Differentiation between High- and Low-Grade Astrocytoma Using a Human Recombinant Antibody to the Extra Domain-B of Fibronectin

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Different fibronectin (FN) isoforms are generated by the alternative splicing of the primary FN transcript. We previously demonstrated that the isoform containing the extra domain B sequence of fibronectin (B-FN), a complete type-III-homology repeat, is a marker of angiogenesis that accumulates around neovasculature only during angiogenic processes. We produced a single-chain human recombinant antibody (scFv), L19, which reacts specifically with B-FN and selectively targets tumor vasculature *in vivo*. We used this scFv and an antibody against a pan-endothelial marker (Factor VIII) in a double-staining procedure on specimens of low- and high-grade astrocytomas to determine the percentage of B-FN-positive vessels, (denominating the resulting value angiogenic index [AI]). Compared to vascular density and proliferative activity (evaluated using antibodies to Factor VIII and Ki67, respectively), AI correlated better with tumor grade ($1.6 \pm 2.6\%$ and $92.0 \pm 8.7\%$ of B-FN-positive vessels in low- and high-grade astrocytomas, respectively) and was a more precise diagnostic tool than either of the two conventional methods. In fact, discriminating analysis using these three parameters showed that only AI accurately classified 100% of the cases studied, compared to 64% and 89% correctly diagnosed by vascular density and of proliferating cells, respectively. (*Am J Pathol* 2002, 161:1695-1700)

Tumors cannot grow without a pronounced remodeling of the extracellular matrix (ECM) of the surrounding normal tissue in which the tumor grows. Remodeling of the ECM takes place through two main processes: proteolytic degradation of the normal tissue's pre-existing ECM components, and neo-synthesis of new ECM components by both

neoplastic and stromal cells. The interplay between these two processes results in the generation of a neoplastic ECM whose components may differ, qualitatively and quantitatively, from their nonneoplastic counterparts. Moreover, this provisional ECM also provides a permissive environment for tumor progression of which angiogenesis is a pivotal step.^{1,2}

One constituent of this provisional ECM is B-FN, the fibronectin (FN) isoform containing the extra domain B (ED-B), a complete type three repeat made up of 91 amino acids.³ B-FN is, with very rare exceptions (eg, the female reproductive system where tissue remodeling and angiogenesis are recurrent physiological processes) virtually undetectable in normal adult tissues.⁴ It is, by contrast, noticeably up-regulated in fetal and neoplastic tissues.⁴ Because B-FN accumulates around neovasculature during angiogenic processes but not around mature vessels it is an ideal marker of angiogenesis and its visualization by immunohistochemistry can yield information on the level of angiogenic activities taking place within a tissue.⁵

We produced a human recombinant antibody, L19, that reacts specifically with the ED-B domain of FN,⁶ and have recently demonstrated that radiolabeled L-19 is able to selectively and specifically target tumor vasculature not only in experimental animal models but also in cancer patients.⁷⁻¹⁰ Moreover, L19 has recently been shown to dramatically enhance the therapeutic index of cytokines and other bioactive molecules when used to deliver these to tumor neo-vasculature.¹¹⁻¹⁵ The possibility of using L19 to target tumors in patients both for *in vivo* diagnostic and therapeutic purposes requires meticulous preliminary studies on the biodistribution of the antigen, ED-B, in various types of tumors. Here, using a double-staining procedure, we determined the percentage of ED-B-positive vessels (denominating the resulting value as the angiogenic index [AI]) in specimens of low- and high-grade astrocytomas. We then compared these results with the vascular density and the proliferative activity assessed within the same tissues using the Ki67 anti-

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body. High-grade astrocytomas showed a strikingly greater AI than low-grade forms, and this was independent of the vascular density. This observation could underpin relevant *in vivo* diagnostic and therapeutic approaches for astrocytic tumors.

Materials and Methods

Normal and neoplastic tissues were obtained from samples taken during the course of therapeutic surgical procedures. The samples investigated included 29 low-grade (grade I to II) astrocytomas (25 diffuse astrocytomas and 4 pilocytic astrocytomas) and 28 high-grade (grade III to IV) astrocytomas (15 glioblastomas and 13 anaplastic astrocytomas). The grading of the examined tumors followed the World

Health Organization classification.^{16,17} Each sample was immediately frozen in liquid nitrogen. Six- μ m-thick cryostat sections were stained with hematoxylin and eosin, and additional frozen sections were used for immunohistochemical staining after fixation in absolute cold acetone for 10 minutes. Immunohistochemistry studies and double-staining experiments were performed according to Castellani and colleagues.⁵ To avoid false-negative results arising from the heterogeneous distribution of FN within the tissues, at least three nonconsecutive sections of the biopsy were analyzed.

