Light and Electron Microscopic Studies on the Localization of Hyaluronic Acid in Developing Rat Cerebellum

J. A. Ripellino, M. Bailo, R. U. Margolis, and R. K. Margolis*

Department of Pharmacology, New York University Medical Center, New York, New York 10016; and * Department of Pharmacology, State University of New York, Health Science Center at Brooklyn, Brooklyn, New York 11203

Abstract. The hyaluronic acid-binding region was prepared by trypsin digestion of chondroitin sulfate proteoglycan aggregate from the Swarm rat chondrosarcoma, and biotinylated in the presence of hyaluronic acid and link protein. After isolation by gel filtration and HPLC in 4 M guanidine HCl, the biotinylated hyaluronic acid–binding region was used, in conjunction with avidin-peroxidase, as a specific probe for the light and electron microscopic localization of hyaluronic acid in developing and mature rat cerebellum.

At 1 w postnatal, there is strong staining of extracellular hyaluronic acid in the presumptive white matter, in the internal granule cell layer, and as a dense band at the base of the molecular layer, surrounding the parallel fibers. This staining moves progressively towards the pial surface during the second postnatal week, and extracellular staining remains predominant through postnatal week three. In adult brain, there is no significant extracellular staining of hyaluronic acid, which is most apparent in the granule cell cytoplasm, and intra-axonally in parallel fibers and some myelinated axons. The white matter is also unstained in adult brain, and no staining was seen in Purkinje cell bodies or dendrites at any age.

The localization of hyaluronic acid and its developmental changes are very similar to that previously found in immunocytochemical studies of the chondroitin sulfate proteoglycan in nervous tissue (Aquino, D. A., R. U. Margolis, and R. K. Margolis. 1984. J. Cell Biol. 99:1117-1129; Aquino, D. A., R. U. Margolis, and R. K. Margolis. J. Cell Biol. 99:1130-1139), and to recent results from studies using monoclonal antibodies to the hyaluronic acid–binding region and link protein. The presence of brain hyaluronic acid in the form of aggregates with chondroitin sulfate proteoglycans would be consistent with their similar localizations and coordinate developmental changes.

We have previously demonstrated a striking decrease in the concentration of hyaluronic acid during the early postnatal development of rat brain (Margolis et al., 1975), and suggested that during this period, hyaluronic acid may play an important role in determining the size and composition of the extracellular space, and the course of neuronal migration and differentiation.

Previous studies on the localization of hyaluronic acid have usually relied upon conventional histochemical staining techniques using cationic dyes such as Alcian blue in conjunction with enzymatic digestions of tissue sections. These methods, however, generally lack both sensitivity and specificity for differentiating between various cationic molecules, and frequently cannot be used at the electron microscopic level. Hyaluronic acid does not appear to occur in covalent linkage with protein (i.e., as a proteoglycan), and due to the highly regular and conserved nature of this glycosaminoglycan, it has not been possible to generate high affinity antibodies to undegraded hyaluronic acid using conventional techniques. Since these obstacles precluded use of the immunocytochemical approach previously used in this laboratory for localization of the chondroitin sulfate proteoglycan of brain (Aquino et al., 1984a, b), we have developed a sensitive and specific biological probe, based on the biotinylated hyaluronic acid–binding region (HABR) prepared from chondroitin sulfate proteoglycan aggregate isolated from a transplatable rat chondrosarcoma (Ripellino et al., 1985). The present report describes the application of this probe for the localization of hyaluronic acid at the light and electron microscopic levels in developing and mature rat cerebellum, whose cytoarchitecture is well documented (Palay and Chan-Palay, 1974).

Materials and Methods

Isolation of Biotinylated Hyaluronic Acid–Binding Region

Proteoglycan aggregates (containing hyaluronic acid and link protein) were isolated by CsCl density gradient centrifugation after extraction from a transplatable (Swarm) rat chondrosarcoma (Faltz et al., 1979). The proteoglycan aggregate (5 mg/ml) was digested for 8 h at 37°C in 0.1 M Tris acetate buffer, pH 7.3, with diphenyl carbamyl chloride-treated trypsin.

