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ARTICLE

Distribution Pattern of Tenascin-C in Glioblastoma: Correlation with Angiogenesis and Tumor Cell Proliferation

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Tenascin-C (TN-C) is an extracellular matrix protein which participates in different processes like normal fetal development, wound healing, inflammation, keloids and rheumatoid arthritis. Furthermore, the immunostaining for TN-C is seen in the stroma of various malignant tumors as in glioblastoma multiforme (GBM), however, the significance of these findings is still not clear. In this study 62 GBM samples were analyzed immunohistochemically for distribution patterns of TN-C and correlated with angiogenesis and tumor cell proliferation. Tenascin-C in GBM localizes in two compartments, perivascular and intercellular space. Intercellular tenascin-C (TN-C ic) showed focal distribution in 66%, and diffuse one in 34% of cases. Perivascular tenascin-C (TN-C pv) showed strong correlation with microvascular density (MVD) and vascular endothelial growth factor (VEGF) expression. Moreover, it seems that TN-C pv enhanced the effect of VEGF. Intercellular TN-C did not correlate with MVD and VEGF expression, but showed strong correlation with proliferation index. Furthermore, tumors with diffuse TN-C ic expression had higher proliferation indices than tumors with focal TN-C expression. Our results indicate that TN-C plays a role in angiogenesis and tumor cell proliferation, but beside the intensity of expression, the distribution patterns are also important in these processes. This study also suggests that perivascular and intercellular TN-C compartments have probably different sources and different roles in GBM. (Pathology Oncology Research Vol 11, No 4, 229–235)

Key words: tenascin, glioblastoma multiforme, angiogenesis, proliferation

Introduction

Glioblastoma multiforme (GBM) is the most common and most aggressive primary brain tumor. Histologically, it is an anaplastic, highly cellular tumor, which is distinguished from the anaplastic astrocytoma (grade III astrocytoma) by the presence of necrosis and a prominent vascular proliferation.

GBM is characterized by a high level of expression of ECM protein tenascin-C (TN-C), a glycoprotein that is normally found in various tissues during embryogenesis. In adult tissues it is either absent or only a faint expression is detectable in kidney, skin, mammary gland, periosteum, ligaments, tendons and myotendinous junctions. It reappears, however, in diverse reactive conditions such as wound healing, inflammation, keloids and rheumatoid arthritis.[7,24] Strong immunostaining is often seen in the stroma of various malignant tumors. [4,14,22]

This protein was at first called glioma mesenchymal extracellular matrix antigen, then myotendinous antigen, hexabrachion protein, cytotactin, neuroactin, and tenascin (from the Latin verbs "tenere" – to hold and "nascere" – to be born), a name that describes its presence in tendon, ligaments and developmental tissues.[5] TN-C molecule, a six-armed glycoprotein, is composed of a radially arranged hexamer. Each monomer consists of four structural domains: a hydrophobic N-terminal domain, epidermal growth factor-like repeats, fibronectin type III-like repeats and a C-terminal fibrinogen-like domain.[24] Numerous studies suggest that TN-C is involved in various processes such as cell adhesion, migration and growth, but its role in these processes is still controversial. In some circumstances it can be adhesive, while in others it is antiadhe-

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sive;[8,24] it can stimulate or inhibit migration of the same cell type (glial cells),[9,18,31] and it can induce cell proliferation in cell cultures (fibroblasts, laryngeal carcinoma cells, glioblastoma cells)[11,13,22] or inhibit it (fibroblasts).[6,21]

In most parts of the normal brain TN-C is not detected. Only weak immunopositivity is shown in the white matter of the telencephalic lobes and in the first layer of the gray matter. A strong to moderate expression is present in the temporal lobe, but there is no TN-C staining in the vessels in these areas.[29] On the contrary, a strong TN-C expression is present in most cases of GBM, especially around hyperplastic blood vessels, but the importance of these findings is not yet clear.[1,15] Therefore, the aim of this study was to analyze the distribution pattern of TN-C in GBM, and to correlate these data with proliferative activity (investigated by the MIB-1 antibody against Ki-67), expression of VEGF and microvessel density (MVD, determined by anti-CD105 antibody). Endoglin or CD105 is a homodimeric membrane glycoprotein, a member of transforming growth factor-beta 1 (TGF-β1) receptor complex.[16] Endoglin is preferentially expressed in proliferating endothelial cells and leukemia cells but weakly or not at all in cells of most normal tissues. Thus, it is a specific marker for tumor neovascularization, defined as a new proliferation-associated marker of endothelial cells.[17,26]

In this study we have demonstrated that distribution patterns of TN-C in GBM, defined as perivascular and intercellular, correlate with tumor cell proliferation and angiogenesis. Moreover, we investigated the glomeruloid vascular pattern appearance in GBM with focal and diffuse TN-C immunostaining, and found that TN-C distribution has a significant influence on glomeruloid vascular arrangement.

