Comparative immunohistochemical staining of atherosclerotic plaques using F16, F8 and L19: Three clinical-grade fully human antibodies

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Abstract

OBJECTIVE: F16, F8 and L19 are three fully human monoclonal antibodies, specific to splice isoforms of tenascin-C and fibronectin, which stain sites of active tissue remodeling and which are currently in Phase I and II clinical trials as radio-immunoconjugates and immunocytokines in patients with cancer and arthritis. The characterization of atherosclerosis using these antibodies may open novel pharmacodelivery options for the imaging and treatment of cardiovascular conditions. It may also allow a better assessment of the corresponding immunoconjugates in polymorbid patients with atherosclerotic plaques. METHODS: We performed a comparative immunohistochemical analysis with the F16, F8 and L19 antibodies in 28 freshly frozen human carotid plaques and in 11 normal arteries. Furthermore, we assessed the localization of the antibodies in relation to the infiltrating macrophages, vasa vasorum and Ki67-positive proliferating cells of the plaque. RESULTS: The F16 antibody, specific to the extra-domain A1 of tenascin-C, stained plaques with a selective and intense pattern, while F8 and L19, specific to the EDA and EDB domains of fibronectin, respectively, exhibited a less selective and intense staining. In immunofluorescence, F16 was found to bind regions rich in macrophages, vasa vasorum and proliferating cells, while showing no detectable vs. weak staining of normal arteries and of quiescent plaque structures. CONCLUSION: The human monoclonal antibody F16 stains areas of active tissue remodeling in atherosclerotic plaques and may thus deserve to be investigated as a suitable building block for the development of radiopharmaceuticals for plaque imaging or for the antibody-based targeted delivery of therapeutic agents to atherosclerotic lesions.
Comparative immunohistochemical staining of atherosclerotic plaques using F16, F8 and L19: three clinical-grade fully human antibodies

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KEYWORDS
Atherosclerosis; Plaques; Human monoclonal antibodies; Extra-domain A1 of tenascin-C; Extra-domain B of fibronectin; Extra-domain A of fibronectin; Vascular targeting.

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FIGURES/TABLES

Five figures are present in the manuscript.

There are also 2 supplementary figures and 3 supplementary tables.
ABSTRACT

Objective:
F16, F8 and L19 are three fully human monoclonal antibodies, specific to splice isoforms of tenascin-C and fibronectin, which stain sites of active tissue remodeling and which are currently in Phase I and II clinical trials as radio-immunoconjugates and immunocytokines in patients with cancer and arthritis. The characterization of atherosclerosis using these antibodies may open novel pharmacodelivery options for the imaging and treatment of cardiovascular conditions. It may also allow a better assessment of the corresponding immunoconjugates in polymorbid patients with atherosclerotic plaques.

Methods:
We performed a comparative immunohistochemical analysis with the F16, F8 and L19 antibodies in 28 freshly frozen human carotid plaques and in 11 normal arteries. Furthermore, we assessed the localization of the antibodies in relation to the infiltrating macrophages, vasa vasorum and Ki67-positive proliferating cells of the plaque.

Results:
The F16 antibody, specific to the extra-domain A1 of tenascin-C, stained plaques with a selective and intense pattern, while F8 and L19, specific to the EDA and EDB domains of fibronectin respectively, exhibited a less selective and intense staining. In immunofluorescence, F16 was found to bind regions rich in macrophages, vasa vasorum and proliferating cells, while showing no detectable vs. weak staining of normal arteries and of quiescent plaque structures.

Conclusion:
The human monoclonal antibody F16 stains areas of active tissue remodeling in atherosclerotic plaques and may thus deserve to be investigated as a suitable building block for the development of radiopharmaceuticals for plaque imaging or for the antibody-based targeted delivery of therapeutic agents to atherosclerotic lesions.
1. INTRODUCTION

The use of monoclonal antibodies for the pharmacodelivery of therapeutic agents (e.g., drugs, cytokines, radionuclides, photosensitizers, pro-coagulant agents) at sites of disease is becoming an attractive pharmaceutical modality in the field of cancer and chronic inflammatory disorders, with the aim to develop drugs that act at the site of disease while sparing normal tissues [1-4].