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Figure 1. Light micrograph showing staining of hyaluronic acid (left) and chondroitin sulfate proteoglycan (right) in 15-20 μm Vibratome sections of 7 d-old rat cerebellum. The most intense staining is seen in the presumptive white matter (wm, not included in field at left) and the granule cell layer (gc), as well as a dense band at the base of the molecular layer (ml) immediately above the unstained Purkinje cells (pc). Bar, 50 μm.

(Sigma Chemical Co., St. Louis, MO; Type XI, 4 μg/mg proteoglycan) to yield the HABR as a complex with hyaluronic acid and link protein. The digestion was terminated by addition of a threefold excess of soybean trypsin inhibitor (Sigma Chemical Co.; Type I-S). The hyaluronic acid-binding complex was then biotinylated and separated from hyaluronic acid as described previously (Ripellino et al., 1985).

Lyophilized hyaluronic acid-binding complex was redissolved in 50 mM Tris–HCl buffer (pH 7.0 at 25°C) containing 4 M guanidine HCl, filtered, and fractionated by HPLC on a Spherogel TSK 3,000 SW column (Beckman Instruments, Palo Alto, CA). The column was eluted with the same buffer at 0.5 ml/min, and the effluent was monitored at 280 nm. This procedure gave a good separation of HABR from a second peak containing link protein. (The identity and purity of these two fractions were confirmed by SDS-PAGE). The fractions containing HABR were pooled, dialyzed against distilled water, and lyophilized in aliquots for storage at −80°C.

Staining of Tissue Sections

The biotinylated HABR was used exactly as described previously for the hyaluronic acid-binding complex (Ripellino et al., 1985), at a protein concentration of 50 and 100 μg/ml for light and electron microscopy, respectively. Since we noted that after 5-6 mo of storage at −80°C it was frequently necessary to use a higher concentration of HABR to obtain a given intensity of staining, it is advisable to use relatively fresh preparations of the probe whenever possible.

We also observed considerable variability between different lots of Streptomyces hyaluronidase with respect to the amounts of contaminating protease activity. However, addition of ovomucoid (trypsin inhibitor Type IV-O, Sigma Chemical Co.; 250 μg/ml) to the other protease inhibitors used previously completely inhibited protease activity (assayed using [methy1-3H]-methemoglobin) present at 100 TRU hyaluronidase/ml, even in those preparations with the highest levels of protease contamination. Since ovomucoid had no effect on hyaluronidase activity or on staining in the absence of hyaluronidase, it was routinely included when hyaluronidase treatment was used to assess staining specificity.

Staining of chondroitin sulfate proteoglycan was performed using the peroxidase-antiperoxidase procedure in conjunction with F(ab')2 fragments prepared from an antiserum to the proteoglycan, as described previously (Aquino et al., 1984a).

Results

Comparison of the Hyaluronic Acid-binding Complex with the Hyaluronic Acid-binding Region

Initial studies were carried out using biotinylated hyaluronic acid-binding complex (i.e., the hyaluronic acid-binding region + link protein) prepared as described previously (Ripellino et al., 1985). However, later lots of clostripain used for digestion of the proteoglycan aggregates contained one or more additional protease activities which resulted in a hyaluronic acid-binding region fragment of ~55 kD, rather than the usual 65-kD fragment obtained by digestion with
trypsin or earlier lots of clostripain. (Similar results have recently been reported by Stevens and Hascall, 1986.) At certain ages (and especially in adult brain), the hyaluronic acid–binding complex produced some apparently nonspecific staining, which was not prevented by pretreatment of tissue sections with *Streptomyces* hyaluronidase. This staining may be due to sites on the link protein which are exposed in the somewhat smaller hyaluronic acid–binding complex obtained by use of newer lots of clostripain, since it is not seen when one uses the biotinylated HABR alone, after separation from link protein by HPLC. The HABR has therefore been used in all of our subsequent studies, and gives results identical to those obtained with hyaluronic acid–binding complex prepared with earlier lots of clostripain.

