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Enhanced expression of the developmentally regulated extracellular matrix molecule tenascin following adult brain injury

(brain wounds/astrocytes/regeneration/*in situ* hybridization/immunocytochemistry)

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ABSTRACT **Tenascin is an extracellular matrix molecule synthesized and released by young astrocytes during embryonic and early postnatal development of the nervous system, and it is concentrated in boundaries around emerging functional neuronal units. In the adult nervous system, tenascin can be detected only in very low levels. Distinct spatial and temporal distributions of tenascin during developmental events suggest a role in the guidance and/or segregation of neurons and their processes within incipient functional patterns. We show here, using *in situ* hybridization and immunocytochemistry, that stab wounds of the adult mouse cerebellar and cerebral cortices result in an enhanced expression of tenascin in a discrete region around the lesion site that is associated with a subset of glial fibrillary acidic protein-positive astrocytes. Tenascin up-regulation in the lesioned adult brain may be directly involved in failed regeneration or indirectly involved through its interactions with other glycoconjugates that either inhibit or facilitate neurite growth.**

Extracellular matrix (ECM) molecules may have important roles during embryonic development, possibly acting as permissive substrates that help guide cells and their processes to their targets (1). Other types of ECM molecules that have only recently been described in the central nervous system (CNS) [e.g., sulfated proteoglycans (2)] could have inhibitory functions and form barriers to growth.

The tenascin molecule is an oligomeric glycoprotein constituent of the ECM that carries the carbohydrate epitope characteristic of the L2/HNK-1 family of adhesion molecules (3, 4). It is referred to variously (3) as hexabrachion, glioma mesenchymal extracellular matrix protein, J1, or cytotactin. In the developing CNS, tenascin is synthesized and expressed by young astrocytes possibly mediating certain neuron–glia interactions (5). Even though the molecule is widely distributed in many different tissues during development and hyperplasia (e.g., in cartilage, regions of epithelial–mesenchymal interactions, tumors) (3), in the CNS it exhibits site-restricted expression (6, 7). This latter attribute of tenascin distribution prompted the designation of these regions as "boundaries," where dense accumulations of this and other glycoconjugates (i.e., glycoproteins, glycolipids, or glycosaminoglycans) cordon off emerging neuronal arrays (8). The possible biological actions of tenascin have been assayed in culture paradigms using neural (e.g., neural crest, neurons, and astrocytes) and nonneural (e.g., fibroblasts) cells, with evidence for inhibition and promotion of migration and process outgrowth (9–12).

The presence or absence of tenascin in or around lesion sites may have important implications for the sequelae of CNS injury. Here, we examine the effects of cerebellar and cerebral cortical lesions on tenascin expression in the adult mouse. The current study presents *in situ* hybridization and immunocytochemical data showing an enhanced expression of astrocytic tenascin associated with adult cortical stab wounds. Even though the function of the molecule during development and in adult brain wounds is still uncertain, it may act in association with other glycoconjugates to modulate neuritic patterning by either inhibiting or facilitating growth.

METHODS

Lesions. Adult (>4 weeks) ICR or C57BL mice ($n > 50$) were anesthetized with intraperitoneal injections of Avertin and secured in a stereotaxic device. Lesions were made by obliquely inserting a 30-gauge needle into either the lateral hemisphere of the cerebellum along the rostrocaudal axis or the parietal and frontal cerebral cortices rostrocaudally. Following survival times of 1 hr to 3 weeks the animals were given a lethal dose of Avertin and perfused through the left ventricle with a mixture containing 4% paraformaldehyde in phosphate-buffered saline. The brains were removed and stored overnight in perfusate.

Immunocytochemistry. *Developmental series.* The brains of postnatal day 7 (P7) and adult mice were removed and stored overnight in perfusate. Following fixation, the brains were cut with a vibratome into 40-μm sections. Sections were processed for immunoperoxidase, as described, with either a monoclonal (5, 6, 9) or a polyclonal (9, 13) antibody to tenascin or glial fibrillary acidic protein (GFAP; Lipshaw Manufacturing, Detroit).