In situ hybridization was performed as previously described.¹⁸ Both sense and anti-sense probes entirely covered the ED-B repeat, but only the anti-sense probe gave a hybridization signal. A probe to human endoglin was used to recognize vascular structures on serial sec-

Figure 1. Serial sections of a glioblastoma (**A** and **B**), normal brain (**C** and **D**), and a pilocytic astrocytoma (**E** and **F**) stained with the scFv L-19 specific for B-FN (**A**, **C**, **E**) and with the anti-factor VIII mAb (**B**, **D**, **F**). Scale bars, 10 μ m.

tions.¹⁹ Discriminating analysis was applied to compare the ability of the three tested parameters (percentage of the ED-B-positive vessels, vascular density, and number of cells in proliferation) to assign patients to the correct group, ie, either high-grade or low-grade astrocytoma.²⁰

The anti-human Von-Willebrand-factor monoclonal antibody (mAb) (DAKO-Factor VIII) and the mAb specific for proliferating cells, Ki-67, were both purchased from DAKO (Carpinteria, CA). The mAb M2 to the FLAG tag

peptide was purchased from Kodak (New Haven, CT). The scFv L-19 to the domain ED-B of FN was purified as previously reported.⁶

Results

Figure 1 shows that the B-FN isoform is detectable in vascular structures of glioblastoma sections, but not in sections of normal brain tissue or grade I pilocytic

Figure 2. Serial sections of anaplastic astrocytoma showing neoplastic and normal tissue (**A** and **B**) stained with the anti-factor VIII mAb and with scFv L-19, respectively. Only the vessels within the tumors were stained by the scFv L-19. Two different magnifications (**C** and **D**) of a glioblastoma multiforme specimen showing neoplastic and normal tissue double stained with the scFv L-19 (brown) and with anti-factor VIII mAb (red). Two different magnifications (**E** and **F**) of an *in situ* hybridization experiment using human glioblastoma cryostat sections with the DIG-labeled cRNA ED-B repeat probe. A positive signal was visible in the endothelial cells. Scale bars, 10 μ m.

Table 1. The Number of Ki67-Positive Cells, the Number of Vessels (Factor VIII-Positive), and the Percentage L-19-Positive Vessels Were Calculated in Five Different Areas of Each Specimen at $\times 250$ Microscopy Magnification

	No. of cases	% of B-FN-positive vessels per HMF*	No. of vessels per HMF*	No. of Ki67-positive cells per HMF*	Survival of patients at 24 months
Low grade	29	1.6 ± 2.6	17.7 ± 11.7	1.0 ± 1.9	90%
High grade	28	92.0 ± 8.7	40.7 ± 28.4	34.2 ± 26.8	12%
Discriminating analysis: % of cases correctly classified		100	64	89	

The average and standard deviation were calculated for each parameter. Discriminating analysis was applied to evaluate the percentage of cases correctly classified for each of the three parameters tested.

*HMF; high magnification field.

astrocytoma. Moreover, double-staining experiments using L19 and a mAb to factor VIII on glioblastoma sections containing both tumor and normal brain demonstrated that the vessels of the normal tissue surrounding the tumor are devoid of B-FN (Figure 2; A to D). Using L-19 in high-grade astrocytoma we observed a positive reaction both in vascular structures showing endothelial cells in proliferation and in structures not showing endothelial cell proliferation. We also performed *in situ* hybridization experiments and found that vascular cells are responsible for the production of this FN isoform (Figure 2, E and F).

On the basis of this observation we quantitatively evaluated the percentage of B-FN-positive vessels in high-grade (28 cases) and low-grade (29 cases) astrocytoma specimens. Our results (Table 1) showed that $92 \pm 8.7\%$ of vessels were positive for B-FN in high-grade astrocytoma sections, whereas only $1.6 \pm 2.6\%$ of vessels were positive in low-grade astrocytoma sections. We also determined the vascular density and the number of prolif-

erating cells per microscope field using the antibody Ki67. Discriminating analysis using these three parameters revealed that only the AI allowed the correct classification of 100% of the cases studied (Table 1). Figure 3 shows the results obtained in each specimen of high- and low-grade astrocytoma. Although considerable overlapping of results for proliferating cells and vascular density was observed, no overlapping was seen when measuring the percentage of B-FN-positive vessels.

A preliminary retrospective analysis considering whether the percentage of B-FN-positive vessels can be used to predict outcome among patients with tumor of the same grade yielded negative results.

