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Research article

# Expression of hyaluronan (hyaluronic acid) in the developing laminar architecture of the human fetal brain

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SUMMARY

Hyaluronan (also called hyaluronic acid or HA) plays a key role in the morphogenesis of the brain, but little is known about its expression in the human fetal neocortex. Using immunohistochemical methods, we assayed the expression of HA, glial fibrillary acidic protein, vimentin, nestin, and proliferating cell nuclear antigen in paraffin-embedded histologic sections of 8 mid-term fetuses (estimated gestational age, 12–16 weeks; crown-rump length, 75–120 mm). At 12–13 weeks, HA was expressed strongly along the membranes of many cells in the cortical plate and the layer 1 or marginal zone, but showed weak, spotty expression in a fiber-rich layer adjacent to the cortical plate, called the cortical stratified transitional field-1 (STF-1 or a primitive form of the subplate). At 15–16 weeks, HA was expressed in the layer 1 and in the early subplate or presubplate, but less strongly in cells of the possible STF-5 near the subventricular zone. However, the positive observation in STF-5 was probably a result of individual difference in development. The developing cortical plate seemed to produce HA in the presubplate to harbor axonal plexus of various afferent systems, while Cajal–Retzius cells were likely to accumulate HA in the layer 1. The HA-rich zones, those sandwiched the cortical plate, might avoid further migration of cortical cells.


## 1. Introduction

Hyaluronan (also called hyaluronic acid or HA), a nonsulfated linear glycosaminoglycan, has been found to facilitate cell movement in the fetal brain by weakening cell attachment to adhesive substrates and by creating hydrated pathways for migrating cells (reviewed by Bignami et al., 1993). To our knowledge, however, HA distribution has not been assessed in the developing laminar or stratified architecture of the fetal cerebrum in any mammal (Meyer et al., 2000; Bayatti et al., 2008; Clowry et al., 2010). Limited information is available on the laminar distribution of proteoglycans in the visual cortex of adult cats (granular cell layer; Lander et al., 1997) and in the telencephalic vesicle of a 17-day rat fetus (Bignami et al., 1993).

Recently, in the postnatal development and adult morphology of the brain and spinal cord, many research groups have paid attention to HA as a major component of perineuronal nets that isolate synapses and hinder lateral diffusion of postsynaptic receptors to control or stabilize the synaptic plasticity (Frischknecht and Seidenbecher, 2008; Kwok et al., 2011). HA in perineuronal nets is anchored to HA synthase-3 on the cell membrane of interneurons (Giamanco and Matthews, 2012). During postnatal development, however, other fiber-like HA structures distinct from perineuronal nets were observed in the putative white matter of mouse cerebellum (Baier et al., 2007). These findings suggested that HA-containing extracellular structures were not limited to perineuronal nets, especially during fetal brain development. We therefore investigated whether HA structures, other than perineuronal nets, are present in the human fetal neocortex. We also analyzed the topographical relationship between HA expression and cortical laminar structure, by staining tissue samples with antibodies to glial fibrillary acidic protein (GFAP), vimentin, nestin and proliferating cell nuclear antigen (PCNA).

According to excellent atlases by Bayer and Altman (2005), in the fetal human neocortex at 12–16 weeks of gestation, the laminar

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structure or cortical stratified transitional field (STF) are identified as follows: (1) the layer 1: the most superficial layer facing the primitive meningis; (2) the cortical plate; (3) STF 1: the superficial fibrous layer or the putative subcortical white matter (possibly corresponding to the primitive form of the subplate in Ulfig et al. (2000), Bystron et al. (2008), and Judaš et al. (2010); (4) STF 2: the upper cellular layer or the last sojourn zone before cells translocate to the cortical plate; (5) STF 3: the honeycomb trilaminar matrix of cells and fibers only in granular cortices; (6) STF 4: the complex middle layer or the putative deep white matter; (7) STF 5: the deep cellular layer or the first sojourn zone to appear outside the germinal matrix; (8) STF 6: the late-forming deep layer of callosal fibers outside the germinal matrix; (9) the subventricular zone or SVZ; (10) neuroepithelium or NEP.

## 2. Materials and methods

Paraffin-embedded specimens were utilized, obtained from 8 mid-term fetuses of estimated gestational age 12–16 weeks and crown-rump length (CRL) of 75–120 mm, including 3 fetuses of gestational age 12–13 weeks and 5 of gestational age 15–16 weeks. These fetuses, obtained by induced abortion, had been donated by the mothers and their families to the Department of Anatomy of Chonbuk National University in Korea, after the mothers were personally informed by an obstetrician about the possibility of donating the fetus for research; no attempt was made to encourage donation. Because of randomization of specimen numbering, it was not possible to trace any of the families concerned.

Use of these fetal specimens for research was approved by the ethics committee of Chonbuk National University, which did not require that the corresponding committee in Japan be informed about this research project. This study was performed in accordance with the provisions of the Declaration of Helsinki 1995 (as revised in Edinburgh 2000).

Each donated fetus was fixed in 10% w/w neutral formalin solution for more than 1 month; and divided into the head and neck, the thorax, the abdomen, the pelvis and the four extremities. All of these body parts were decalcified by incubating them at 4 °C in 0.5-mol/l EDTA (pH 7.5; decalcifying solution B; Wako, Tokyo) for 1–3 days, depending on the size of the specimen. The head and neck specimens were sectioned sagittally or horizontally at 20–50 μm intervals, depending on the size of the sections. Sections included not only the brain but also the surrounding structures, including eyes and ears. Thus, each sample included skull base cartilage, a positive control for HA staining. Most sections were stained with hematoxylin and eosin (HE), with others used for immunohistochemistry.