Adult lesions. Vibratome, coronal sections of lesioned cerebral and cerebellar cortices were processed for indirect immunofluorescence with polyclonal antibodies to GFAP (Lipshaw Manufacturing) or tenascin as described (5, 6, 14). Following incubation in fluorescently labeled secondary antibodies (anti-mouse fluorescein isothiocyanate or anti-rabbit 7-amino-4-methylcoumarin-3-acetic acid), the sections were viewed under a fluorescence microscope. Other antibodies, used as controls, included a monoclonal antibody to the J1 160/180 molecule, another developmentally regulated, glycoconjugate expressed by oligodendrocytes (15); neural cell adhesion molecule (16); polysialylated neural cell adhesion molecule (17); and L1 (18). Some sections were incubated in secondary antibody only.

***In Situ* Hybridization.** Sections from lesioned adult cerebral cortex and cerebellum and from normal P7 cerebellum were

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Abbreviations: ECM, extracellular matrix; CNS, central nervous system; GFAP, glial fibrillary acidic protein; P, postnatal day.

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hybridized with sense and antisense digoxigenin-labeled cRNA probes specific for mouse tenascin mRNA (U.D., unpublished observations). Briefly, sections were hybridized, further processed with an alkaline phosphatase-labeled antibody to digoxigenin (Boehringer Mannheim), and allowed to color react with nitroblue tetrazolium (see ref. 19 for a detailed protocol). Following photodocumentation, some sections were also processed for indirect immunofluorescence with an antibody to GFAP. P1–P7 cerebellum was processed concurrently as positive control as there is abundant expression of the tenascin protein and message at this age (19). Specificity of the hybridization signal was established by using a sense cRNA probe having the same activity and signal intensity as the antisense probe.

RESULTS

Normal Developmental Expression of GFAP and Tenascin. The expression of GFAP and tenascin during normal development in the mouse cerebral cortex has been well characterized (6, 8, 14, 20). Here we describe GFAP and tenascin expression during a limited period of normal development in the mouse cerebellum (also see ref. 19). On P7, GFAP (Fig. 1*A*) and tenascin (Fig. 1*C*) immunoreactive Bergmann glial processes traverse the molecular layer, where they can be seen in the external granule cell layer and abutting the pial surface. Labeling can also be seen in the granule cell layer and white matter in these sections. *In situ* hybridization at this age with the tenascin cRNA probe labels a distinct band of cells near the Purkinje cell layer; based on their position at this developmental age, we presume these cells to be Golgi epithelial cells, the parent cell bodies of Bergmann glial processes (21) (Fig. 1*B*) (also see ref. 19). Less intensely labeled probe-positive cells also can be seen in the internal granule cell layer on P7 (Fig. 1*B*). At this same age, tenascin immunocytochemistry reveals a prominent band of labeling in the molecular layer, as well as along radial processes, presumed to be Bergmann glial fibers, within the molecular layer (Fig. 1*C*).

In the adult, very little GFAP labeling of normal Bergmann glial processes or Golgi epithelial cells is seen; likewise, no obvious staining is present within the granule cell layer (Fig. 1*D*). This normal level of attenuated sensitivity can be appreciated if one compares the level of GFAP immunostaining associated with "reactive" Bergmann glial processes following a lesion in the adult cerebellum, such that affected and unaffected folia are adjacent (see Fig. 1*D*; compare with Fig. 2*A*). Tenascin expression in the normal adult cerebellum, using the immunoperoxidase method, is low (Fig. 1*E*; compare with Fig. 2 *B* and *C*, which also show reduced tenascin staining in the adult, with immunofluorescence, in areas away from a lesion site).

Lesions of the Adult Cerebellum. The lesion cases presented in Figs. 2 and 3 present data from 3-day postlesion survival cases. Stab lesions in the adult cerebellum result in an area of gliosis around the wound (Fig. 2*A*). Antibodies to GFAP reveal reactive astrocytes in all layers of the cerebellum. Golgi epithelial cells and Bergmann processes are seen in the molecular layer; GFAP-positive vellate and/or smooth protoplasmic astrocytes (21) are seen in the granule cell layer (Fig. 2 *A* and *G*). Finally, immunoreactive astrocytes are seen in the cerebellar white matter. Double immunofluorescence of these same sections with the tenascin antibody (Fig. 2*B*) shows that, in close proximity to the lesion site, a subset of GFAP-positive astrocytes is also located within areas of tenascin immunoreactivity. Although some tenascin immunoreactivity is seen in all layers of the lesioned cerebellum, there is a strikingly dense band of immunoreactivity in the molecular layer that seems to correspond exactly with a band of GFAP-positive Golgi epithelial cells and their Bergmann processes (compare Fig. 2 *A* with *B*). Adjacent folia, seemingly uninvolved in the lesion, show no increased GFAP or tenascin immunoreactivity.

