Brain-specific hyaluronate-binding protein: An immunohistological study with monoclonal antibodies of human and bovine central nervous system

(hyaluronectin/extracellular matrix/astrocytes)

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ABSTRACT  Hyaluronectin is a protein isolated from acid extracts of human brain by affinity chromatography on immobilized hyaluronate. With polyclonal antibodies, it was immunohistologically localized in the rat at the nodes of Ranvier of central and peripheral myelinated fibers and in mesenchymal tissues. Compared to adult rat, hyaluronectin-immunoreactive material was more abundant in embryonal rat brain and mesenchyma. We report a different localization in human and bovine tissues with monoclonal antibodies reacting with human hyaluronectin by NaDodSO4/PAGE and immunoblotting but not staining rat tissues by immunohistology. In human and calf the antigen reacting with hyaluronectin monoclonal antibodies was brain specific, while several peripheral tissues were stained by the polyclonal antibodies. In human and bovine central nervous system monoclonal antibodies stained white matter and tissues formed predominantly by glial fibers (e.g., subependymal glia). In white matter hyaluronectin-immunoreactive material formed a delicate mesh surrounding individual myelinated fibers, a pattern compatible with the distribution of fine astroglial processes in this location. Gray matter did not stain with monoclonal antibodies, the granular layer of the cerebellum excepted. The findings suggest that human hyaluronectin is heterogeneous and comprises at least two fractions. The main fraction is a brain-specific protein, probably produced by white matter astrocytes. Another fraction cross-reacting with rat is more abundant in embryonal tissues, including mesenchyma and brain.

Little is known concerning the extracellular matrix of mature brain. In fact, the topic is not even mentioned in two recent reviews of the subject (1, 2). On the basis of immunohistological studies, several extracellular matrix proteins have been proposed as the substrate allowing cell migration and axonal growth in immature brain: fibronectin (3), laminin (4), and chondroitin sulfate proteoglycan (5). However, the findings have not been independently confirmed to date and negative results have been published concerning the presence of laminin and fibronectin in brain development (6, 7). As to chondroitin sulfate proteoglycan, it was found to be exclusively intracellular in adult brain and mainly localized in axons (8).

We now report an immunohistological study with monoclonal antibodies suggesting that a protein fraction isolated from acid extracts of human postmortem (autolyzed) brain may provide a tool to study the composition of the extracellular matrix in mature and developing brain. This fraction, defined on the basis of its affinity for hyaluronate, was isolated previously by Delpech and his collaborators and called hyaluronectin (9).

Previous studies on the immunohistological localization of hyaluronectin were conducted with polyclonal antibodies and on rat tissues. In adult rat, the protein was found at the nodes of Ranvier of central and peripheral myelinated fibers and in some cerebral and cerebellar neurons (10), a finding consistent with the localization of hyaluronate in oligodendroglial cells in primary dissociated cultures of newborn rat brain (11). Moreover, the antibodies also stained nonneural tissues, including the interstitial tissues of rat renal medulla and of human mammary gland (12). Compared to the limited amount of hyaluronectin-immunoreactive material in adult rat tissues, large amounts were found with the polyclonal antibodies in the rat embryo, both in mesenchyma and brain (13). In rat embryo brain, hyaluronectin was found in radial glia and in the primordial plexiform layer of the developing cerebral cortex (14).