**Comparison of Developmental Changes in the Localization of Hyaluronic Acid and Chondroitin Sulfate Proteoglycan**

Light microscopy of 7-d–old cerebellum showed that staining of both hyaluronic acid and chondroitin sulfate proteoglycan was most intense in the presumptive white matter and in the granule cell layer, as well as in the form of a dense band at the base of the molecular layer (Fig. 1). This dense band of staining moves progressively towards the pial surface, which it has almost reached by 12 d postnatal, at which time there is a pattern of reticular staining in the granule cell layer and a persistence of hyaluronic acid staining in the white matter (Fig. 2). In adult cerebellum, the most intense staining of hyaluronic acid is apparent as a reticular pattern in the granule cell layer, whereas staining of chondroitin sulfate proteoglycan is stronger in the molecular layer (Fig. 3). By this age, staining of both hyaluronic acid and chondroitin sulfate proteoglycan has disappeared from the white matter.

**Electron Microscopic Localization of Hyaluronic Acid**

Electron microscopy demonstrated that the staining in the granule cell and molecular layers at 7 d represents extracellular hyaluronic acid surrounding the granule cells and parallel fibers (Fig. 4). This predominantly extracellular staining is seen through 20 d postnatal, by which time weak cytoplasmic staining of granule cells begins to appear (Figs. 5 and 6). In adult brain, the most intensely stained areas are the rims of granule cell cytoplasm surrounding a large nucleus (Figs. 7 and 8), which gives the reticular appearance seen by light
microscopy, and intra-axonal and membrane staining of certain axons. This latter staining is especially prominent in the parallel fibers (which are the granule axons), although some myelinated axons are also stained (Figs. 9-11).

**Discussion**

In previous immunoelectron microscopic studies on the localization of the chondroitin sulfate proteoglycan in immature (7 d postnatal) rat cerebellum (Aquino et al., 1984a, b), we demonstrated almost exclusively extracellular staining in the granule cell and molecular layers. Staining was also extracellular and/or associated with plasma membranes in the region of the presumptive white matter. At 14 and 28 d postnatal there was a significant decrease in extracellular space and staining, and by 21 d distinct cytoplasmic staining of neurons and astrocytes appeared. This intracellular staining further increased by 33 d so as to closely resemble the pattern seen in adult central nervous tissue, where the proteoglycan was found to be exclusively intracellular in cerebellum, cerebral, brain stem, and spinal cord. Some neurons (such as granule cells) and astrocytes showed strong cytoplasmic staining, and there was heavy axoplasmic staining of many (but not all) myelinated and unmyelinated fibers. Staining was also seen in retinal neurons and glia (ganglion cells, horizontal cells, and Müller cells), but several central nervous tissue elements were consistently unstained, including Purkinje cells, oligodendrocytes, myelin, optic nerve axons, nerve endings, and synaptic vesicles. Analyses of the proteoglycans isolated from 7-d-old and adult brain demonstrated that they have essentially identical biochemical compositions, immunochemical reactivity, size, charge, and density. Our findings therefore indicated that the antibodies used in those studies recognized the same macromolecule in both early postnatal and adult brain, and that the localization of this proteoglycan changes progressively from an extracellular to an intracellular location during brain development.

Our present studies on the localization of hyaluronic acid in developing rat cerebellum, using an entirely different histochemical technique, have yielded results very similar to those previously found for the chondroitin sulfate proteoglycan, insofar as during postnatal brain development there is...
a progressive change from an extracellular to an intracellular localization. In mature brain, both hyaluronic acid and chondroitin sulfate proteoglycan are especially prominent in the granule cell cytoplasm and in the axoplasm of many myelinated and unmyelinated axons, whereas neither were apparent on electron microscopic examination of other types of neurons, such as Purkinje cells. Although we did not observe hyaluronic acid staining in any clearly identifiable glial cells, it is likely that some of the apparently "extracellular" staining seen in certain fields was present in fine astrocytic processes.