Sections processed only with fluorescently labeled secondary antibody do not reveal increased staining near the lesion site (Fig. 2*D*). Additionally, lesioned tissue processed for immunocytochemistry of other neural antigens, such as polysialylated neural cell adhesion molecule, fails to show significant immunoreactivity and the staining pattern around the lesion site resembles the control shown in Fig. 2*D* (i.e., no staining or "halo" around the wound).

In situ hybridization of lesioned adult cerebellum with a tenascin cRNA probe shows faintly labeled cell bodies near the Purkinje cell layer throughout the cerebellum (Fig. 2*E*). In the vicinity of the lesion, there are very densely labeled cell bodies near the Purkinje cell layer and occasionally in the molecular layer and the white matter (Fig. 2 *E* and *F*). It is interesting that in adult cerebellar lesions we can detect tenascin protein in all cerebellar layers (Fig. 2*B*), but *in situ* hybridization indicates that only the Golgi epithelial cell population and occasional cerebellar white matter astrocytes express tenascin message. Away from the lesion site and the densely probe-positive cells that surround the wound, the row of normal Golgi epithelial cells is seen to exhibit light labeling (e.g., see Fig. 2*E*). We presume this to be the baseline level of tenascin expression in normal Golgi epithelial cells. Subsequent immunostaining of these same sections with an antibody to GFAP reveals that virtually all of the

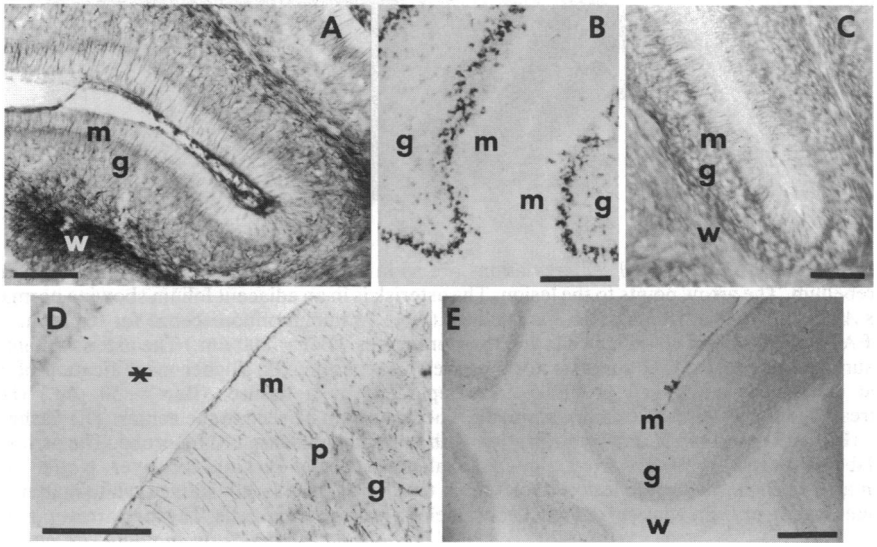

FIG. 1. GFAP and tenascin expression in the developing and adult unlesioned and lesioned mouse cerebellum. m, Molecular layer; g, granule cell layer; p, Purkinje cell layer; w, white matter. (*A*) Immunoperoxidase of GFAP on P7 shows many immunostained astrocytes in all cerebellar layers. (Bar = 100 μm.) (*B*) *In situ* hybridization with tenascin cRNA probe on P7 shows cell bodies of Golgi epithelial cells and granule cell layer astrocytes. (Bar = 100 μm.) (*C*) Tenascin on P7 shows various amounts of the protein in different layers. (Bar = 100 μm.) (*D*) GFAP immunostaining in a lesioned adult cerebellum. The asterisk shows the "normal" level of GFAP in a folium uninvolved in the lesion. (Bar = 200 μm.) (*E*) Immunoperoxidase for tenascin antibody in normal adult mouse cerebellum shows a low level of staining in the cerebellum, particularly the molecular layer. (Bar = 200 μm.)