F16, F8 and L19 are three fully human monoclonal antibodies developed by our laboratory, which recognize markers of angiogenesis and of tissue remodeling and which are currently used for the pharmacodelivery of radionuclides or cytokines in patients with cancer and arthritis [5-14]. F16 recognizes the alternatively-spliced A1 domain of tenascin-C, while F8 and L19 recognize the alternatively-spliced EDA and EDB domains of fibronectin, respectively [15-17]. These components of the modified extracellular matrix are known to be virtually undetectable in normal human tissues (exception made for the endometrium in the proliferating phase), but to be over-expressed during fetal development and at sites of active tissue remodeling, with a vascular and/or stromal pattern of staining [18]. Seven derivatives of F16, F8 and L19 [F16-IL2, F16-131I, F16-124I, F8-IL10, L19-IL2, L19-131I, L19-TNF] are currently being investigated in Phase I and Phase II clinical trials in cancer and in rheumatoid arthritis [1,14,19].

Tissue remodeling and angiogenesis (e.g., *vasa vasorum*) are important processes during atherosclerotic plaques formation [20-21]. Indeed, we have previously shown that monoclonal antibodies to splice isoforms of tenascin-C and fibronectin can selectively localize at atherosclerotic plaques *in vivo* following intravenous administration, using ApoE knock-out mice fed with a cholesterol-rich diet as animal model [22-23]. A direct comparative immunohistochemical analysis of the F16, F8 and L19 antibodies in human atherosclerotic plaques has not been reported so far. Such studies are complicated by the fact that these antibodies do not work well in formalin-fixed, paraffin-embedded specimens, but rather require the analysis of freshly frozen tissue [24-25]. A detailed knowledge of the ability of the three antibodies to react with atherosclerotic plaques in patients would be important not only in view of possible imaging and pharmacodelivery applications in the cardiovascular field [26], but also in consideration of the fact that these clinical-stage antibodies may be administered to polymorbid cancer patients and may thus target cytokines or radionuclides to sites of atherosclerosis. In spite of promising results with the L19-based targeted delivery of interleukin-2,
which was reported to reduce atherosclerotic plaques in the ApoE -/- mouse model [27], at this moment in time we do not have a detailed knowledge of whether the antibody-based targeted delivery of pro-inflammatory (e.g., IL-2, TNF) or anti-inflammatory (e.g., IL-10) cytokines to atherosclerotic plaques may have a beneficial or detrimental effect.

In this article, we describe a comparative analysis of the F16, F8 and L19 antibodies in 28 freshly frozen carotid plaques and in 11 normal arteries, using immunohistochemical and immunofluorescence procedures. The best staining results were obtained with the F16 antibody, which was found to strongly react at certain areas of plaques rich in macrophages, vasa vasorum and proliferating cells, while showing no detectable vs. weak staining of normal arteries and of plaque areas not involved in active remodeling.
2. MATERIALS AND METHODS

2.1. Subjects characteristics

The study group comprises 28 carotid atherosclerotic plaques, collected during carotid endarterectomy (CEA) from 20 patients, and 10 fragments of normal external iliac artery from patients treated for abdominal aortic aneurysm. Also a normal pulmonary artery from the lobectomy specimen of a patient who was operated for non-small cell lung cancer was used. Tissue specimens were immediately processed according to the guidelines of the Swiss Society of Pathology for frozen sections. Plaque or normal artery fragments were snap-frozen in OCT (optimal cryo-temperature) medium and stored at -80°C. Data collected included age, gender, clinical history, macroscopic evaluation at the moment of surgery and classification into stable and unstable plaques.

2.2. Antibodies

The F16 antibody, specific to the extra-domain A1 of tenascin-C, the F8 antibody, specific to the extra-domain A of fibronectin, and the L19 antibody, specific to the extra-domain B of fibronectin, have been described before [15-17].

2.3. Immunohistochemistry

For immunohistochemistry, the F16, F8, and L19 antibodies were used in biotinylated small immunoprotein (SIP) format [16-17, 28-29]. Aliquots of antibodies were prepared from a single batch, stored at 4°C, and were used only once, thus contributing to excellent reproducibility of immunohistochemical results [30].