As shown in Figs. 1 and 2, hyaluronic acid and chondroitin sulfate proteoglycan followed an essentially identical time course of developmental changes in different cerebellar regions during the first postnatal month, by which time the localization of both was the same as that seen in adult brain. It should be noted that our biotinylated probe (either HABR,
or the hyaluronic acid-binding complex, which includes link protein) is not merely binding to link protein in chondroitin sulfate proteoglycan aggregates, since staining can be completely prevented by treatment of tissue sections with protease-free *Streptomyces* hyaluronidase.

These results add further support to the unusual cytoplasmic localization of hyaluronic acid and chondroitin sulfate proteoglycan in adult brain, since they were obtained using quite different experimental techniques, and indicate a coordinate developmental regulation of both glycosaminoglycans. Moreover, as previously noted with respect to the chondroitin sulfate proteoglycan (Aquino et al., 1984b), the developmental changes which we observed and the ultimate cytoplasmic localization of hyaluronic acid cannot merely be attributed to a lack of specificity of our biotinylated probe, since in such a case one would expect to find both intracellular and extracellular staining at most or all ages examined, whereas there is only a brief developmental period during which this staining pattern was seen.

The most likely explanation for our findings is that hyaluronic acid and the chondroitin sulfate proteoglycan occur together in the form of proteoglycan aggregates, similar to those known to be present in cartilage and other connective tissues (Hascall and Hascall, 1982). We previously reported that only a small degree of aggregation (~10%) could be demonstrated when the chondroitin sulfate proteoglycan of brain was mixed with various proportions of hyaluronic acid under associative conditions, and that the elution profile of the proteoglycan was not noticeably affected by gel filtration in the presence of 4 M guanidine HCl (Kiang et al., 1981). More recent attempts to demonstrate a greater degree of aggregation using electrophoresis on composite agarose/polyacrylamide gels (Heinigard et al., 1985) were also unsuccessful, and aggregates of typical morphology were not apparent on electron microscopic examination (Buchwalter and Rosenberg, 1982) of proteoglycan-cytochrome c monolayer spreads on nitrocellulose films (Buckwalter, J. A., and R. U. Margolis, unpublished results). However, by SDS-PAGE followed by immunoblotting, we were able to detect a 65-kD component of the brain chondroitin sulfate proteoglycan which was recognized by the 12/21/1-C-6 monoclonal antibody (Caterson et al., 1986) to the hyaluronic acid binding-region of cartilage, muscle, aorta, and other proteoglycans, and a major 45-kD component was detected by the 9/30/8-A-4 monoclonal antibody (Caterson et al., 1985), which recognizes two epitopes in the polypeptide portion of rat chondrosarcoma link protein (Margolis, R. K., and R. U. Margolis, unpublished results). In recent studies, we have found that both the pattern and developmental changes in the staining of rat cerebellum by these two monoclonal antibodies closely parallels that seen for hyaluronic acid and chondroitin sulfate proteoglycan.

Our findings therefore suggest that the chondroitin sulfate proteoglycan of brain may form aggregates with hyaluronic acid in situ, even though little aggregation can be demonstrated in vitro. The low degree of aggregation observed in our assays may be due to proteolysis occurring either intracellularly or during the isolation procedure, even when this is carried out in the presence of the usual combination of protease inhibitors, whose addition does not noticeably affect the molecular size or other properties of the proteoglycan (Kiang et al., 1981). The fact that the 65-kD component of the brain proteoglycan, which reacts with antibodies to the hyaluronic acid-binding region, is the same size as the hyaluronic acid-binding region obtained by trypsin treatment of cartilage chondroitin sulfate proteoglycan subunits, lends added weight to the possibility that it represents a proteolytic degradation product of the native proteoglycan. It is also likely that hyaluronectin, a hyaluronic acid-binding protein of similar molecular size which has been purified from an acid extract of brain (Delpech et al., 1987), may be de-