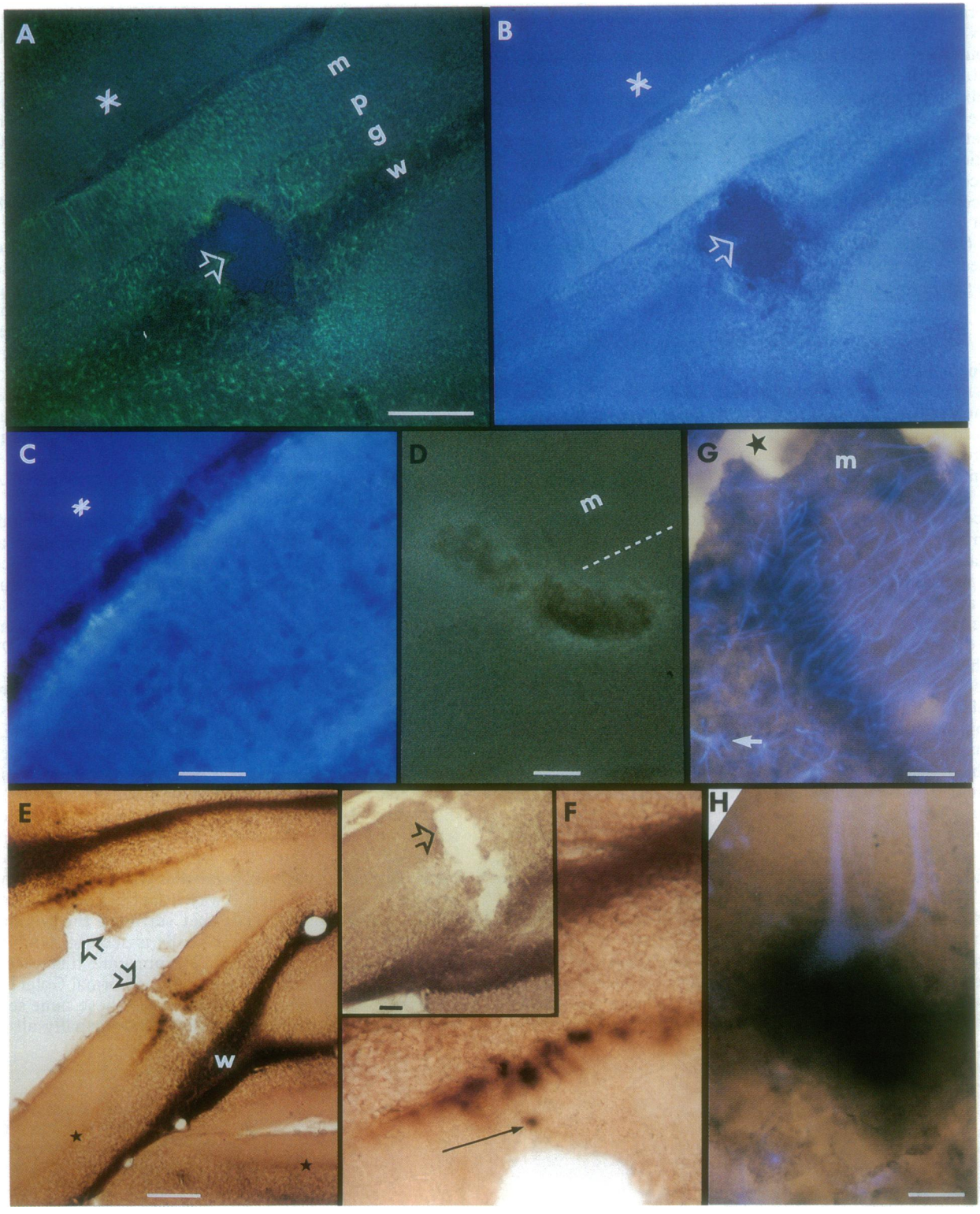

FIG. 2. GFAP and tenascin in lesioned adult cerebellum, 3-day survival. Abbreviations are as in Fig. 1. (*A*) Fluorescein isothiocyanate immunofluorescence for GFAP in lesioned adult cerebellum. The arrow points to the lesion. The asterisk is in an adjacent folium showing normal levels of GFAP. (Bar = 200 μm.) (*B*) Same field as *A*, 7-amino-4-methylcoumarin-3-acetic acid (AMCA) immunofluorescence for tenascin. A dense band corresponds to the area occupied by GFAP-positive Golgi epithelial cells and their processes. (Bar = 200 μm.) The asterisk points out a lesion-uninvolved folium, separated by a fissure and meninges, that contains normal levels of tenascin. (*C*) Higher magnification of *B* comparing tenascin expression in lesion-involved and lesion-uninvolved (asterisk) folia separated by a