Histochemical Localization of Hyaluronic Acid in Vitreous during Embryonic Development

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PURPOSE. To determine the chronology of the appearance and localization of hyaluronic acid (HA) in mouse vitreous during the embryonic and early postnatal stages of development and in human vitreous during early embryonic development.

METHODS. A histochemical method using the specific affinity for HA of a bovine cartilage proteoglycan was used on mouse eyes at embryonic (11 to 18 days) and early postnatal (8 and 18 days) stages. The same technique was applied to human embryonic eyes of 6, 8, 9, and 10 weeks.

RESULTS. In the mouse, HA is detected early (12-day embryo stage) in the equatorial vitreous and in the internal portion of the corresponding retinal epithelium, behind the anterior rim of the optic cup. Later in development, HA staining extends temporarily to the posterior vitreous and to the internal layers of the posterior retinal epithelium. In human embryos, HA staining is clearly visible in the posterior vitreous and in the equatorial vitreous, where it is more intense at all the developmental stages. From the 8-week stage onward, the internal layers of the developing retina are also heavily stained.

CONCLUSIONS. HA appears very early in developing vitreous of mice and humans, and staining is observed first and predominantly in the equatorial portion of the vitreous. In contrast to human embryos, HA staining in the posterior mouse fetal vitreous is only faint and transient. In both species, staining of the internal layers of the retinal epithelium is detected in the presumptive ciliary body region and in the more posterior retina. The observed temporal and regional simultaneous localization of HA staining in the vitreous and the internal layers of the retinal epithelium is suggestive of a possible role for these cells in the production of the molecule.


Growth of the eye must occur to exact specifications because the various tissues of this organ must bear precise geometric relations to one another if optic and photoreceptive functions are to be achieved. Emmetropization is a complex control mechanism of eye growth, partly genetically and partly visually regulated, in which the important role of the vitreous portion of the eye has been clearly established. However, the nature of the afferent and efferent pathways of this process is still a mystery, and the different molecular effectors influencing eye growth have not been identified. Protein concentrations of the vitreous do not seem to influence directly the size of the eye. Demonstration of the necessity of a positive intravitreal pressure for the development of the eye was performed by Coulombre, and intracocular pressure in general has been suggested to play a role in the development of myopia, later in children. In mammals, hyaluronic acid (HA) has already been proposed as one of the possible molecular instruments of fine regulation of vitreous volume and pressure because of its varying size and its biochemical characteristics, but until now, according to biochemical data, HA was supposed to appear late in development and to increase only after birth in bovine and human vitreous. However, histochemical studies performed in human and mouse embryos using indirect techniques have shown that there are many waves of glycosaminoglycan (GAG) synthesis in the vitreous during embryogenesis and that one of these GAGs could be HA. The purpose of this work was to study distribution of HA in mouse and human embryonic vitreous using a proteoglycan-derived probe that has been demonstrated to be more specific and sensitive than indirect histochemical techniques. A better knowledge of the distribution of this molecule in the early vitreous could help to clarify its function during eye growth.

MATERIALS AND METHODS

Mouse Embryos

Groups of five Swiss female mice caged overnight with one male were checked every morning for vaginal plugs. Taking the day of vaginal plug discovery as day 0 of gestation, 4 embryos were collected at each of 11, 12, 13, 14, and 18 days and were immediately immersed in fixative. Four eyes isolated from 2½-week-old mice were also collected; they were opened through the cornea, and the anterior aspect of the lens capsule was incised for aspiration of the hard lens nucleus before fixation. All animal handling was in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Two different fixatives were tested: buffered formaldehyde containing 1% (wt/vol) cetyl pyridine chloride with fixation times ranging from 3 hours to 3 weeks and Sainte-Marie’s solution (99 volume 95% ethanol:1 volume 99% glacial acetic acid) with a fixation time of 1 hour. After dehydration through graded ethanol, the specimens were embedded in paraffin.