Ten μm thick tissue sections were treated with ice-cold acetone, rehydrated in Tris buffer solution (TBS: 50 mM Tris, 100 mM NaCl, 0.001% Aprotinin, pH 7.4) and blocked with TBS 20% fetal calf serum. Biotinylated SIP(F16), SIP(F8) and SIP(L19) were added onto the sections in a final concentration of 2 μg/ml and detected using a streptavidin-alkaline phosphatase complex (Biospa, Milano, Italy). Fast Red (Tablets Set, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was used
as phosphatase substrate and sections were counterstained with Gill’s hematoxylin no. 2 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). For every immunohistochemical experiment a negative control was performed by omitting the primary antibody (Suppl. Fig. 1).

The optic microscope Zeiss Axiovert S100TV and the Zeiss Axiovision Release 4 acquisition software were used to evaluate the expression of the A1 domain of tenascin-C and the EDA and EDB domains of fibronectin, as revealed by the staining given by the F16, F8 and L19 antibodies, respectively.

The staining intensity was semi-quantitatively scored using four possible levels: no staining, weak, moderate and strong (0, 1, 2, 3). A reference “training” table was created before immunohistochemical results, following the recommendations of a certified pathologist (A.S.) (Suppl. Fig. 2). Using the training table as reference, sections stained with the F16, F8 and L19 antibodies were scored blindly by two independent investigators (M.P. and C.S.). The results were then compiled as average of the two scores, indicating the standard deviation for each analysis. Significance levels for pairwise comparisons between the scores of the three antibodies were calculated using Student’s t-test.

2.4. Immunofluorescence studies

The most representative stable and unstable plaques were analyzed for the amount of macrophage infiltration, *vasa vasorum* and proliferating cells in correlation with the staining pattern of the F16 antibody, against the A1 domain of tenascin-C. The immunofluorescent staining of plaque sections was performed using antibodies against the following antigens: F4/80 (monoclonal rat anti-human F4/80, clone BM8, AbCam, Cambridge, UK) for the detection of macrophages, vWF (polyclonal rabbit anti-human vWF, Dako Cytomation, Glostrup, Denmark) for the detection of *vasa vasorum*, Ki67 (monoclonal mouse anti-human Ki67, clone B126.1, AbCam, Cambridge, UK) for the visualization of proliferating cells. In all cases, a co-staining for the A1 domain of tenascin-C was performed with the biotinylated SIP(F16) antibody (final concentration of 1.6 μg/ml).

Plaque sections of 10 μm thickness were treated with ice-cold acetone, rehydrated in PBS, blocked with PBS + 10% donkey serum + 10% goat serum, incubated with the primary antibodies (in a PBS 12% BSA solution) and detected using fluorescent Alexa 488 coupled to secondary antibodies.
(donkey anti-rat, goat anti-rabbit, goat anti-mouse, BD Biosciences Pharmingen, Allschwil, Switzerland) or Alexa 594 coupled to streptavidin, in a PBS 12% BSA solution.

The microscope Zeiss Axioskop 2 mot plus, with the fluorescence lamp HXP 120 Kubler Codix and the acquisition software Zeiss Axiovision Release 4 were used to detect the plaque-infiltrating macrophages, *vasa vasorum* and proliferating cells, in correlation with the expression of the A1 domain of tenascin-C.
3. RESULTS

3.1. Clinico-pathological data

Twenty patients that were referred to the Clinic for Cardiovascular Surgery of the University Hospital of Zurich for carotid endarterectomy (CEA) were enrolled in the study. Patients with carotid artery restenosis were excluded. Written informed consent was obtained from all patients. The most frequent indication for CEA was symptomatic carotid artery stenosis. Before operation, all patients underwent Duplex ultrasound (to define the degree of stenosis and the morphologic characteristics of plaques), or computer tomography angiography (CTA), as well as a computer tomography or magnetic resonance brain scan. All patients after acute stroke or transient ischemic attack (TIA) (n=18; 90%) were operated within two weeks after the event occurred.

Carotid plaques were collected during carotid endarterectomy (Fig. 1a). All but 3 CEAs were performed in local anesthesia. All surgeries were realized using the carotid bifurcationplasty. After dissection of the common (CCA), external (ECA) and internal carotid artery (ICA), heparin administration and clamping, longitudinal arteriotomy is started in the CCA proximal to the lesion and extended cephalic through the plaque to the relatively normal artery. Another arteriotomy (the “fork” fashion) extends cephalic to the distal ECA and match the ICA in a similar fashion. Using dissector, optimal CEA is performed between the inner and the outer medial layer. The proximal end of the plaque is sharply cut in CCA. CEA is continued distally up to carotid bifurcation and then divided sharply with scissors in the bulb, so that the endarterectomy of ECA and ICA can be performed independently. If there is unusual extension of the plaque distally, the plaque is sharply divided and, using the tucking suture, fixed to the distal intima.