MATERIALS AND METHODS

Antigen Preparation. Hyaluronectin was isolated from human brain as reported (9, 15). Samples of cerebral hemispheres were obtained within 4–20 hr after death and frozen at −70°C after removal of leptomeninges. In some cases the white matter was dissected and used as the source of hyaluronectin. In a typical experiment 50 g of tissue was extracted at 0°C in 2 vol of HCl (final pH 2.1) in the presence of 0.36 mM phenylmethylsulfonyl fluoride and 0.02% sodium azide (acid extract in Figs. 1 and 2). Supernatant was made 100 mM with NaCl and brought to pH 5.6, then pH 7.2, and in both cases clarified by centrifugation. Supernatant was adsorbed at 4°C on 30 ml of hyaluronate-Sepharose, prepared according to Tengblad (16) from 7.5 g of aminohexyl-Sepharose, 0.5 g of sodium hyaluronate (Sigma grade III-S), and 0.38 g of coupling reagent and acetylated according to Baues and Gray (17). The adsorbed material was successively washed with 10 vol of sodium phosphate-buffered saline and 25 vol of buffered 0.8 M NaCl, both containing phenylmethylsulfonyl fluoride and azide. The hyaluronectin fraction was eluted with 4 M guanidine-HCl in 50 mM sodium acetate, pH 5.8, dialyzed against phosphate-buffered saline and, when necessary, concentrated by pressure dialysis on a Diafo YM30 membrane. Average yield was 5 mg of protein per 100 g of brain tissue.

NaDodSO4/PAGE was run as reported (18) in 8% gels with 0.1% NaDodSO4. Samples were reduced with dithiothreitol. Immunostaining of electroblots was done according to Towbin et al. (19) as reported (20).

Immunohistology. Immunohistology was carried out on acetone-fixed or alcohol-fixed cryostat sections (10 min at 4°C) of rat, calf, and human tissues stained by indirect immunofluorescence or by the avidin–biotin complex immunoperoxidase method. Rats were asphyxiated with carbon dioxide and the cerebellum was rapidly dissected after

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decapitation. Rat embryos (14–15 days gestational age) were removed from time-pregnant mothers (Charles River Breeding Laboratories) and the head was cut in horizontal sections including the cerebral hemispheres. The sections were kept frozen until used. Calf tissues were obtained still warm after slaughter, placed directly on cryostat chucks with mounting medium, and frozen on dry ice. Samples of the following tissues were examined: brain (cerebral cortex and white matter, caudate nucleus, thalamus, cerebellum, pons), spinal cord (upper cervical segment), eye (including optic nerve), peripheral nerve (third cranial nerve), nasal cartilage, liver, kidney, testis, and aorta. Human tissues were obtained 12 hr after death. The patient, a 59-year-old man, had squamous cell carcinoma of the lung with no brain metastases and died of bronchial hemorrhage. The same tissues were sampled as in calf except that the eye was not removed and tracheal cartilage rather than nasal cartilage.

**Antibodies.** The six monoclonal antibodies used in this study were prepared as reported (21) from three fusions obtained from three mice in two separate immunization experiments. Each mouse was injected with 0.5 mg of hyaluronectin over a period of 5 weeks. Supernatants were screened on cryostat sections of adult rat cerebellum, 14- to 15-day rat embryo cerebral hemispheres, and calf cerebellum. Calf cerebellum was selected because it was stained by the antisera like human cerebellum and with a different pattern compared to rat. Sections stained with mouse and rabbit hyaluronectin antisera raised in rabbits and mice, respectively, served as standards. Antisera were obtained from mice used for the preparation of the monoclonal antibodies. Rabbit antisera were a gift of B. Delpech.

**RESULTS**

On NaDodSO4/PAGE human brain hyaluronectin migrated as a major band at 59 kDa relative to egg albumin and bovine serum albumin (Fig. 1A). Identical results were obtained with a sample provided by B. Delpech. On immunoblotting with monoclonal antibody supernatants, the bulk of the antigenic reactivity corresponded to the major band; a band of slightly higher apparent molecular weight and a variable number of faster migrating bands were weakly reactive (Fig. 1B). With antisera a series of weakly reactive bands of higher apparent molecular weight were also present (not illustrated). While human brain acid extracts prior to affinity chromatography gave patterns similar to the pattern of the hyaluronectin fraction, NaDodSO4 extracts of human white matter did not show on immunoblotting a band corresponding to the main band of the hyaluronectin fraction (Fig. 1B). With both monoclonal and polyclonal hyaluronectin antibodies, a weak band was observed corresponding to the position occupied in the hyaluronectin fraction by the band of slightly higher apparent molecular weight than the major band. Fig. 2 shows that the hyaluronectin fraction was not contaminated with glial fibrillary acidic protein, the subunit of astrocyte-specific intermediate filaments (22).