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2. Ripellino, J. A., R. U. Margolis, and R. K. Margolis. Immunocytochemical localization of hyaluronic acid-binding region and link protein epitopes in brain. Abstracts, Annual Meeting of the Society for Complex Carbohydrates, (Bethesda, MD) November 5–7, 1987.
derived from the chondroitin sulfate proteoglycan. Considered together, our results suggest that hyaluronic acid occurs in brain as a component of chondroitin sulfate proteoglycan aggregates, which would account for their very similar locations during early postnatal cerebellar development, and in adult brain.

The considerable cellular specificity in the localization of hyaluronic acid and chondroitin sulfate proteoglycans in brain suggests that their functional role(s) there may be quite different from those proposed for other tissues. For example, in mature brain staining of both hyaluronic acid and chondroitin sulfate proteoglycan is consistently seen in granule cell bodies and in their axons extending into the molecular layer (i.e., the parallel fibers), but was never apparent on electron microscopic examination of Purkinje cell bodies or dendrites. Unfortunately, we currently have no information which would help in identifying what these possibly unique functional roles might be.

Our evidence for the presence of hyaluronic acid and chondroitin sulfate proteoglycans in the cytoplasm raises a number of still unresolved questions concerning their sites of biosynthesis and mechanism of entry into this subcellular compartment. The results of our studies using a polyclonal antiserum to the chondroitin sulfate proteoglycan, a biotinylated nonantibody probe for hyaluronic acid, and two monoclonal antibodies to components of chondroitin sulfate proteoglycan aggregates (the hyaluronic acid–binding region of the core protein and link protein) all strongly support this unusual localization even though the specific methodology used is different in each case. Moreover, since our original report concerning the presence of chondroitin sulfate proteoglycans in the cytoplasm of mature central nervous tissue (Aquino et

3. Although by light microscopy some Purkinje cells showed varying degrees of staining with antibodies to the chondroitin sulfate proteoglycan, many Purkinje cells within the same folium were completely unstained, and stained Purkinje cells were never seen by electron microscopy, nor were these cells stained by the HABR at either the light or electron microscopic levels. It is unclear whether the light microscopic staining of some Purkinje cells by antibodies to the chondroitin sulfate proteoglycan actually indicates biochemical heterogeneity, as has previously been reported for other Purkinje cell antigens (Chan-Palay et al., 1981; Ingram et al., 1985).

Figure 6. Extracellular staining of hyaluronic acid between granule cells in 20-d-old cerebellum. Weak cytoplasmic staining of granule cells is also apparent at this age. Bar, 1 μm.
Figure 7. Staining of hyaluronic acid in granule cell cytoplasm (and one nucleus) of adult brain. Bar, 1 μm.
Figure 8. Cytoplasmic staining of hyaluronic acid in adult granule cell. Bar, 0.5 μm.

Figure 9. Intra-axonal and membrane staining of parallel fibers in the molecular layer of adult cerebellum. Bar, 1 μm.
al., 1984a), several other studies have appeared which bear on the question of glycosylated macromolecules in the cytoplasm. Two laboratories have reported that nuclear pore complex glycoproteins contain cytoplasmically disposed O-glycosidically-linked N-acetylglucosamine residues (Holt et al., 1987; Hanover et al., 1987), and a cytosolic rat kidney α-D-mannosidase which differs in specificity from the lysosomal mannosidase suggests a role of the cytosolic enzyme in glycoprotein catabolism (Tulsiani and Tust, 1987). It is likely that further studies of these different systems will help elucidate the mechanisms involved in the glycosylation of cytoplasmic proteins.

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