fissure. (Bar = 50 μm.) (*D*) Immunofluorescence of lesioned adult cerebellum treated in the absence of primary antibody. The lesion can be seen in the center. The dashed line indicates the position of the Purkinje cell layer. (Bar = 50 μm.) (*E*) *In situ* hybridization with tenascin antisense cRNA probe. The arrows point out the lesion site. The star indicates lightly labeled, normal probe-positive Golgi epithelial cells near the Purkinje cell layer. Notice the increased labeling of cells near the lesion site (open arrows show the needle lesion track within two folia). The darkly stained white matter is nonspecific labeling. (Bar = 200 μm.) (*F*) Higher magnification of *E* showing several intensely labeled probe-positive cells. The long arrow points

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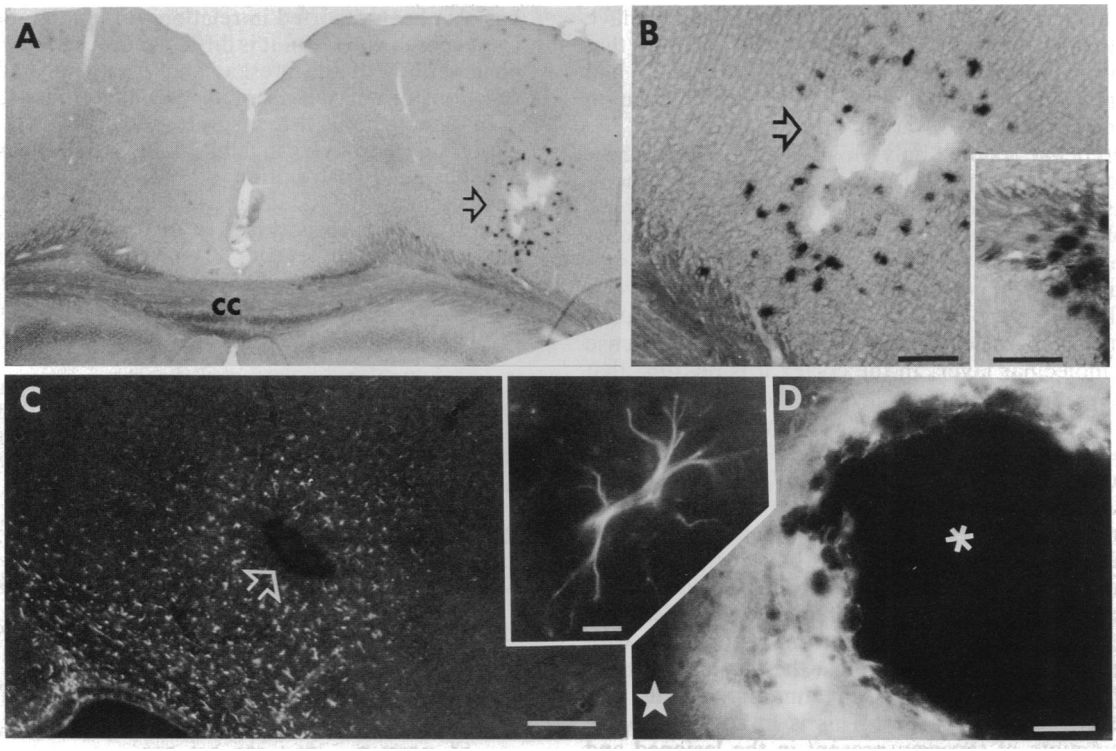

