Immunohistochemical staining of TN, fibronectin (FN), and GFAP in serial sections of glioblastoma. **A** TN was expressed in the basement membrane zone of tumor vessels. **B** FN was not expressed in the same vessels (*arrows*). **C** GFAP was expressed in only a few of the tumor cells around the TN-positive vessels (*arrows*). **A–C** × 200

Fig. 3A–C. Immunohistochemical staining of TN, FN, and GFAP in serial sections of anaplastic astrocytoma. The tumor vessel, which did not express TN (**A**, *arrows*), was stained for FN (**B**). A positive fuzzy staining of FN in the perivascular tumor matrix was also visualized (**B**). GFAP was positively stained in most astrocytic tumor cells around this vessel (**C**). **A–C** × 100

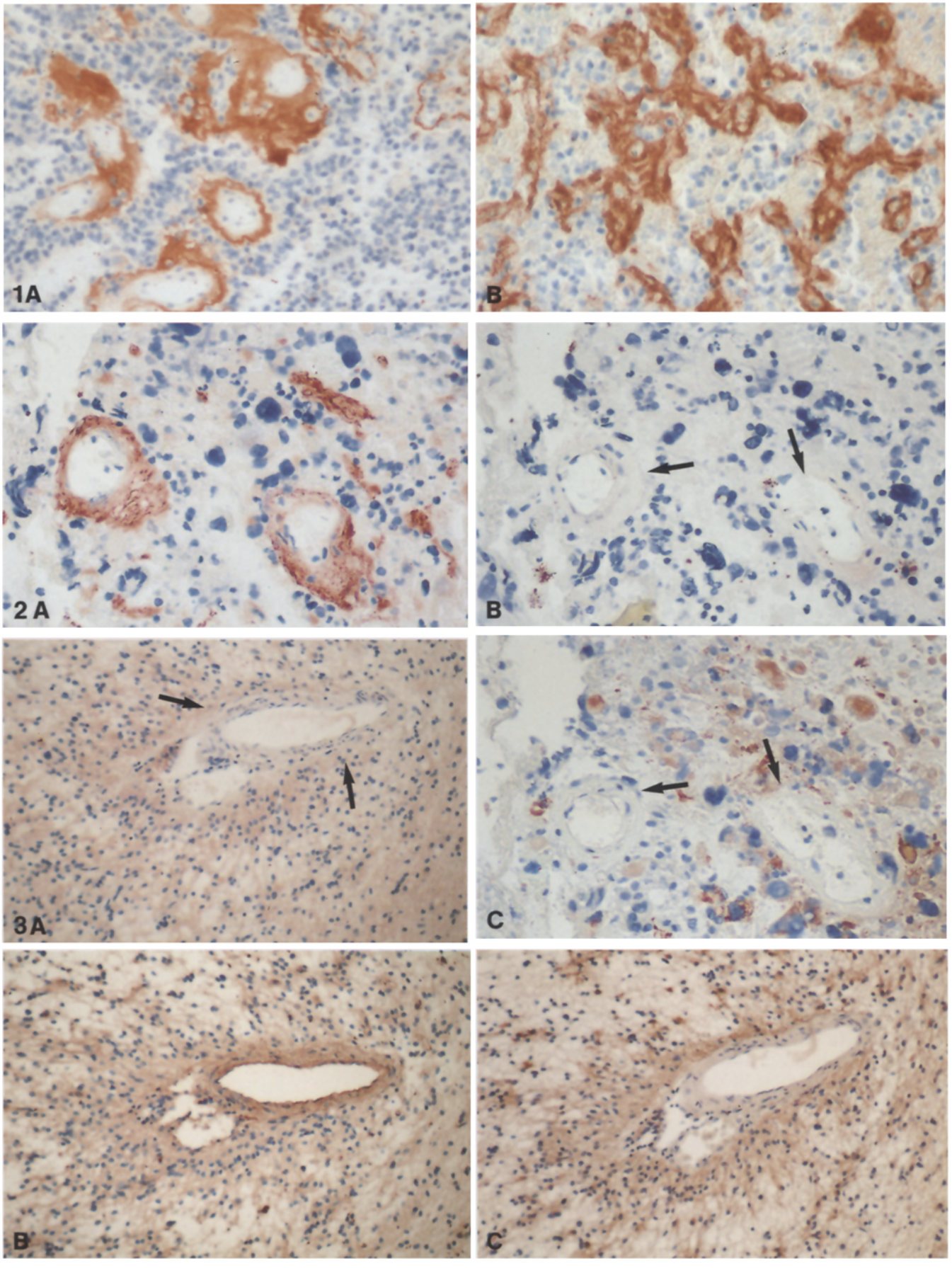
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Table 1. Results of tenascin (TN) and fibronectin (FN) staining of human glioma tissue sections