Table 1 summarizes the immunohistological findings obtained with polyclonal and monoclonal antibodies. Hyaluronectin monoclonal antibodies did not stain rat tissues. With rabbit and mouse antisera the findings were similar to those previously reported (10, 13, 14). In adult rat, the polyclonal antibodies decorated small dots in the white matter (Fig. 3A), thus suggesting that the antigen cross-reacting with rat was localized in oligodendrocytes, in accordance with a study conducted in primary cultures of neonatal rat brain (11). In 14- to 15-day rat embryos, large amounts of immunoreactive material were observed in the primordial plexiform layer of the cerebral cortex (13, 14) (Fig. 3B).

With monoclonal antibodies, the immunoreactivity was strictly confined to the central nervous system in the man and calf. Conversely, several other tissues were stained by the polyclonal antibodies: peripheral nerve (nodes of Ranvier and basal laminae), cartilage (chondrocytes), renal medulla (interstitial tissues), and testis (interstitial tissues and granular material within seminiferous tubules). With both monoclonal and polyclonal antibodies we were not able to assess

<table>
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<th>Antibodies</th>
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<td>White matter (around myelinated axons), periventricular glia, gray matter (diffuse and perineuronal)</td>
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<td>Polyclonal</td>
<td>Oligodendrocytes and Schwann cells (nodes of Ranvier)</td>
<td>Radial glia, cerebral cortex (external plexiform layer), mesenchyma</td>
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<td>Negative</td>
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<tr>
<td>Monoclonal</td>
<td>Negative</td>
<td></td>
<td></td>
<td>White matter (around myelinated axons), periventricular glia, granular layer of cerebellum</td>
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*Similar results in human tissues.
the immunoreactivity of the aorta due to the autofluorescence of elastic fibers.

In human and calf brain and spinal cord, the findings with monoclonal and polyclonal antibodies were similar, although there were additional localizations with the antisera. These were more apparent in gray matter which, as a general rule, did not stain with the monoclonal antibodies. In cerebral and cerebellar white matter, the antibodies decorated a fine mesh surrounding small cavities like a honeycomb. The localization of the immunoreactive material was more easily assessed in transverse sections of long fiber tracts within the brain stem and the spinal cord. Here it became apparent that the antigen surrounded individual myelinated fibers (Fig. 4). Tissues predominantly formed by glial fibers (subependymal glia, glia limitans on the surface of spinal cord and spinal cord septa, pontine raphe) were also stained (Figs. 4 and 5). Calf eye, including retina, was negative, while in optic nerve the staining pattern was similar to that observed in white matter. Ependyma as well as glial fibrillary acidic protein-positive stellate astrocytes and Bergmann radial fibers in the molecular layer of the cerebellum were not stained by either monoclonal or polyclonal antibodies.

Gray matter did not stain with the monoclonal antibodies with one notable exception—i.e., the granular layer of the cerebellum, although the staining was definitely less intense than in white matter (Fig. 6A). In this location the immunoreactive material surrounded groups of granule cell and the basal surface of individual Purkinje cells (Fig. 6B).

Polyclonal antibodies stained gray matter diffusely, so that the sharp contrast between negative gray matter and positive white matter, a typical finding with the monoclonal antibodies (Fig. 7), was not apparent. Moreover, the polyclonal antibodies stained the periphery of large neurons in brain stem and spinal cord, a finding previously reported by Delpech et al. (10) for rat cerebral cortex and cerebellar deep nuclei.

**DISCUSSION**

We will discuss the localization of hyaluronectin in human and calf on the basis of our findings with monoclonal antibodies since the additional localizations observed with the polyclonal antibodies could be due to hyaluronectin heterogeneity. As an example, hyaluronectin immunoreactivity was strictly brain specific with the monoclonal antibodies, while staining of several peripheral tissues, including
peripheral nerve, was observed with the polyclonal antibodies.