Discussion

Expression of the ED-B domain of FN is regulated by alternative splicing, a process that is modulated by cyto-

Figure 3. Left: Number of Ki-67-positive cells calculated in five different areas of each specimen of high- and low-grade astrocytomas using the mAb specific for proliferating cells. Middle: Number of vessels calculated in five different areas of each specimen of high- and low-grade astrocytomas using the mAb anti-factor VIII. Right: Percentage of B-FN-positive vessels calculated in five different areas of each specimen of high- and low-grade astrocytomas double stained using the scFv L-19 and the mAb anti-factor VIII. Although considerable overlapping of the results of high- and low-grade astrocytomas for proliferating cells and vascular density was observed, no overlapping was seen when measuring the percentage of B-FN-positive vessels.

kines and extracellular pH.^{3,21,22} The fact that this domain is 100% homologous in all mammalian species thus far tested offers both advantages and disadvantages. One major drawback initially lay in the impossibility to prepare mAbs that directly reacted with the ED-B domain because of immunotolerance. In earlier studies on ED-B expression in various normal and pathological tissues this problem was circumvented using the mAb BC-1.⁴ BC-1 is able to react with an epitope present on all of the different FN isoforms, but it is cryptic when ED-B is omitted and is unmasked when the domain is present.²³ Thus, BC-1 is able to indirectly detect B-FN expression. BC-1 has been used extensively until now for studies on ED-B expression in normal and pathological tissues.^{24–27} Nevertheless, because of all of the possible interactions of FN molecules in a tissue, we could not assume *a priori* that the epitope recognized by BC-1 could become unmasked even in the absence of the ED-B domain. To overcome these problems we used a phage display library to generate human antibodies able to react directly with the ED-B domain.²⁸ The dissociation constant of one of these antibodies, L19, was increased to 54 pmol through maturation procedures.⁶

L19 has been extensively characterized in immunohistochemical experiments, confirming the earlier observation with the mAb BC-1 that ED-B is a marker of tissue remodeling and, in particular, of angiogenesis.²⁸ Furthermore, using radiolabeled L19 we demonstrated the selective cancer-targeting properties of the scFv in tumor-bearing mice as well as in patients.^{7–10} Here, using such a recombinant antibody, we show that B-FN could be considered a clear-cut marker of differentiation between low-grade and high-grade astrocytoma, as well as between tumor and surrounding normal cerebral tissue. In fact, discriminating statistical analysis showed that the percentage of ED-B-positive vessels was the only parameter among those tested (ED-B-positive vessels, vascular density, and number of cells in proliferation) able to correctly assign 100% of the cases. Furthermore, L-19 clearly discriminates pilocytic astrocytoma (AI = $3.1 \pm 2.0\%$) from grade IV astrocytoma (AI = $94.7 \pm 5.1\%$), which can occasionally be confused because both may present necrosis, high vascular density, and mitoses.

We recently provided evidence that L19 radiolabeled with ^{125}I and injected intravenously in cancer patients selectively accumulates in tumors, including glioblastoma.⁷ This property could offer an appropriate diagnostic tool to differentiate high-grade from low-grade astrocytoma. Moreover, because it is known that a number of low-grade astrocytomas (B-FN-negative) may develop into anaplastic astrocytomas (B-FN-positive), the follow-up of low-grade astrocytoma by immunoscintigraphy using radiolabeled L19 would seem fitting. Such an approach would provide a viable alternative to the more invasive cerebral stereotactic biopsy, which entails a risk of morbidity as well as of mortality mainly because of hemorrhaging.²⁹ Beyond the intrinsic risks associated with stereotactic biopsies, and although these techniques can be used to obtain several biopsy specimens from different areas of a given lesion, it has been argued that small tissue specimens may not be consistently rep-

resentative of the degree of malignancy. Indeed, it has been demonstrated that the grading of glial tumors obtained by stereotactic biopsy may lead to a significant underestimation of the degree of malignancy. Furthermore, a considerable variability in histological grading on small astrocytoma specimens has been reported, not only among different observers but, throughout time, between multiple readings by the same observer.³⁰ This is of particular relevance because the highest level of variability was observed between low-grade and anaplastic astrocytoma, in which the grading has profound implications on the adopted therapy and, consequently, on the patient's outcome.³⁰ Immunohistochemical analysis of specimens could drastically reduce the level of uncertainty, because the AI, measured as the percentage of B-FN-positive vessels, is a much better indicator of malignancy than the commonly used parameters of vascular density and number of Ki67-positive cells. Moreover, the fact that the human antibody L19 specifically targets the vascular structures of high-grade astrocytoma paves the way for a noninvasive differential diagnosis between high- and low-grade astrocytoma by immunoscintigraphy. It also heralds a number of attractive treatment approaches, including the selective delivery of therapeutic substances to the tumor vasculature of high-grade astrocytoma, a disease that is still practically untreatable.

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