Heparin Is a Unique Marker of Progenitors in the Glial Cell Lineage*

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The oligodendrocyte-type-2 astrocyte progenitor cells (precursors of oligodendrocytes and type-2 astrocytes) are an excellent system in which to study differentiation as they can be manipulated in vitro. Maintenance of oligodendrocyte-type-2 astrocyte progenitor cells requires basic fibroblast growth factor, a growth factor whose action normally depends on a heparan sulfate coreceptor. Biochemical analysis revealed a most surprising result: that the oligodendrocyte-type-2 astrocyte progenitors did not synthesize heparan sulfate, the near ubiquitous N-sulfated cell surface polysaccharide, but the chemically related heparin in a form that was almost completely N- and O-sulfated. The heparin was detected in the pericellular fraction of the cells and the culture medium. In contrast the differentiated glial subpopulations (oligodendrocytes and type-2 astrocytes) synthesized typical heparan sulfate but with distinctive fine structural features for each cell type. Thus heparin is a unique differentiation marker in the glial lineage. Previously heparin has been found only in a subset of mature mast cells called the connective tissue mast cells. Its presence within the developing nervous system on a precise population of progenitors may confer specific and essential recognition properties on those cells in relation to binding soluble growth and/or differentiation factors and the extracellular matrix.

In the developing rat optic nerve, bipotential glial progenitor cells (O-2A progenitors) give rise to both oligodendrocytes, which myelinate central nervous system axons, and type-2 astrocytes, which have been proposed to contact the axons between adjacent myelinated regions at nodes of Ranvier (1). Small numbers of O-2A progenitors are found in the nerve as early as embryonic day 15 (2). They continue to proliferate for several weeks (3, 4) and differentiate in the early postnatal period oligodendrocytes appearing before the type-2 astrocytes (5).

The O-2A progenitors represent one of the few cell types in which most aspects of differentiation and proliferation can be manipulated in a controlled in vitro environment. O-2A progenitors grown in chemically defined medium in the absence of mitogen differentiate rapidly into oligodendrocytes without additional signals (6–8). If, however, they are exposed to appropriate inducing factors like fetal calf serum or bone morphogenic protein-2, the cells differentiate into astrocytes (6, 9–11). Differentiation can be inhibited and O-2A progenitor proliferation maintained by basic fibroblast growth factor (bFGF) used either alone or in combination with PDGFA (12).

It is now well established that many growth factors and cytokines act on target cells via a dual receptor system in which one relatively low affinity receptor facilitates transfer to a higher affinity species essential for cell signaling. In the case of bFGF, and other members of the fibroblast growth factor family, the low affinity receptor is heparan sulfate (HS) (13–15) a near ubiquitous sulfated polysaccharide present on the cell surfaces and in the extracellular matrix as a component of proteoglycans. A number of growth factors such as vascular endothelial growth factor, hepatocyte growth factor, and heparin binding growth-associated molecule (16–18) are also dependent on HSs, whereas the effectiveness of others is enhanced by their interaction with HS possibly due to a resultant increase in their pericellular concentration. The long splice variant of PDGFA falls into the latter category (19, 20).

Specific HS species are required to bind to these growth factors (17, 21–24). Permutations in HS structure arise from non-random but variable O-sulfation of saccharides within N-sulfated sections (sulfated domains) of the sugar chain and their immediate flanking sequences. These hypervariable sulfated regions are separated by extended sequences of N-acetylated saccharides of low or zero sulfation (reviewed in Ref. 25). This pattern distinguishes HS from the chemically related heparin, which is essentially highly sulfated along its entire length and in contrast to the ubiquitously distributed HS has so far been found only in a subgroup of mast cells, called the connective tissue mast cells (26).

Studies from a number of groups have shown that the patterns of expression of HSPG core proteins and the structure of HS chains are developmentally regulated and tightly controlled at the cellular level (27, 28). Such changes modify cell responses to extrinsic growth and differentiation factors and may provide guidance cues for axonal pathfinding in the nervous system (29). In the following investigation, the primary O-2A cells were utilized to study changes in HS composition during differentiation of the bipotential progenitor cells into.
the two functionally and morphologically distinct glial cell lineages. A remarkable switch from heparin expression in the O-2A progenitors to distinctive HS species in the oligodendrocytes and astrocytes was observed.