Materials and methods

Tissue preparation

Surgical specimens from 62 cases of GBM were fixed in 10% formalin and embedded in paraffin. Serial 4-μm sections were prepared from each sample and used for routine H&E staining and immunohistochemistry. Tumors were histopathologically classified according to the WHO Classification of Brain Tumors.

Immunohistochemical analysis

For immunohistochemical staining of TN-C, endoglin, Ki-67 and VEGF, streptavidin-biotin staining method was used (LSAB, DakoCytomation Corporation, Carpinteria, USA). TN-C immunostaining was detected by a mouse mAb (1:70 dilution; DakoCytomation). For endoglin immunostaining, mouse anti-CD105 mAb (clone SN6h) was used (1:10 dilution; DakoCytomation). For assessment of proliferation index we used the mouse mAb MIB-1 recognizing Ki-67 (1:50 dilution; DakoCytomation). For VEGF immunostaining, a mouse anti-VEGF mAb (C-1) was used (1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, USA).

Formalin-fixed and paraffin-embedded tissue sections were deparaffinized in xylene, and rehydrated through 100%, 96% and 70% ethanol. For TN-C and endoglin staining, digestion with proteinase K enzyme (DakoCytomation) was performed for 10 min at room temperature. For immunostaining with MIB-1 and anti-VEGF (C-1), microwave antigen retrieval was performed (4 x 5 min in 10 mM citrate buffer). All immunostained sections were counterstained with hematoxylin.

Counting and assessment of immunostaining

Two pathologists assessed all parameters. Perivascular (TN-C pv) and intercellular (TN-C ic) immunostaining for tenascin were evaluated separately, by using a semiquantitative scale: weak (1), less than 10% of stromal blood vessels or intercellular interstitium with TN-C positivity; moderate (2), 10-50% of stromal blood vessels or intercellular interstitium with TN-C positivity; strong (3), more than 50% blood vessels or intercellular interstitium with TN-C positivity.

For quantification of MVD, the most vascular areas (the so-called hotspots) were located in the tumor at low magnification. Blood vessels visualized by anti-CD105 mAb were counted at 400-fold magnification. Any positive endothelial cell or group of cells in contact with a spot was counted as an individual vessel. The mean of counts from three areas was calculated and used in statistical analysis.

The MIB-1 nuclear staining was quantitated on the Image Analysis System. For each case, 1000 cells were counted and the percentage of stained cells was expressed as the proliferation index.

VEGF immunostaining was also evaluated by using a semiquantitative scale: score 1, less than 25% VEGF-positive tumor cells; score 2, 25-50% VEGF-positive cells; score 3, more than 50% VEGF-positive cells.

Statistical analysis

The correlation between TN-C distribution patterns and the other parameters determined was evaluated by using the Spearman test and multivariate analysis (multiple regression test). Mean differences in MVD counts and Ki-67 were compared with the use of t test. The relationship between TN-C distribution and glomeruloid vascular arrangement was analyzed by using the χ^2 test.

Results

Expression of TN-C and VEGF, and MVD

In concordance with previous studies, we detected high perivascular and intercellular tenascin expression in GBM. Namely, only 9.7% (6/62) and 17.7% (11/62) of the

tumors showed weak TN-C pv and TN-C ic expression, respectively. Strong TN-C pv expression was observed in 56.5% (35/62) of GBM. TN-C distribution, especially in the intercellular compartment, was very heterogeneous. Approximately an equal number of tumors exhibited moderate (28 tumors) and strong (23 tumors) TN-C ic immunostaining, but most of them had focal distribution pattern (41/62, 66%), while the rest had diffuse distribution pattern (21/62, 34%).