Endarterectomy was performed with minimal manipulation. After removal of the plaques, all specimens were photo documented, so that a macroscopic re-evaluation was possible. According to the traditional classification of the American Heart Association, plaques were characterized as stable if they macroscopically presented a prominence without endothelial disruption (Fig. 1b, left). Plaques were considered unstable if there was at least one of the following: disruption of the surface, intraplaque hemorrhage or thrombosis [31] (Fig. 1b, middle).
The study group consisted of 28 (common, external or internal) carotid artery plaques from 20 patients. All ICA or ECA plaques were unstable (n=20). Eight CCA specimens showed smooth plaque surface and were classified as stable (n=8). The patients’ male/female ratio was 3:1. The average age at the moment of surgery was 70 ± 9.14 (standard deviation). All patients were symptomatic: 30% had a transient ischemic attack (TIA) and 60% had a stroke (Suppl. Table 1).

Ten specimens of normal arteries (control group) were obtained from patients treated for abdominal aortic aneurysm. In four patients a redundant segment of the external iliac artery was resected (Fig. 1b, right). In the other six patients a circular specimen of the external iliac artery wall was excised with 5.0 mm aortic hole punch, creating a uniform circular hole for the vascular anastomosis. Also a normal artery from the lung of a patient who was operated for non-small cell lung cancer was used as negative control (Suppl. Table 2). Age, sex, risk factors and treatments did not differ between the study and the control groups (Suppl. Table 3).

3.2. Immunohistochemistry on human carotid atherosclerotic plaques and on normal arteries

We assessed the expression of the A1 domain of tenascin-C and of the EDA and EDB domains of fibronectin in sections of 28 human carotid atherosclerotic plaques and of 11 fragments of normal arteries.

In order to study the expression of splice isoforms of tenascin-C and fibronectin in atherosclerosis, we used identical concentrations of the F16, F8 and L19 antibodies, which recognize their cognate antigen with similarly high binding affinity (kinetic dissociation constants \(k_{\text{off}}\) towards the respective cognate antigen \(<10^{-2}\) s\(^{-1}\) in real-time interaction analysis experiments performed on a BIAcore 3000 instrument) [15-17], and which had previously been reported to stain antigen-rich tumors with comparable intensity [30].

F16 and F8 were found to stain both stable and unstable plaques with similar patterns (Fig. 2). F16 gave a strong staining in the majority of plaques (75% ± 5). F8 displayed moderate and strong stainings (52% ± 2.5 and 41% ± 2.5, respectively), while L19 gave a general weak staining (79% ± 0) (Fig. 3a). F16 displayed a weak (54% ± 5) or no detectable (46% ± 5) staining of normal arteries and
an excellent contrast between quiescent and actively-remodeled plaque regions (Fig. 3b). F8 and L19 also preferentially stained atherosclerotic lesions compared to normal arteries, yet less intensely and selectively (Fig. 3b). All pairwise comparisons between the scores of the three antibodies resulted highly significant.