**EXPERIMENTAL PROCEDURES**

**Materials**—PDGF-A and bFGF for cell culture were gifts from C. George-Nascimento and L. Cousens (Chiron Corp., Emeryville, CA), recombinant human insulin growth factor-1-a gift from Genentech, ciliary neurotrophic factor from Frank Collins (Synergen) and recombinant human leukemia inhibitory factor from John Heath. Recombinant bFGF used in the affinity experiments was given by David Fensom (Liverpool, United Kingdom (UK)). Poly-L-lysine (M, 175,000), bovine pancreatic insulin, human transferrin, progesterone, putrescine, t-thyroxine, selenium, soybean trypsin inhibitor, and keratanase (endo-β-galactosidase, EC 3.2.1.103) were obtained from Sigma (Poole, UK). Bovine serum albumin was from Miles Laboratories Inc.

b[3H]Glucomannose hydrochloride (20–45 Ci/mol) was obtained from NEN Life Science Products (Stevenage, UK), Heparinase I (EC 4.2.2.7) and chondroitinase ABC (EC 4.2.2.4) were from Seikagaku Kogyo Co. (Tokyo, Japan). Heparinase II (no EC number assigned) and heparinase III (EC 4.2.2.8) were obtained from Grampian Enzymes (Orkney Island, UK). Bio-Gel P10 and Affi-Gel 10 were purchased from Bio-Rad (Hemel Hempstead, UK), Sephadex CL-6B and CL-4B from Amersham Pharmacia Biotech (St. Albans, UK), and Sephadex PA1 analytical columns from DIONEX (Camberley, Surrey, UK).

Trizol, Superscript II, oligo(dT) primers, and Elongase polymerase were from Life Technologies, Inc. The Advantage PCR pure kit was from CLONTECH (Palo Alto, CA).

**Purification of O-2A Progenitor Cells**—O-2A progenitor cells were isolated from the corpus callosum from 7-day-old rats in order to obtain the number of cells for the biochemical assays. The procedure used was as described previously for the isolation of progenitors from the optic nerve (6, 7). Progenitor cells were purified using a specific antibody capture assay adapted to the O-2A lineage, which reproducibly yields cell populations that are 95–100% pure (31). The O-2A progenitor cells were then either maintained as progenitors in the presence of 10 ng/ml PDGF-A and 10 ng/ml PDGF-B (12). Differentiation to oligodendrocytes was achieved by culture in the presence of PDGF-A alone (32) and to oligodendrocytes and astrocytes (data not shown) was found in the progenitors, the most common HSPG types by RT-PCR. Expression of syndecan-1, 2, 3, and 4 and glypican was found in the progenitors, the majority of GAGs were found in the pericellular fraction. A remarkable switch from heparin expression in the O-2A progenitors to HS species in the oligodendrocytes and astrocytes was observed for the isolation of progenitors from the optic nerve (6, 7). Progenitor cells were purified using a specific antibody capture assay adapted to the O-2A lineage, which reproducibly yields cell populations that are 95–100% pure (31). The O-2A progenitor cells were then either maintained as progenitors in the presence of 10 ng/ml PDGF-A and 10 ng/ml PDGF-B (12). Differentiation to oligodendrocytes was achieved by culture in the presence of PDGF-A alone (32) and to type-2 astrocytes by addition of 10% fetal calf serum (6). Cell populations that were generated by in vitro differentiation were harvested at a time point where the differentiation process is complete to ensure 99–100% pure homogenous populations (7 days for oligodendrocytes and 14 days for astrocytes). This purified fraction was dispersed on glass cover slips in 24-well plates pretreated with fibronectin (0.1 mg/ml) and covered with 0.1% agarose. Each well was covered with a cover slip and incubated for 16 h at 37 °C. Similarly, CS/DS was identified by their low pH nitrous acid (33).

**Radiolabeling and Preparation of Glycosaminoglycans**—HS and heparin were detected by their sensitivity to low pH nitrous acid (33). Heparin binding by chondroitinase ABC (EC 4.2.2.4) and keratanase (EC 3.2.1.103) was obtained from Sigma (Poole, UK). Bovine serum albumin was from Miles Laboratories Inc.