Due to prominent neovascularization in GBM, all 62 samples stained positive for VEGF. In only 6 of 62 cases (9%), a minority of tumor cells showed cytoplasmic staining (weak VEGF expression, score 1 or VEGF 1), while most tumors (91%) had moderate (VEGF 2, 24 cases) or strong (VEGF 3, 32 cases) expression.

CD105-positive MVD ranged from 18-99 microvessels. Most tumors (44/62, 71%) had channeled microvascular spaces, without glomeruloid bodies. Glomeruloid vascular proliferation appeared in 18 of 62 tumors (29%).

Comparison between TN-C pv, VEGF expression and MVD

Expression of TN-C pv classified as weak, moderate and strong was correlated with mean CD105-MVD, as shown in *Table 1*. It is notable that tumors with strong TN-C pv expression contained significantly more newly-formed blood vessels (61.89±19.76) than tumors with moderate (40.58±10.48) or weak (34.05±8.65) TN-C pv expression, and the difference was significant ($p<0.05$). A significant correlation was found between TN-C pv expression and CD105-MVD ($R=0.59$, $p<0.001$).

VEGF expression was classified by using a semiquantitative scale (score 1, 2 and 3), and it was correlated with mean CD105-MVD. As it can be seen in *Figure 1*, a strong correlation was found between these two parameters ($R=0.40$, $p=0.001$). In our samples, most tumors with strong TN-C pv expression exhibited a strong VEGF expression (*Figure 2*). This correlation proved significant ($R= 0.37$, $p=0.003$; *Figure 3*). Moreover, we compared CD105-MVD in the group of tumors with strong VEGF- (score 3) but weak to moderate TN-C pv expression, as well as in tumors with strong VEGF- and strong TN-C pv expression. In the former group, the mean CD105-MVD was 39.99±8.40, while in the latter it was 66.54±20.09 (*Figure 4*), the difference was significant ($p=0.001$). These results suggest that TN-C pv and VEGF collaborate in the process of angiogenesis, and it seems that TN-C enhanced the effect of VEGF.

Table 1. **Perivascular expression of tenascin-C (TN-C pv) and CD105-positive microvessel density (CD105-MVD) in patients with glioblastoma multiforme**

No. of patients	*TN-C pv*	*CD105-MVD (mean ± SD)*
6	weak	34.05 ± 8.65
21	moderate	40.58 ± 10.48
35	strong	61.89 ± 19.76

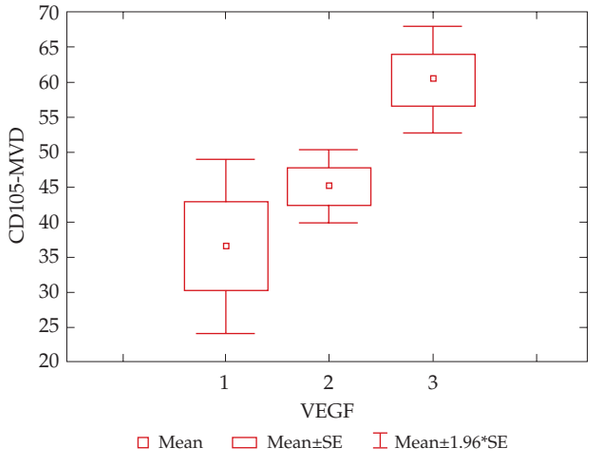

Figure 1. *Correlation between VEGF expression (score 1, 2, 3) and CD105-MVD ($R=0.40$, $p=0.001$)*

Comparison between TN-C ic, VEGF expression, MVD and proliferation index

Intercellular expression of tenascin-C (TN-C ic) did not correlate with either VEGF expression ($R=0.23$, $p=0.11$) or CD105-MVD ($R=0.23$, $p=0.06$). A positive correlation was observed between TN-C ic expression and the proliferation index ($R=0.40$, $p=0.001$). Namely, tumors with weak TN-C ic expression had a lower proliferation index (percentage of Ki-67-positive cells; 15.88±8.22) than those with moderate TN-C ic expression (22.64±9.22), and tumors with strong TN-C ic immunostaining had the highest proliferative activity (29.93±13.26) (*Table 2*). In the next step, distributions of TN-C ic immunostaining were distinguished as focal (heterogeneous) and diffuse (homogeneous). Ki-67 count was 21.15±12.66 in the former and 30.19±10.18 in the latter group. This difference was significant ($p=0.003$), indicating a role of TN-C ic in tumor cell proliferation (*Figure 5*).