In brain and spinal cord, hyaluronectin appeared to be a white matter protein with two important exceptions—i.e., tissues formed predominantly by glial fibers (periventricular glia, pontine raphe, glial septa, and glia limitans in spinal cord) and the granular layer in the cerebellar cortex. In cerebral and cerebellar white matter, the fine mesh of immunoreactive material was difficult to localize, but the findings in the long fiber tracts in brain stem and spinal cord indicated that hyaluronectin surrounded individual myelinated axons. The staining of tissues formed by glial fibers suggested that hyaluronectin immunoreactivity in white matter could be related to the fine astrocytic processes that also surround individual myelinated axons. In fact, there was a striking correspondence between the distribution of hyaluronectin and glial fibrillary acidic protein immunoreactivities in these locations (white matter, periventricular glia, pontine raphe, spinal cord glia limitans, and septa), but there were also important differences, indicating that hyaluronectin is not a cytoplasmic protein like glial fibrillary acidic protein. In addition to the fine fibrillar network, glial fibrillary acidic protein antibodies decorate stellate astrocytes in white matter—i.e., the astrocyte perikaryon and the main processes from which the fine processes surrounding myelinated axons arise (see, for example, figures 6 and 7 in ref. 23). Stellate astrocytes were not seen with hyaluronectin polyclonal and monoclonal antibodies in white matter, nor were they seen in the other regions of the brain where they are prominently stained by anti-glial fibrillary acidic protein—e.g., hippocampus and spinal cord gray matter (23). Furthermore, glial fibrillary acidic protein-positive Bergmann glia were not stained in the molecular layer of the cerebellum by either monoclonal or polyclonal hyaluronectin antibodies. We thus believe that hyaluronectin is predominantly a product of white matter astrocytes and that ultrastructural studies will show its localization on the surface of white matter astrocytes and in the extracellular space. White matter astrocytes share a common precursor with oligodendrocytes (24) and appear relatively late in development—i.e., at the time of myelination (25).

The localization of hyaluronectin in human and calf cerebellum was almost identical to that of hyaluronate in 2-week postnatal rat cerebellum at the light microscopic level (com-

**Fig. 5.** Indirect immunofluorescence staining of calf subependymal glia with hyaluronectin monoclonal antibodies. (A) Glia underlying the floor of the IV ventricle. (B) Glia surrounding the central canal of the spinal cord. Ependyma (E) is not stained. (×280.)

**Fig. 6.** Staining of calf cerebellum with hyaluronectin monoclonal antibodies. (A) The granular layer (G) is weakly stained compared to white matter (W). (Indirect immunofluorescence; ×280.) (B) The granular layer hyaluronectin-immunoreactive material surrounds groups of granule neurons and the basal surface of individual Purkinje cells (P). (Immunoperoxidase staining; ×280.)
paras Fig. 6 with figure 2 in ref. 26). In this study, hyaluronate was localized in tissue sections by using the hyaluronate binding region prepared by clostripain digestion of chondroitin sulfate proteoglycan in conjunction with biotinylation and avidin-peroxidase staining. Hyaluronate was found in the extracellular space at the ultrastructural level (26). A similar localization was also obtained when hyaluronectin/anti-hyaluronectin soluble complexes were used as a hyaluronate probe and localized by indirect immunofluorescence in rat cerebellum (27). In accordance with our suggestion that human hyaluronectin does not cross-react with rat except for minor fraction(s), the distribution of the hyaluronectin/anti-hyaluronectin complex was more extensive compared to the staining observed with hyaluronectin antisera and appeared to correspond to the extracellular space.

In conclusion, we believe that hyaluronectin isolated from human brain by affinity chromatography on hyaluronate is heterogeneous and comprises at least two fractions. The main fraction is a brain-specific white matter protein probably produced by astrocytes and deposited in the extracellular space. A fraction or fractions that are minor components in adult brain are present in larger amounts in the embryo, predominantly in mesenchyma and brain, as evidenced by immunohistological studies conducted with polyvalent anti-

sera. Immature glia express vimentin rather than glial fibrillary acidic protein and may thus be considered mesenchymal cells on the basis of intermediate filament typing (22). It is tempting to speculate that immature glia produce a mesenchymal form of hyaluronectin and that the transition to a brain-specific form may be related to neurogliial differentiation.

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