Histochemical Method

HA was detected using a biotinylated form of bovine nasal cartilage proteoglycan (hyaluronic acid binding proteoglycan...
region, HABPR) that binds to HA with high affinity and specificity. The probe, prepared as described by Lütjen-Drecoll et al. was a kind gift from Professor Kiell Madsen (Pharmacia AB, Uppsala, Sweden). HA was visualized by staining the probe using the ABC technique and conjugated peroxidase. Two sets of serially adjacent sections of 10 μm were cut and mounted on precleaned microslides. All slides were dewaxed for 10 minutes in each of two changes of toluene, hydrated in graded series of ethanol, and washed. The sections were incubated for 30 minutes in methanol with 0.3% H₂O₂ to block endogenous peroxidase activity. One set of sections was incubated for 2 hours in a humidified chamber at 37°C in phosphate-buffered saline (PBS) with 50 U/ml of Streptomyces hyaluronidase (Sigma H 1136; Sigma Aldrich Fine Chemicals, Bornem, Belgium) as negative control. The other set of sections was incubated under the same conditions in buffered saline only. Both sets of slides were then rinsed in PBS (2 times for 5 minutes) and incubated in serum albumin (10 mg/ml) for 30 minutes to block nonspecific binding of the probe. After rinsing (2 times for 5 minutes in PBS), the sections were incubated for 2 hours at room temperature with HABPR-biotin (20 μg/ml) in PBS. They were then rinsed (2 times for 5 minutes) in PBS, incubated for 30 minutes with Vectastain Elite ABC Reagent (Vector Laboratories Inc., Burlingame, CA) and rinsed (2 times for 5 minutes). The sections labeled with peroxidase were visualized with substrate solution prepared as follows: 10 mg 3-amino-9-ethylcarbazole in 6 ml DMSO was added to 40 ml acetate buffer, pH 5, containing 5 μl H₂O₂. The sections were incubated in this solution for 5 minutes and rinsed in tap water. They were counterstained in methyl green solution (0.075% at pH 4.7) for several minutes, mounted under glass coverslips in glycerin-gelatin, and examined by light microscopy.

**Human Embryos**

The eyes of four human embryos, issued from abortion at 6, 8, 9, and 10 weeks of development, were collected and fixed in buffered formaldehyde containing 1% (wt/vol) cetyl pyridine chloride for 3 weeks. After fixation, these specimens were treated exactly like the mouse embryos. A defect in phenylalanine hydroxylase had been diagnosed in the 9-week-old embryo, but its morphologic aspect was normal.

**Results**

Two different fixatives were tested to achieve optimal staining results. Both of them were recommended by authors who had used the same biotinylated hyaluronan probe to detect hyaluronan in tissue sections. No differences in staining were observed between specimens fixed in either solution, but the fixation time in formaldehyde containing cetyl pyridine chloride must be at least 3 weeks to obtain sufficient fixation of the morphologic structure of the embryos. Treatment with Streptomyces hyaluronidase prevented staining completely. The results described here involve the posterior part of the eye, not the cornea, iridocorneal angle, or iris.

**Mouse Embryos**

In 11-day-old embryos the vitreous space appears very narrow and contains many fibers and a few vessels. The lens is still vesicular and has not completely separated from the ectoderm. No staining of hyaluronic acid is detectable in the eye rudiment at this stage. In 12- and 13-day embryos, the volume of the vitreous is increased, and positive staining of hyaluronic acid appears in the vitreous region surrounding the equatorial part of the lens. The internal portion of the retinal epithelium, situated behind the anterior rim of the optic cup, along the perieuquatorial vitreous, is also stained (Figs. 1A, 1B). In the 14-day embryos, the perieuquatorial vitreous staining is much more intense and extends more posteriorly. Faint staining of the fibrillar material of the posterior vitreous is also detectable.

In the retinal epithelium, staining extends more deeply and slightly more posteriorly than in earlier stages. In the 18-day embryos, the iris is well developed, growing from the anterior border of the optic cup, and a slight swelling of this border is visible, indicating the developing ciliary body. The neural retina consists of an outer nuclear layer, a transient intermediate anuclear layer, and an inner nuclear layer. Staining of the posterior vitreous material has almost disappeared; however, positive staining remains detectable in a small portion of the perieuquatorial vitreous, near the surface of the iris base, and of the forming ciliary body (Fig. 1C). In the retina, a faint staining in the inner nuclear and intermediate anuclear layers is also visible. In the 15-day-old mice, weak staining is detectable at the surface and deep in the ciliary body. There is no more staining in the retinal layers or in other parts of the vitreous. The zonular fibers, which are clearly visible at this stage, are also negatively stained.