By investigating the appearance of glomeruloid vascular pattern in cases of GBM with focal and diffuse TN-C ic immunostaining, we found that tumors with focal TN-C ic expression were more prone to have glomeruloid vascular arrangements (16/41, 39%) than those with diffuse expression (2/21, 9.5%) where the channeled vascular spaces predominated (*Figures. 2,6*). This finding indicates that TN-C distribution is important in the process of modulation of vessels, and that a diffuse distribution is accompanied by a rare

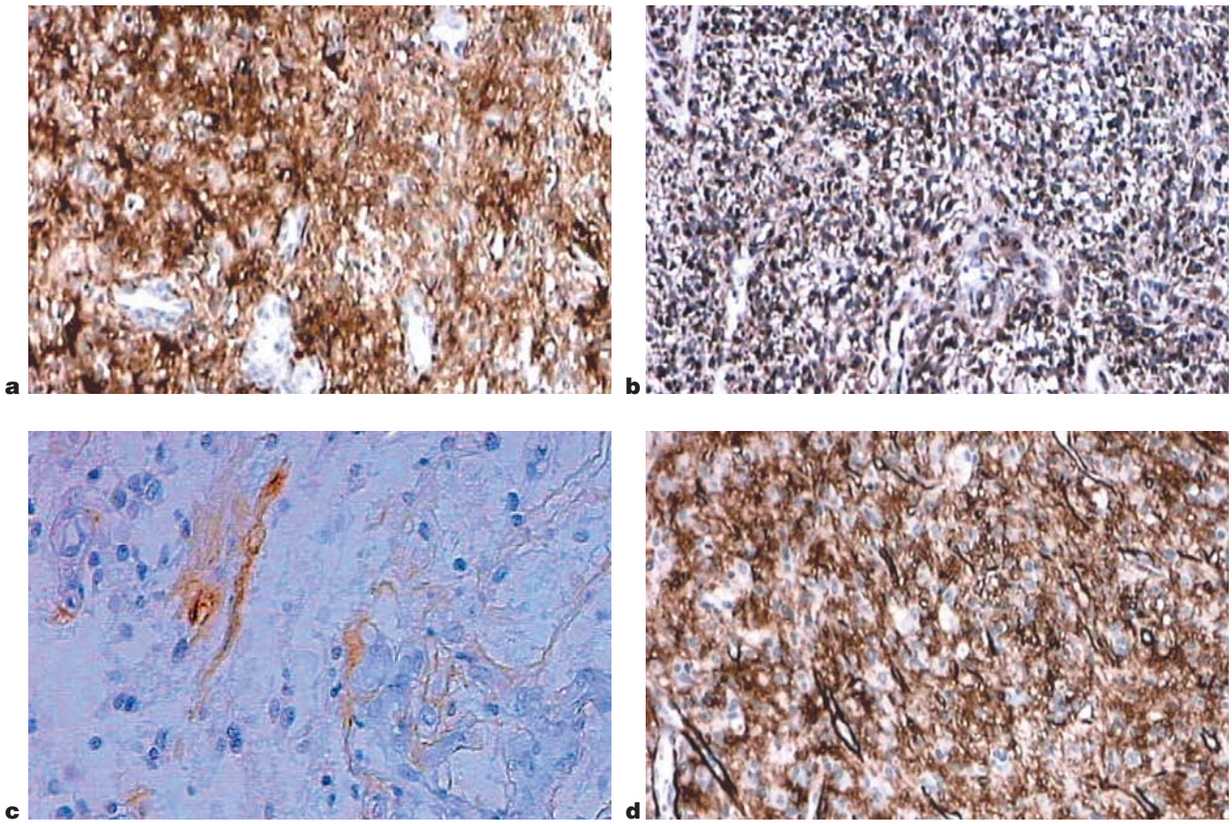

Figure 2. *Immunohistochemical staining for tenascin-C (TN-C) and VEGF in glioblastoma multiforme (GBM) (x100). Serial sections from the same sample of GBM with strong intercellular and perivascular TN-C expression* ***(a)*** *and strong cytoplasmic VEGF expression in tumor cells* ***(b)****. Tumors with focal intercellular TN-C expression (TN-C ic) are more prone to have glomeruloid vascular arrangements* ***(c),*** *while in tumors with diffuse TN-C ic expression channeled vascular spaces predominate* ***(d).***

glomeruloid microvascular proliferation. On the other hand, the intensity of TN-C pv expression did not correlate with the appearance of glomeruloid vessels (there was no significant difference in the occurrence of glomeruloid pattern between tumors with weak, moderate or strong TN-C pv expression).