FIG. 3. (*A*) Tenascin mRNA in lesioned adult cortex (3-day survival). The arrow indicates the cortical lesion site. cc, Corpus callosum. (*B*) Higher magnification of *A*. The arrow points to the lesion site. (*Inset*) Lesions involving the white matter result in probe-positive cells. (Bar = 100 μm.) (*C*) GFAP immunofluorescence in lesioned adult cortex. Reactive astrocytes surround the lesion (arrow) with a much wider distribution area than the tenascin probe-positive cells seen, for example, in *A*. (Bar = 200 μm.) (*Inset*) GFAP-positive, reactive cortical astrocyte near the lesion. (Bar = 10 μm.) (*D*) Tenascin immunofluorescence (asterisk indicates the cortical lesion) is present as an extremely small halo just around the lesion, and labeling abruptly drops off to background (star). (Bar = 20 μm.)

densely probe-positive cells near the lesion site also have GFAP-positive processes emanating from them (Fig. 2 *G* and *H*). Control sections, hybridized with sense cRNA, fail to show labeling of any cell bodies (Fig. 2*F*, *Inset*). The dark staining of the cerebellar white matter is a nonspecific artifact of the *in situ* technique.

Lesions of Adult Cerebral Cortex. GFAP immunocytochemistry of stab lesions in the adult cerebral cortex reveals a halo of reactive astrocytes around the lesion site that extends inferiorly into the subcortical white matter (Fig. 3*C*). As seen in cerebellar lesions, some of the GFAP-positive astrocytes around the wound fall within an area of tenascin immunoreactivity (Fig. 3 *C* and *D*). In general, it appears that the amount of tenascin immunostaining in a cerebral cortical stab wound is significantly less than that seen in a comparably sized cerebellar lesion (Fig. 3*D*). *In situ* hybridization of lesioned cerebral cortex reveals the presence of tenascin mRNA-positive cells surrounding the wound (Fig. 3 *A* and *B*). These probe-positive cells have a distribution that appears to correspond to the distribution of the tenascin protein as seen with the anti-tenascin antibody (compare Fig. 3 *B* with *D*). A few very lightly stained probe-positive cells also can be seen in cortical areas some distance from the lesion, presumably representing either normal baseline levels of tenascin mRNA or cells that are indirectly related to the lesion (e.g., cells not in the primary lesion site that may be involved in secondary degenerative events). As in the cerebellum, some probe-positive cell bodies can be seen in the fiber tracts in those cases where the lesion infringed upon the subcortical white matter (Fig. 3*B*, *Inset*). As seen in the cerebellar lesions, cortical sections processed with the sense cRNA probe failed to display any labeled cells.

Although we have not performed an extensive time course analysis, we have examined cases with survival times ranging from 1 hr to 3 weeks, and it appears that the time course of enhanced tenascin expression is similar to that of GFAP immunoreactivity—that is, at 1 hr there is little or no up-regulation of either protein, and, by 3 weeks, the labeling around the wound site has diminished to a barely detectable level (data not shown).

DISCUSSION

Stab wounds of the adult mouse cerebellar and cerebral cortices result in an enhanced expression of tenascin considerably in excess of unlesioned brain. This finding was demonstrated most consistently using *in situ* hybridization with a cRNA probe to tenascin. It is our impression that, immunocytochemically, the protein sometimes exhibited variable levels of expression across cases. It is important to use *in situ* hybridization and immunocytochemistry whenever one makes penetrating lesions of the brain because cellular sources of molecular expressions are difficult to resolve with immunocytochemistry alone; fibroblasts have been reported to produce tenascin (3), and these and other cells, such as vascular-derived macrophages or endothelial