Classification of 56 gliomas	Reactivity[a]			
	TN		FN	
Astrocytoma	0/8	(0%)	8/8	(100%)
Anaplastic astrocytoma	8/15	(53.3%)	12/15	(80.0%)
Glioblastoma	28[b]/33	(84.8%)	22/33	(66.7%)

[a] Results are expressed as the number of tissue section presenting TN- or FN-positive tumor vessels/total number of tissues examined

[b] Of these 28 samples, at least 12 glioblastomas exhibited TN staining also in the extracellular matrix of the tumor tissue

glioblastomas, there was marked variation in the intensity of staining in different regions of the same section. FN was also detected in the vessels of brain tissue adjacent to the tumor in all specimens examined. As was observed previously [4, 16], faint fuzzy staining of FN was found in the extracellular space around FN-positive tumor vessels, which were distinguishable from background staining (Fig. 3B). FN was not detected in the cytoplasm of tumor cells or of normal glial or neuronal cells. The results of immunoperoxidase staining are summarized in Table 1.

Immunoperoxidase staining of LN

LN was detected in all vascular and pial-glial basement membrane zone of both tumor and brain tissues (not shown). The intensity of LN expression was not related to the degree of tumor anaplasia. No intracytoplasmic reaction of anti-LN antisera was observed either in tumor cells or in normal glial or neuronal cells.

Expression of TN and FN in the same samples

The expression of TN in the tumor vessels was compared with that of FN in serial sections from anaplastic astrocytomas and glioblastomas (Fig. 4). In 7 of 15 anaplastic astrocytomas (46.7%) and 19 of 33 glioblastomas (57.6%), various degrees of both TN and FN were expressed. In these samples, however, most of the TN-positive tumor vessels did not express FN (Fig. 2A,B), and most of the FN-positive vessels were negative for TN staining (Fig. 3A,B). In addition, TN and FN were expressed exclusively of each other in all areas on serial sections from 6 anaplastic astrocytomas and 12 glioblastomas. That is, 1 anaplastic astrocytoma (6.7%) and 9 glioblastomas (27.3%) expressed TN, but not FN. In contrast, 5 anaplastic astrocytomas (33.3%) and 3 glioblastomas (9.1%) expressed FN, but not TN. Two anaplastic astrocytomas and two glioblastomas expressed neither TN nor FN.