Discussion

Although the adhesion-modulatory ECM protein TN-C appears in many injuries and diseases, its role in neoplasms is of particular interest. TN-C plays a certain role in modulating cell adhesion, migration and growth. Numerous studies have been conducted on this subject, but the results are controversial and the functional role of TN-C is still unclear.

It is well known that TN-C immunopositivity is present in the ECM in astrocytic brain tumors, and the intensity of TN-C staining correlates with the tumor grade.[11,12,19] Many researchers found a correlation between TN-C expression, angiogenesis and tumor cell proliferation in GBM,[11,12,33] but opposite results have also been reported.[2]

In the present study a high level of perivascular and intercellular TN-C expression was observed in GBM. Furthermore, a very heterogeneous TN-C distribution was detected, especially with regard to different level of staining intensity in the intercellular space. Moreover, in the intercellular compartment two different distribution patterns were observed, most tumors exhibiting focal (inhomogeneous) TN-C ic expression, and a minority expressing diffuse (homogenous) staining.

The process of angiogenesis requires a direct interaction of endothelial cells with their surrounding matrix. The results of this study demonstrated that tumors with strong TN-C pv expression have a significantly higher CD105-MVD (newly formed blood vessels). These results suggest that TN-C is synthesized not only by glioma cells but also by endothelial cells, and thus concentrates around blood vessels.[28,33] Its possible role could be explained by the findings of some authors who showed that TN-C disrupts focal adhesions and the actin-based cytoskeleton, and induces cellular rounding and detachment, which is required for the first step of angiogenesis (disconnecting endothelial

cells from the "mother" vessels).[24] Since TN-C possesses antiadhesive and adhesive activities, it may induce reattachment of endothelial cells to ECM, and migration. These different TN-C effects on endothelial cells are possible through its interaction with different endothelial integrins ($\alpha 2\beta 1$) that has a pro-migratory function and av-containing integrin with anti-migratory tendency).[28] $\alpha v\beta 3$ is selectively expressed on growing neovasculature but not on quiescent blood vessels.[1] Schenk et al. found that TN-C plays an important role in early angiogenesis by modulating the action of bFGF on endothelial cells, and the fibrinogen globe of TN-C induces detachment of endothelial cells, cytoskeletal reorganization and endothelial sprouting.[25]

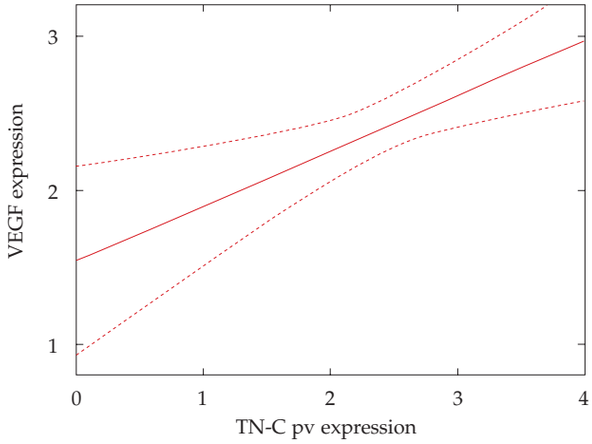

Figure 3. *Correlation between perivascular tenascin-C (TN-C pv; weak – 1, moderate – 2, strong – 3) and VEGF expression (score 1, 2, 3) in glioblastoma multiforme. All results are grouped between the dotted lines, while the full line shows an association between increased expression of TN-C pv and increased expression of VEGF (R=0.37, p=0.003).*