**Affinity Chromatography**—To prepare a bFGF affinity gel column, 200 µg of recombinant bFGF was mixed with 200 µg of heparin in 100 µl of coupling buffer (0.1 M HEPES, 80 mM NaCl, pH 7.0) and incubated for 20 min at room temperature. The bFGF was then bound to Affi-Gel 10, and the column prepared as described for a hepatocyte growth factor (HGF) affinity column linked to an Anachem HPLC system, equilibrated with double-distilled acidified water, pH 3.0. After sample injection in 2 ml of acidified water, the column was washed with 2 ml of acidified water, followed by elution in a two-step gradient of 0–1 M NaCl, pH 3.0, over 45 ml, then 1–2 M NaCl, pH 3.0, over 3 ml. Disaccharides were identified by comparison with elution positions of eight known standards.

**RESULTS**

**Proteoglycan Expression in O-2A Progenitors and Differentiated Cells**—RNA isolated from O-2A progenitors and from cultures allowed to differentiate into oligodendrocytes and astrocytes was analyzed for expression of the most common HSPG types by RT-PCR. Expression of syn- dican 1, 2, 3, and 4 and glypican was found in the progenitors, oligodendrocytes, and astrocytes. There was no indication of serglycin expression by RT-PCR using two different sets of mouse primers, as unlike the other proteoglycans mentioned there was no rat serglycin DNA sequence information available. RT-PCR analysis also showed that all of the three cell types expressed the bFGF receptor, FGFR2 type IIIC, with FGFR1 type IIIC additionally expressed by the oligodendrocytes (data not shown).

**GAG Composition of the O-2A Progenitors, Oligodendrocytes, and Astrocytes**—Metabolically radiolabeled GAG saccharide chains were isolated and analyzed from primary cultures of O-2A progenitor cells and the differentiated cell types. In all cultures the majority of GAGs were found in the culture me-
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**Fig. 1.** GAG composition of glial progenitors and differentiated cells. 3H-Radiolabeled GAG chains were extracted from the medium (M), pericellular (P), and intracellular (I) fractions of primary O-2A progenitors, and from the oligodendrocytes and astrocytes into which a proportion of them had been differentiated in culture. HA (open bar) was separated from the other GAGs by anion exchange chromatography and its presence confirmed by testicular hyaluronidase digestion. CS/DS (solid bar) were removed from the remaining sulfated GAGs and their levels determined by breakdown with specific enzymes.

A tiny amount of keratan sulfate found in the pericellular fraction of one preparation of progenitors was similarly removed by specific enzyme breakdown. The remaining material was confirmed to be HS/heparin (striped bar) by nitrous acid treatment. The histogram depicts mean values from three preparations of cells. The standard errors for each data category are as follows: O-2A progenitors, HA, M 0 003, P 0 008, I 0; HS, M 0 069, P 0 011, I 0; CS, M 0 028, P 0 023, I 0. Oligodendrocytes, HA, M 0 005, P 0 005, I 0 005; HS, M 0 026, P 0 010, I 0; CS, M 0 16, P 0 020, I 0. Astrocytes, HA, M 0 012, P 0 004, I 0 009; HS, M 0 035, P 0 002, I 0; CS, M 0 005, P 0 003, I 0.

**Fig. 2.** CL6B-Sepharose gel filtration of HS/heparin chains. The size distribution of the 3H-radiolabeled HS/heparin purified from O-2A progenitor (solid line), oligodendrocyte (dotted line), and astrocyte (dashed line) extracts were compared by CL6B gel filtration chromatography. The position of the column void volume (VO) and total volume (VT) is indicated. The CL6B-Sepharose gel filtration analysis has been carried out several times on the three preparations of the different cell types in Fig. 1.

Disaccharide Composition—Disaccharides were prepared from the three cell types by combined heparinase digestion and separation on Bio-Gel P10, followed by strong anion-exchange HPLC. Analysis of the disaccharide composition (Fig. 4A) confirms the distinctive heparin nature of the progenitor HS, which contained 73% trisulfated disaccharides (UA(2S)-GlcNSO3(6S)) and no N-acetylated disaccharides (Table I). The contrast to the disaccharide elution profiles of the differentiated cell types is immediately apparent (Fig. 4, B and C), where a range of all of the eight disaccharide types commonly seen in HS were present (Table I). In both cases there was over 30% non-sulfated disaccharides and less than 15% were trisulfated. The disaccharide composition of the astrocytes was very similar to that found in human skin fibroblast HS (36). The oligodendrocytes HS disaccharide composition was distinct from the astrocytes, with a significantly higher level of the trisulfated disaccharide.