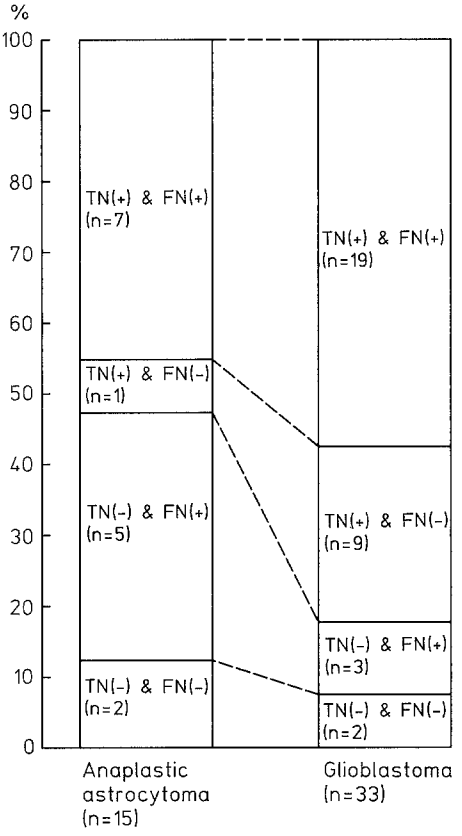

Fig. 4. Expression of TN in the tumor vessels compared to that of FN in serial sections of anaplastic astrocytomas and glioblastomas. TN (+) & FN (+): Various degrees of both TN and FN were expressed in the tumor vessels. However, most of the TN-positive vessels did not express FN, and the FN-positive ones did not express TN. *TN(+) & FN(−):* TN, but not FN, was expressed in the tumor vessels. *TN(−) & FN(+):* FN, but not TN, was expressed in the tumor vessels. *TN(−) & FN(−):* Neither TN nor FN was expressed in the tumor vessels

Immunoperoxidase staining of GFAP

In both anaplastic astrocytomas and glioblastomas, only a few of the tumor cells around the TN-positive but FN-negative tumor vessels expressed GFAP (Fig. 2C). This protein was also slightly expressed in tumor cells surrounded by a TN-positive tumor matrix, and in cells whose cytoplasm was positively stained for TN. On the other hand, even in the same section from each tumor, most tumor cells around TN-negative but FN-positive vessels exhibited GFAP in their cytoplasm (Fig. 3C).

Discussion

Expression of TN, FN, and LN with regard to histological malignancy

In the present study, TN was found to be expressed chiefly in the basement membrane zone of tumor vessels, as reported previously [5, 28]. The intensity of the stain for TN correlated well with the degree of

endothelial proliferation of the tumor vessels. TN was also detected in the extracellular space around tumor cells in at least 12 glioblastomas. Although the TN staining in the extracellular space was, more or less, found in other glioblastomas and anaplastic astrocytomas, we could not strictly distinguish these staining from background staining. We demonstrated that the expression of TN correlates well with the degree of histological malignancy of human gliomas. TN was not detected in any of the 8 astrocytomas or in brain tissue, whereas 8 of 15 anaplastic astrocytomas (53.3%) and 28 of 33 glioblastomas (84.8%) expressed TN. These findings are consistent with the facts that the presence of endothelial proliferation, as well as those of necrosis and increased mitotic activity, is regarded as a reliable sign of poor prognosis in patients with malignant glioma [13, 32], and that TN is expressed intensely in the capillary basement membranes and/or stromal elements of several highly anaplastic tumors, whereas this glycoprotein is little expressed, if at all, in differentiated tumors [1, 3, 6, 22, 24].

Although glioblastomas expressed TN to a high degree [5, 28], there was 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 suggests that the expression of TN may be due to the biological heterogeneity of malignant gliomas.

In contrast to TN, the degree of FN expression in tumor vessels was inversely related to the malignancy of human gliomas; FN was expressed in all 8 astrocytomas, and 12 of 15 anaplastic astrocytomas (80.0%), whereas it was expressed in only 22 of 33 glioblastomas (66.7%). As observed in TN staining, marked variation in the intensity of immunostaining was found in case of FN staining among different tumor samples and different regions on the same section of individual tumors, which may be due to the biological heterogeneity of malignant gliomas. Although previous reports described consistent FN expression in all gliomas examined [4, 28], our findings may be supported by the fact that malignant transformation often reduces or eliminates FN production in vitro [12, 39].

Unlike TN and FN, it seems that the expression of LN in human gliomas is not related to the degree of histological malignancy and anaplasia, since LN was detected to the same degree in the basement membrane zone of all samples examined.