3.3. Microscopic analysis of plaque-infiltrating macrophages, vasa vasorum and proliferating cells

In order to assess whether the plaque staining observed with the promising F16 antibody occurred at sites of infiltrating macrophages, vasa vasorum and cells in rapid proliferation as defined by Ki67 staining [32], we analyzed plaque sections by multi-color immunofluorescence. Figure 4 shows representative serial sections of a macroscopically classified stable plaque. Regions of intense F16 staining (Fig. 4a) were found to be associated with a dense network of F16-positive extracellular matrix fibers, which surrounded macrophage infiltrates, Ki67-positive cells and vasa vasorum (Fig. 4b). Similar findings were observed for a plaque that had been classified as unstable (Fig. 5a,b). By contrast, plaque areas that were not stained with the F16 antibody were typically characterized by dense collagenous fibrosis and calcifications.
We have characterized in freshly frozen human carotid atherosclerotic plaques the staining patterns of the human monoclonal antibodies F16, F8 and L19, which are currently being investigated in clinical trials in patients with cancer and arthritis. A first goal of the study was to learn more about the expression of splice-isoforms of tenascin-C and of fibronectin, following preliminary reports from our group that had indicated antigen expression in atherosclerotic plaques of the ApoE -/- mouse model and in certain pathological specimens [22-23]. This information is important in consideration of the fact that certain patients enrolled in clinical studies with F16-IL2, F16-\(^{131}\)I, F16-\(^{124}\)I, F8-IL10, L19-IL2, L19-\(^ {131}\)I and L19-TNF may have undetected plaques, for which it is reasonable to expect a pharmacological effect from the therapeutic agent. Indeed, it has recently been reported that L19-IL2 reduces the number of atherosclerotic plaques in ApoE -/- mice fed with a cholersterol-rich diet [27].

As monoclonal antibodies to splice-isoforms of tenascin-C and fibronectin (in particular the F16 antibody) appear to recognize certain structures in human atherosclerotic plaques, which are associated with macrophage infiltration, \textit{vasa vasorum} and increased cellular proliferation, it would be conceivable to use such antibodies for imaging purposes or for pharmacodelivery applications. In contrast to the situation in oncology, where lesions can be visualized thanks to radio-labeled antibodies [33], the use of immuno-PET procedures for the visualization of atherosclerotic plaques may present challenges in relation both to the size and the body location. While it should be feasible to use PET methodologies to image plaques in the carotid and at branches of the aorta, the visualization of atherosclerosis in coronary arteries is complicated by their small size and by the movement of the heart. In principle, the combination of antibody-based infrared fluorophore delivery to atherosclerotic plaques and of intravascular imaging devices could be considered [34-36].

The molecular differentiation of plaques at risk from stable plaques remains an important, yet elusive challenge of cardiovascular research. These studies are complicated not only by the lack of suitable markers and antibodies, but also by the need of better imaging modalities and the requirement to monitor patients over a long period of time, while plaques may still rupture abruptly.

In this project, we characterized human plaques that were substantially different in terms of size, location and macroscopic appearance at surgery [31]. Even though the degree of F16-positivity...
differed from plaque to plaque (Fig. 2 and 3), no striking staining difference was observed between plaques that had been characterized as stable or unstable at surgery. Certain parameters (e.g., thickness of fibrous cap, macrophage infiltration, vasa vasorum, foam cells, calcification) have previously been associated with the probability of plaque rupture [21]. In this study, we observed plaques with thick fibrous cap or rich in macrophages which were, nonetheless, strongly positive for F16 staining.

The F16, F8 and L19 antibodies have demonstrated their ability to selectively localize in vivo to neo-vascular structures in tumor-bearing animals and in patients with cancer [6, 16-17, 19, 29, 37]. For that reason, these antibodies have been labeled with the beta-emitting radionuclide $^{131}$I or fused to the strong pro-inflammatory cytokines IL2 and TNF, and have entered Phase I and Phase II clinical trials in oncology [6-7, 14, 38]. By contrast, the antibody-mediated pharmacodelivery of the anti-inflammatory cytokine IL10 is about to be investigated in patients with rheumatoid arthritis, as the unconjugated recombinant human IL10 had exhibited encouraging therapeutic results in this setting [39]. What antibody-based pharmacodelivery strategies should be considered in order to prevent unstable plaque formation and rupturing or to mediate plaque remodeling? There is a general believe that pro-inflammatory cytokines could promote the transformation of stable into unstable plaques [21], and the pharmacodelivery of anti-inflammatory cytokines may thus be preferable. However, the antibody-based delivery of IL2 has recently been reported to reduce the number of atherosclerotic plaques in the ApoE -/- mouse model [27]. Interestingly, the use of anti-fibrin antibodies for the delivery of hirudin has been reported to potently inhibit fibrin deposition on experimental clot surfaces in baboon plasma [40], while an enhanced thrombolytic and anti-thrombotic potency has been reported for the targeted delivery of recombinant uPA to thrombi. Thus, antibody-based pharmacodelivery strategies may be more suitable for the prevention of thrombotic complication after plaque rupture, rather than for the promotion of plaque dissolution: a very ambitious goal in cardiovascular research.