Affinity of the HS for bFGF—As bFGF is known to be a key
growth factor for regulating the proliferation and differentiation of the O-2A cell type (12), the relative affinities of the different glial HS types for a bFGF Affi-Gel column were compared. In all cases the majority of HS eluted between 0.15 and 1.6 M NaCl, with a small amount of HS binding at high affinity up to at least 1.6 M NaCl (Fig. 5). A control column did not exhibit any binding of HS above 0.15 M NaCl (data not shown). The progenitor heparin showed stronger binding to the bFGF Affi-Gel than the oligodendrocyte HS with a greater proportion of the material eluting at 0.8 M NaCl and above, while the astrocyte HS elution profile was more analogous to the progenitors. The bFGF affinity of the astrocyte HS was not significantly different from mouse fibroblast HS (Fig. 5B) consistent with their similarity in composition (Fig. 3; Ref. 34). CS/DS from the progenitors and oligodendrocytes showed much weaker binding to the bFGF Affi-Gel, the majority of material eluting between 0.15 and 0.4 M NaCl with no binding seen above 0.6 M NaCl (data not shown).

**DISCUSSION**

The results in this paper clearly indicate that O-2A progenitor cells produce heparin (Figs. 3 and 4). Heparin has only previously been found in connective tissue mast cells. This finding was both interesting and unexpected, and on differentiation to either type-2 astrocytes or oligodendrocytes there was a switch to HS expression. Furthermore neuroepithelial cells, which are thought to be developmental precursors to the O-2A progenitor cells (38–40), produce HS rather than heparin (28).

During heparin/HS biosynthesis (for reviews, see Refs. 25, 41, and 42), the N-deacetylase/N-sulfotransferase (NDST) enzymes commit the polymeric precursor heparan, composed of repeat sequences of GlcAβ1–4GlcNAc, to heparin or HS by catalyzing the conversion of an appropriate proportion of GlcNAc residues to GlcNSO3. The simplest explanation of the present results would be that the O-2A progenitors express the NDST isoform isolated from the heparin-synthesizing mouse mastocytoma (NDST2), which catalyzes conversion of over 80% of the GlcNAc residues to GlcNSO3, and the differentiated cells express the rat liver isoform (NDST1) assumed to be important.

**FIG. 3.** Depolymerization of HS/heparin by nitrous acid treatment or heparinase III enzyme digestion. 

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**FIG. 4.** Analysis of the disaccharide composition of O-2A progenitor, oligodendrocyte, and astrocyte HS/heparin. Disaccharides that were prepared by exhaustive heparinase enzyme digestion of the [3H]HS/heparin from the O-2A progenitors (A), oligodendrocytes (B), and astrocytes (C) were separated on a Pro Pac PA1 strong anion exchange HPLC analytical column (4 × 250 mm) eluted with a NaCl gradient as described under “Experimental Procedures.” The elution positions of the sample disaccharide peaks, identified by scintillation counting of the eluting fractions, were compared with the elution positions of eight known disaccharide standards as follows: 1, hexA-GlcNAc; 2, hexA-GlcNSO3; 3, hexA-GlcNSO3(6S); 4, hexA(2S)-GlcNAc; 5, hexA-GlcNSO3(6S); 6, hexA(2S)-GlcNSO3; 7, hexA(2S)-GlcNAc; 8, hexA(2S)-GlcNSO3(6S), and the sample peaks have been numbered accordingly. The peak depicted by the asterisk is also considered to be standard 1, hexA-GlcNAc, the elution position of which has been found to be extremely sensitive to minute changes in the column environment.