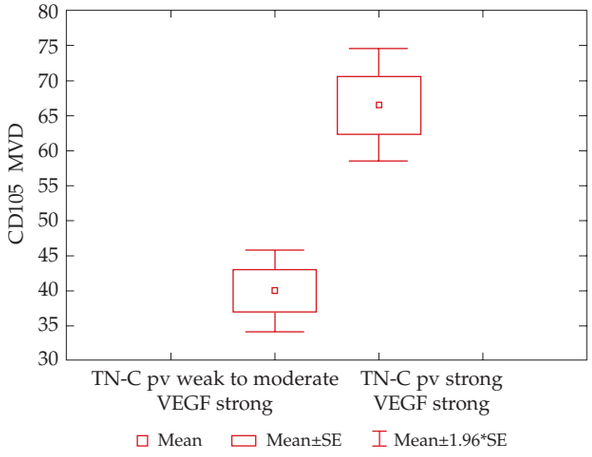

Figure 4. *Perivascular tenascin-C (TN-C pv) expression enhances the effect of VEGF. Tumors with strong VEGF (VEGF 3) and strong TN-C pv expression have more newly formed blood vessels (CD105-MVD) than tumors with strong VEGF but weak to moderate TN-C pv expression.*

Table 2. **Intercellular expression of tenascin-C (TN-C ic) and proliferative activity (Ki-67 labeling) in patients with glioblastoma multiforme**

No. of patients	*TN-C ic*	*Ki-67 (%) (mean ± SD)*
11	weak	15.88 ± 8.22
28	moderate	22.64 ± 9.22
23	strong	29.93 ±13.26

Furthermore, our study has not only shown a significant correlation between TN-C pv expression and the expression of VEGF, one of the strongest angiogenic factors, but it has also demonstrated that VEGF is much more efficient if it is supported by perivascular TN-C: tumors with strong VEGF and strong TN-C pv expression contained more newly formed blood vessels (higher CD105-MVD) than tumors with strong VEGF but weak to moderate perivascular TN-C expression (significantly lower CD105-MVD). These results are supported by other authors who showed that TN-C enhances the proangiogenic effects of various growth factors (VEGF, TGF-β, P1GF).[3] The conclusion was that the upregulation of VEGF and TN-C is spatially and temporally related to neovascularization, as they are both found at the site of neovascularization and detected at the peak of angiogenesis, but not when angiogenesis had ceased.[34] Finally, *Tanaka* et al reported that TN-C regulates tumor angiogenesis through the regulation of VEGF.[27]

It seems that the TN-C ic compartment is not involved in the process of microvascular hyperplasia since it did not correlate with CD105-MVD, but we observed a strong correlation between intercellular TN-C expression and proliferation index. Namely, proliferation index was significantly higher when the intercellular TN-C immunopositivity was stronger. This finding was corroborated by the observation that tumors with a diffuse distribution pattern had a significantly higher proliferation index than those with a focal TN-C ic distribution. Some studies reported that the mitogenic activity of tumor cells is associated with the interaction of TN-C fibronectin type III-like domains and av-containing integrins.[24] Moreover, *Huang* et al. showed that TN-C enhances proliferation of some tumor cell lines, and that it might increase tumor mass by elevating the number of tumor cells.[13] They found that TN-C blocks cell adhesion to fibronectin, and thus prevents syndecan-4 activation (coreceptor in integrin signaling) and inhibits cell adhesion and spreading. This prevents the suppressive effect of fibronectin on tumor cell proliferation.

The hallmark of glioblastoma is microvascular proliferation, in particular glomeruloid vascular proliferation, and

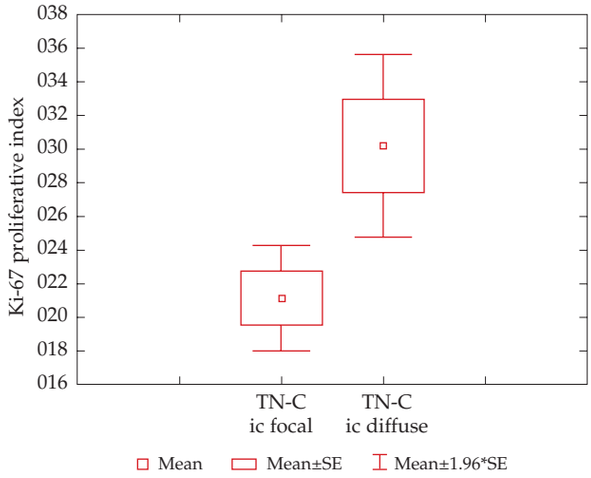

Figure 5. *Connection of proliferative activity (presented as percentage of Ki-67-positive cells) and intercellular expression of tenascin-C (TN-C ic) in glioblastoma multiforme. GBM with diffuse TN-C ic expression have higher proliferation indices than tumors with focal TN-C ic expression.*