Expression of GFAP related with that of TN and FN

We found that most of the tumor cells around the TN-positive tumor vessels and in the TN-positive tumor matrix did not express GFAP. The tumor cells whose cytoplasm was positively stained for TN were also negative for GFAP. On the other hand, GFAP was well exhibited in most tumor cells around the TN-negative vessels and matrix. In contrast, the expression of FN in tumor vessels correlated well with that of GFAP in the tumor cells around the vessels.

The variation of GFAP expression in malignant gliomas is closely related to the heterogeneity and/or sarcomatous change of the tumor cells [32, 35]. Anaplasia not only shows phenotypic modulation but also is accompanied by the development of cell subpopulations with different genetic properties, thus resulting in cellular heterogeneity [19, 35]. The number of GFAP-positive cells is inversely related to the degree of differentiation, and GFAP is often negative in the highly anaplastic cells that proliferate most rapidly [19, 35]. It has been suggested that a negative reaction for GFAP in tumor cells can also be indicative of sarcomatous change, and that both the tumor cells and the mesenchyme of the sarcomatous component strongly express FN [14, 34]. In our study, however, the areas adjacent to the TN-positive region did not show a sarcomatous nature either with the reticulin stain or with the immunostain for FN. Therefore, our results indicate that the expression of TN in human gliomas may correlate with the degree of dedifferentiation, but not with sarcomatous change.

Source of TN in human glioma tissue

Although intracytoplasmic TN staining was identified in a few tumor cells, the source of TN in human glioma tissue in vivo remains unknown. Since human glioma cell lines U251MG and U373MG secrete a large amount of TN in vitro [6, 11], TN expressed at least in the ECM of tumor tissue may be produced by glioma cells themselves. On the other hand, a wide range of human carcinoma cell lines have been assayed for TN secretion, but none produced a detectable amounts [11]. It is also suggested that the tumor cells do not produce TN but induce its synthesis by the mesenchyme in vivo [1, 24]. In this regard, TN in the tumor vessels might be produced either by the tumor cells themselves, or by the vascular components with induction of surrounding tumor cells.

Role of TN in tumor angiogenesis

We found that most of the TN-positive tumor vessels did not express FN, and were surrounded by GFAP-negative tumor cells. On the other hand, TN was rarely detectable in the majority of FN-positive vessels which were surrounded by GFAP-positive tumor cells. This may be related to tumor neovascularization by the following mechanism: the gliomas with anaplastic change may suppress the expression of FN in the tumor vessels, and then produce TN or induce the tumor vessels to express TN. It has been demonstrated that TN is a much less effective substrate for attachment of various cells than FN [8, 9, 23]. Therefore, if TN istead of FN is expressed in the tumor vessels, this may result in loosening of the adhesion of endothelial cells to the surrounding matrix. Consequently, the proliferating endothelial cells may become able to migrate more easily, thus promoting tumor angiogenesis. The association of TN with angio-

genesis may be distinct from that of type VIII collagen, in that type VIII collagen was expressed in the tumor vessels of both high-grade and low-grade gliomas to a high degree [31], while TN was expressed in the tumor vessels of high-grade gliomas, but not low-grade gliomas.

Further studies, including molecular analyses, should be carried out to elucidate the mechanism of tumor angiogenesis, especially by identifying the factor which converts expression of FN to that of TN in tumor vessels. Such an approach may present a new modality of treatment for malignant gliomas.

In conclusion, the results of this study indicate that the degree of histological malignancy and cell dedifferentiation of human gliomas correlate well with the expression of TN, but are inversely related to that of FN. The glioma cells with anaplastic change may suppress the expression of FN in the tumor vessels, and then produce TN or induce the tumor vessels to express TN. Furthermore, it is suggested that expression of TN rather than FN may play a role in the promotion of angiogenesis in malignant gliomas.

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