The primary antibodies used for immunohistochemical staining were (1) rabbit polyclonal anti-human GFAP (1:100; Dako Cytomation, Kyoto, Japan; catalog number Z0334); (2) mouse monoclonal anti-human vimentin (1:10; Dako, Glostrup, Denmark; catalog number M7020); (3) mouse monoclonal anti-human nestin (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, US; catalog number sc23927); (4) mouse monoclonal anti-human PCNA (1:1000; Abcam, Cambridge, UK); (5) mouse monoclonal anti-human growth associated protein-43 or GAP 43 (1:8000; Sigma-Aldrich, St. Louis, US); and (6) rabbit polyclonal anti-human calretinin (1:100; Invitrogen, CA, US). Samples were not pretreated in the autoclave because of the loose nature of the fetal tissues. Following incubation with primary antibody, the sections were incubated with horseradish peroxidase (HRP)-labeled secondary antibody (Histofine Simple Stain Max-PO, Nichirei, Tokyo) for 30 min, followed by incubation with diaminobenzidine (Histofine Simple Stain DAB, Nichirei) for 3–5 min. All samples were counterstained with hematoxylin.

HA-binding proteins appear in the late stage fetus, near the time of myelination (Bignami et al., 1993). Therefore, HA staining was performed using a biotinylated HA-binding protein (2 μg/ml; Seikagaku Corp., Tokyo, Japan) after immersing the sections in chondroitinase ABC (10 microunits/ml; Sikagaku Corp, Tokyo, Japan) in 0.1 M Tris–acetate buffer (pH 8.0, 37 °C) for 30 min (Shibata et al., 2003). The sections were incubated for 30 min with the Histofine SAB kit (Nichirei) for the 3-amino-9-ethylcarbazole (AEC) reaction, yielding a red biotin complex or with Histofine Simple Stain Max-PO (Nichirei) for the DAB reaction with HRP, yielding a dark brown biotin complex. The latter sections were counterstained with hematoxylin.

## 3. Results

Fibers positive for vimentin and nestin were observed running across the neocortex, from the deep neuroepithelium to the superficial amorphous layer (layer 1), in both smaller (12–13 weeks; Fig. 1) and larger (15–16 weeks; Figs. 2 and 3) fetuses. Vimentin-positive fibers, indicative of radial glial cells, were clearly observed (Figs. 1C, and 2C and G) because the fibers reached the cortical plate. However, the fibers did not express GFAP in the cortical plate of smaller fetuses (Fig. 1B). We also attempted to identify stratified structures in the neocortex according to Bayer and Altman (2005). Layer 1, the cortical plate, the subventricular zone and the neuroepithelium were evident in both larger and smaller fetuses. Layer 1 showed strong expression of HA (Fig. 1A and 2F).

In the smaller fetuses (Fig. 1), strong HA expression was observed along the membranes of many cells in the cortical plate and the early subplate, especially in the latter, with weak spotty expression observed in a fiber-rich intermediate layer adjacent to the cortical plate. Likewise, in layer 1, HA expression was not diffuse but appeared to be concentrated around composite cells. Thus, diffuse expression of HA in layer 1 and the subplate appeared to occur around 14 weeks. These HA-binding cells in the cortical plates of smaller fetuses did not express either nestin or GFAP. HA-positive cells, apparent in the putative STF 5 of the larger fetus (see below), were not observed in smaller fetuses. Thus, the other cortical stratified transitional fields (STFs 2–6) could not be distinguished in the smaller fetuses. The early subplate contained axon bundles which were strongly positive for GAP43 (Fig. 1F). Calretinin-positive cells were almost evenly distributed throughout all layers of the neocortex (Fig. 3B).

In the larger fetuses, the subplate or STF 1, the superficial fibrous layer or putative subcortical white matter, was characterized by (1) diffuse expression of HA (Fig. 2F), (2) strong expression of GAP 43 (Fig. 2B), (3) few GFAP-positive fibers (Fig. 2G) and (4) absence of PCNA-positive cells (Fig. 2D). If, in larger fetuses, STF 5, consisting of the deep cellular layer or the first transitional zone to appear outside the germinal matrix, was defined as a thin layer containing HA-bound cells in AEC reaction (Fig. 2F), then STF 2–4 and 6 could be identified based on the different arrangements of GFAP-positive fibers between layers (Fig. 2G). Nevertheless, DAB reaction did not demonstrate HA-positive cells in the probable STF 5 despite the strong positivity in the layer 1 and subplate (Fig. 3A). Moreover, even with AEC reaction, the positive cells in the STF5 were not found in our later trial 6 months after the sectioning (figures, not shown). Calretinin-positive cells were rarely seen and, if present, mostly distributed in the STF 2–6 or the intermediate zone (Fig. 3C).

Laminar expression of HA was not observed in the large olfactory bulb, the thalamus, the ganglionic eminence (the putative basal ganglia) or the brainstem of both larger and smaller fetuses (Fig. 4). In sites other than the neocortex, HA-positive cells were distributed widely and evenly; we were unable to identify a specific topographical relationship between the putative nucleus and HA expression.