necrosis with pseudopalisading. Glomeruloid vascular structures in glioblastoma are complex aggregates of newly formed microchannels lined with hyperplastic endothelial cells that have an altered morphological phenotype. These microchannels are supported by basal lamina and pericytes and are devoid of astrocytic end-feet.[23] Contrary to previously reported data,[33] we have not found any correlation between the intensity of perivascular or intercellular TN-C expression and glomeruloid vascular proliferation. However, our results demonstrated that glomeruloid microvascular proliferation was more often present in glioblastoma exhibiting focal TN-C ic distribution, while in tumors with diffuse TN-C ic distribution channeled blood vessels predominated. This implies that focal expression of TN-C is a possible factor in glomeruloid body forming. Namely, it seems that diffuse TN-C ic distribution is acquired for channeled vascular space modulation. This would be in line with the results of *Castellon* et al. who examined TN-C angiogenic effects in vitro in normal and diabetic retinal endothelial cells, and concluded that TN-C is a better promoter of tube branching and stability than other ECM proteins they tested. TN-C significantly delayed the collapse of capillary-like tubes and decreased tube involution. This effect was mediated by the TN-C receptor αvβ3 integrin, while secondary sprouting seemed to be mediated by α1β1 integrin.[3] Another group of authors studied the immunohistochemical expression of integrins and extracellular matrix proteins in GBM, and found that solid-glomeruloid endothelial proliferations were α2β1, α3β1, αvβ1 negative but channeled-branching and channeled-teleangiectatic vessels were α2β1, α3β1, αvβ1 positive.[30] It is obvious that channeled vascular structures possess integrins that are capable of binding TN-C, which confirms the modulatory role of TN-C in channeled vessel formation. It seems that TN-C has no active role in glomeruloid body formation, as stated by some authors. Studies showing that strong perivascular TN-C immunolocalization could be seen without concomitant endothelial proliferation, glomeruloid formation or vascular tortuosity,[20] support this statement.

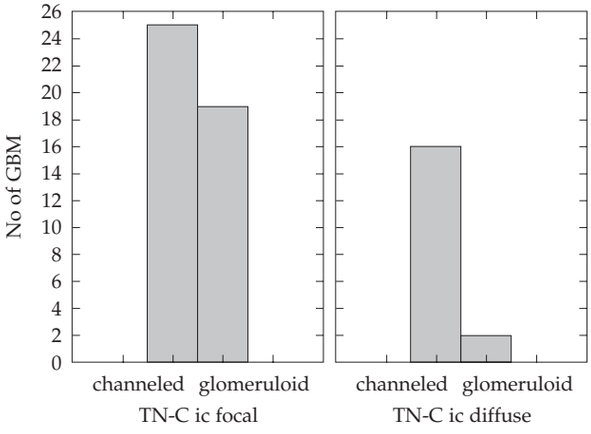

Figure 6. *Number of GBM samples with channeled or glomeruloid vessels in correlation with focal or diffuse intercellular expression of tenascin-C (TN-C ic). Tumors with focal TN-C ic expression have more glomeruloid vascular arrangements than tumors with diffuse TN-C ic distribution.*

In conclusion, our results indicate that TN-C is involved in the process of angiogenesis in GBM, and may have an effect on tumor cell proliferation. We showed that the intensity of TN-C immunostaining is important in these processes, and, furthermore, that the TN-C distribution patterns play a certain role. Since we noticed that TN-C pv expression correlated with angiogenesis but not with the tumor proliferation index, as well as that TN-C ic expression correlated with the proliferation index and not with angiogenesis, our results suggest that these two TN-C compartments have different and distinct activities. It is known that TN-C can be synthesized by both endothelial and glioma cells. It is, therefore, possible that these two compartments have different sources, which implies that endothelial cells and glioma cells are likely to have their own auto-regulatory mechanisms of TN-C regulation. Similarly, it has been reported that tumor cells can control their own destiny by remodeling their ECM through the synthesis of ECM proteins and proteoglycans and by upregulating receptors and proteoglycans on their cell surface.[10]

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