**TABLE I**

**Disaccharide composition of HS from the progenitors and differentiated cells**

Disaccharides were prepared by exhaustive heparinase enzymes digestion of the [3H]HS/heparin from the O-2A progenitors, oligodendrocytes (oligos), and astrocytes, resolved by strong anion exchange HPLC as shown in Fig. 4 and quantified.

| Disaccharide standard | Progenitors (% | Oligos (% | Astrocytes (%) |
|-----------------------|--------------|----------|---------------|
| 1 HexA-GlcNAc         | 35.6         |         | 43.0          |
| 2 HexA-GlcNSO3        | 20.0         |         | 25.1          |
| 3 HexA-GlcNSO3(6S)    | 4.0          |         | 3.6           |
| 4 HexA(2S)-GlcNAc     | 3.2          |         | 3.3           |
| 5 HexA-GlcNSO3(6S)    | 3.5          |         |               |
| 6 HexA(2S)-GlcNSO3    | 17.8         |         |               |
| 7 HexA(2S)-GlcNAc(6S) | 0.5          |         |               |
| 8 HexA(2S)-GlcNSO3(6S)| 3.5          |         |               |
NaCl, increasing in 0.2 M steps from 0.4 M subsequent to a 0.15 M wash. The cell line resulted in highly selective inhibition of the mastocytoma enzyme (NDST2) in a human kidney cell line that the enzymes work in concert to achieve the level of HS biosynthesis (43). However, these enzymes were found only in the medium and pericellular (i.e. trypsin-releasable) fractions (Fig. 1). Furthermore, RT-PCR studies did not detect any expression of the serglycin proteoglycan (data not shown). RT-PCR studies indicated that all four syndecans and glypicans HSPGs are expressed in the O-2A progenitors, oligodendrocytes, and astrocytes but it cannot be certain that these O-2A proteoglycans bear the heparin chains.

A novel HSPG that appears to be important in central nervous system development has been found in the primitive neuroepithelial cells (47), which are precursors to the O-2A cells. Analysis of the HS chains from embryonic day 10 (E10) and day 12 (E12) neuroepithelial precursor cells showed that changes in structure in the form of an increase in chain length and 6-O-sulfation in the more committed E12 cells (28) corresponded to a switch in growth factor response of the cells from bFGF in E10 to aFGF in E12. bFGF is also known to be important for the self-renewal property of the O-2A progenitors (12). In this paper we found that the switch from heparin in the O-2A progenitors to the much lower sulfated HS in the oligodendrocytes corresponded to a significant decrease in the apparent bFGF affinity of the polysaccharide chains (Fig. 5). This might be expected, as both N-sulfation and 2-O-sulfation of the HS chains have been found to be important for bFGF binding (21–23), while 6-O-sulfation appears to be critical for biological activation (48–50). However, the astrocyte HS bound bFGF as efficiently as the more highly sulfated progenitor heparin (Fig. 5), possibly as a result of the prevalence of long sulfated domains (dodecasaccharides and larger) within the astrocyte HS (Fig. 3F).

Although oligodendrocytes are postmitotic (51), they still respond to bFGF by down-regulation of myelin-specific markers, re-entry into the cell cycle without mitosis, and increase in the length of cellular processes (52). The present results indicate that this response to bFGF may be less reliant on the HS coreceptor, which is at low levels (Fig. 1) and has low bFGF affinity (Fig. 5) than other cell systems (13–15) and could possibly be mediated by the signaling receptor FGFR2 type IIIc, which is not found in the the progenitors or astrocytes.

The long splice variant of the other key growth factor in this system, PDGFA (12), has recently been discovered to bind to highly sulfated heparin like oligosaccharides, enriched in the trisulfated disaccharides (24), which form 74% of the O-2A progenitor heparin (Table I). PDGFA is critical for the timing of the O-2A progenitor differentiation into oligodendrocytes and astrocytes (31, 51), and heparin may be essential in regulating the PDGFA response.

The specific temporal expression of heparin, in the O-2A progenitors, during central nervous system development suggests that heparin must have a function critical to this lineage or to the adjacent neurons. It would be interesting to discover whether heparin is also produced by the subset of glioblastoma multiforme tumors believed to be derived from the small population of adult O-2A progenitor cells (53), as it might enhance the activity of the myriad of autocrine growth factors mitogenic to glioblastomas (e.g. Refs. 54–57). Similarly, during injury of the central nervous system where reactive astrocytes are thought to revert to a more primitive state (for reviews, see Refs. 58 and 59), reappearance of heparin expression could possibly aid their rapid response. Further study of the heparin produced by O-2A progenitors could give important insights into the control of heparin and HS biosynthesis and their role in both development and disease states.

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