At present, we do not anticipate a functional role of monoclonal antibodies specific to splice isoforms of fibronectin and tenasin-C, since mice which were knock-out for the EDA or EDB domain of fibronectin, or lacked completely tenasin-C, were found to develop normally [41-42]. Interestingly, EDA -/- and EDB-/- double-knock-out mice exhibited a vascular phenotype, yet with incomplete
penetrance [43]. Moreover, some authors have commented that a non-redundant role of extra-cellular matrix components (e.g., tenascin-C) may not be observed during development, but may become relevant in disease [44-45].

In this article, only symptomatic patients that were referred to the Clinic for Cardiovascular Surgery of the University Hospital of Zurich for carotid endarterectomy (CEA) were analyzed. Future studies will also need to address in more detail possible differences in F16, F8 and L19 staining between symptomatic and asymptomatic patients. A recent report has indicated high levels of EDA in plaques and serum of asymptomatic patients, thus suggesting that this fibronectin splice isoform may be associated with a stable plaque phenotype [26].
5. CONCLUSIONS

Based on the immunohistochemical findings of this study and the extensive researches so far reported in the oncology field, F16 and similar antibodies should be able to selectively localize at sites of atherosclerosis [22-23]. It should thus become possible to investigate whether the antibody-based delivery of bioactive agents (e.g., drugs with cleavable linkers, pro- or anti-inflammatory cytokines, pro- or anti-coagulant agents) is beneficial or detrimental for patients with atherosclerosis. Progress in this field will require the use of fully murine antibody derivatives in mouse models of cardiovascular disease (in order to minimize immunogenic reaction) and, possibly, better animal models of human atherosclerosis. If certain antibody-based therapeutics were proven to be beneficial in animal models with atherosclerotic plaques, it would then become conceivable to investigate these agents (or better, their fully human counterparts) in patients.
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CONFLICT OF INTEREST

Dario Neri is shareholder of Philogen (www.philogen.com), a company that has licensed the F16, F8 and the L19 antibodies from the ETH Zurich.
REFERENCES


FIGURE LEGENDS

**Fig. 1:** (a) Pictures of a carotid endarterectomy (CEA) performed in the Clinic for Cardiovascular Surgery of the University Hospital of Zurich. (b) Left, up and down: pictures of a stable atherosclerotic plaque. Middle, up and down: pictures of an unstable atherosclerotic plaque. Right, up and down: segment of a normal external iliac artery.

**Fig. 2:** Representative immunohistochemical staining results of a normal artery and of some carotid plaques, using the F16, F8 and L19 antibodies, specific to the extra-domain A1 of tenascin-C, and the EDA and EDB domains of fibronectin, respectively. All sections are at 1x magnification.

**Fig. 3:** Analysis of the immunohistochemical staining results obtained with the F16, L19 and F8 antibodies in atherosclerotic plaques (a) and in normal arteries (b). The standard deviation refers to differences in scores obtained from independent assessments. Standard deviation bars are not visible whenever the assessments of the examiners were completely concordant.

**Fig. 4:** Immunohistochemical and immunofluorescence analysis of a macroscopically classified stable plaque (serial sections). (a) Expression of the A1 domain of tenascin-C, as indicated by the staining of the F16 antibody, in comparison to the negative control of the same plaque, where the primary antibody is omitted. (b) Immunofluorescence detection of plaque-infiltrating macrophages, *vasa vasorum* and proliferating cells in correlation with the A1 domain of tenascin-C.

**Fig. 5:** Immunohistochemical and immunofluorescence analysis of a macroscopically classified unstable plaque (serial sections). (a) Expression of the A1 domain of tenascin-C, as indicated by the staining of the F16 antibody, in comparison to the negative control of the same plaque, where the primary antibody is omitted. (b) Immunofluorescence detection of plaque-infiltrating macrophages, *vasa vasorum* and proliferating cells in correlation with the A1 domain of tenascin-C.
SUPPLEMENTARY FIGURE LEGENDS

**Suppl. Fig. 1:** Example of an immunohistochemical experiment. In addition to plaque sections stained with the F16, F8 and L19 antibodies, a negative control of the same specimen was performed by omitting the primary antibody.

**Suppl. Fig. 2:** Reference “training” immunohistochemical table with four possible levels of staining intensity.