FIG. 2. to a displaced Golgi epithelial cell. (*Inset*) Lesioned cerebellum hybridized with sense cRNA probe. The arrow points out the lesion site. (Bar = 50 μm.) (*G*) Double exposure of a section hybridized with antisense cRNA probe and counterstained using anti-GFAP (AMCA). The star marks the lesion site. Notice the blue GFAP-positive processes emanating from a row of darkly stained, probe-positive cell bodies. The white arrow points to a probe-negative, GFAP-positive astrocyte in the granule cell layer. (Bar = 50 μm.) (*H*) Higher magnification of *G* showing GFAP-positive processes originating from a tenascin mRNA-positive cell body that abuts another probe-positive cell body that apparently lacks GFAP processes. (Bar = 10 μm.)

cells, that may or may not express tenascin could have infiltrated the lesion site. The present study shows that tenascin up-regulation in the lesioned adult cerebellum and cerebral cortex can be attributed to a discrete subpopulation of GFAP-positive astrocytes around the wound site.

In the cerebellar lesions, only Golgi epithelial cells show up-regulation of tenascin mRNA, and yet immunocytochemistry reveals tenascin protein present in all cerebellar layers. One possible explanation for this finding is that the probe-positive Golgi epithelial cells and their Bergmann processes release tenascin protein that then diffuses into deeper layers. This hypothesis is supported by the fact that the immunostaining in the granule cell layer is considerably less intense than in the molecular layer. In the cortex it is possible that particular types of astrocytes may be more disposed than others to express tenascin following injury. Perhaps newly generated astrocytes (e.g., in cortex) or a persistently immature class of astrocyte (e.g., radial-like Golgi epithelial cells) is more likely to express tenascin following injury in the adult. In a recent study, the response of tenascin expression following lesions of the adult mouse optic nerve has been observed to be much less intense than in the adult mouse cortex or cerebellum (22). In optic nerve lesions, tenascin was detectable in increased amounts in association with blood vessels and in the meninges near the crush or transection site, whereas very little tenascin immunoreactivity was seen in the nerve tissue proper. This is in contrast to the extensive amounts of tenascin present in the lesioned and regenerating sciatic nerve, as reported by Martini and coworkers (23). It is, therefore, conceivable that different CNS regions show intrinsically distinct responses of their constituent glia toward lesions. Lesion-associated astrocytes may recapitulate the differentiation sequences of biochemical changes seen during development or they may possibly dedifferentiate or divide in response to lesion-associated events, such as the release of substances from injured cells, growth factors and mitogens [e.g., platelet-derived growth factor, for review see ref. 24; cytokines (e.g., interleukin 1), ref. 25], and other vascular or macrophage-derived molecules.

It is interesting that tenascin reappears or is up-regulated in CNS and peripheral nervous system (PNS) responses to injury; however, there are some intriguing differences between the injured adult CNS and the injured adult PNS and the developing CNS in terms of the nature of tenascin expression. Although our results have not warranted quantification, one can easily see that there is a strikingly small amount of tenascin immunoreactivity in the lesioned adult cerebellum compared to the developing cerebellum (19) and the lesioned adult sciatic nerve (23). At present, we do not know why there are various degrees of tenascin production between different nervous system structures. There is precedence for novel glycoconjugate expression by astrocytes in different areas (26), and it is compelling to consider that such variations in protein expression might lead to dichotomous effects on neurite regeneration following injury in the different areas.

As for the putative biological action of tenascin, culture assays indicate that the molecule apparently mediates neuron–astrocyte interactions, but the precise nature of these interactions is still unclear (4, 9, 11, 13, 27). Tenascin has been reported to be a repulsive substrate for several types of CNS neurons; however, the way in which the molecule is presented to cells seems to affect its action (e.g., in soluble form tenascin appears to attenuate neurite outgrowth) (10–12). It is noteworthy that an ambiguity in tenascin function has also been reported in relation to the migration of neural crest cells *in vitro* (10). It is difficult at present to reconcile the observations of the present study with any of the known functions of tenascin from *in vitro* experiments.

In conclusion, the discrete localization of tenascin-expressing astrocytes in adult brain wounds, compared with a widespread distribution of boundary astrocytes during development, suggests that components of the glial scar may be related to a class of astrocytes involved in global brain pattern formation events. Tenascin and tenascin-binding glycoconjugates may act together to affect neurite patterning during development and to influence, through yet undefined mechanisms, the regrowth of neurites in adult brain wounds.

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