**Human Embryos**

At the 6-week stage of human embryos, the vitreous appears dense and homogeneous, crossed over by numerous hyaloid vessels communicating with the anterior mesenchyme. Intense staining of HA can be seen in the whole vitreous and in the anterior mesenchyme. The staining is particularly intense in the perieuquatorial vitreous where fibers seem to converge at the retinal surface (Fig. 1D). Faint staining is visible over the whole retinal surface, even posteriorly, and in the inner third of the neural retinal epithelium where the staining appears to be extracellular. From the 8-week stage onward, the eye volume increases considerably, and the anterior communication of the vitreous with the external mesenchyme is lost. The external portion of the vitreous, near the retinal surface, is more filamentous and homogeneous than the innermost part of the vitreous, which appears more granular. More intense staining is observed in the perieuquatorial region (Fig. 1E). The marginal layer and the inner neuroblastic layer of the neural retina are also heavily stained (Fig. 1F). The position and relative intensity of staining remain unchanged in 9- and 10-week embryos.

**Discussion**

The results of direct histochemical staining of HA depend on the amount of HA molecule in the tissue, its availability to the HA probe used, the specificity of the probe, and the quantity of HA remaining after all the fixation and staining procedures have been completed.

We believe that our staining effectively shows the presence of HA in the early embryonic mouse and human vitreous. The presence of positive or negative staining using the described technique correlates with biochemical data obtained from ocular tissues (sclera and cornea). To obtain the best...
(A, B) Thirteen-day mouse embryo. (A) There is a reddish-brown staining for hyaluronic acid in the vitreous surrounding the equatorial part of the lens and in the internal portion of the retinal epithelium in the same region. (B) Staining is prevented by pretreatment with Streptomyces hyaluronidase.

(C) Eighteen-day mouse embryo. In the vitreous, staining remains detectable only in a small portion of the periequatorial region, near the surface of the iris base, and the forming ciliary body.

(D) Six-week human embryo. The staining for hyaluronic acid is intense in the whole vitreous but particularly in the periequatorial zone. Faint staining is visible over the whole retinal surface and in the inner third of the neural epithelium.

(E, F) Ten-week human embryo. (E) Equatorial portion of the optic cup. There is an intense staining of the vitreous material that appears more filamentous (arrowhead) near the retinal surface and more granular (asterisk) in the innermost part. (F) Posterior part of the optic cup. The vitreous and the marginal and inner neuroblastic layers of the neural retina are heavily stained. Scale bar, (A through F), 100 μm.
preservation possible of the molecule before staining, the fixatives used in this study were chosen according to the recommendations of other authors who had compared different fixation techniques. In addition, the specificity of the probe was controlled by comparison with samples in which staining was completely prevented by incubation with Streptomyces hyaluronidase. Component concentrations and the rheological state of the vitreous differ with age and between species. To the best of our knowledge, the composition of mouse vitreous has never been chemically analyzed, and the nature of its components is still hypothetical. The present study shows that in the developing mouse vitreous, HA staining appears early in the equatorial part and persists longer than in the posterior part where it is never intense and disappears before birth. This decline in staining in the posterior vitreous of older mice embryos and of 2-week-old mice was totally unexpected. It could reflect a true reduction of the production of HA that is replaced by another GAG in the posterior vitreous during development, as already observed in other tissues (such as the cornea, skin, and vessels). Alternatively, it could indicate a change in the biochemical binding of HA to other vitreous components that results in a loss of the molecule during the histologic procedures. Persistence of HA staining in the equatorial part of the vitreous at the latest stages of development is in agreement with the observations of Chan et al. They reported colocalization in rats of HA and chondroitin sulfate proteoglycan at the surface of the zonular fibers, in the basement membrane of the inner ciliary epithelium, only at the pars plana region, and in the equatorial region of the lens capsule. In the retinal epithelium, the staining that we first observed in the region of the presumed ciliary body extends later to the internal layers of the posterior retina; this extension could be correlated chronologically with differentiation of the Müller cells which are suspected to produce HA. The cellular origin of the vitreous and zonular components is still unclear, but it has already been demonstrated in other tissues that HA localized in between early developing epithelial cells is gradually lost from those sites and begins to accumulate in the basement membrane regions of the epithelia concerned. The simultaneity of localization of HA in the vitreous and in the internal retinal layers suggests a possible role of these retinal epithelial cells in the production of the glycosaminoglycan. However, demonstration of this hypothesis requires further experiments.

To the best of our knowledge, there have been only a few studies concerned with the distribution of HA in the posterior segment of the mammalian embryonic eye. Azuma et al. presented histochemical data suggesting that Müller cells could produce HA transiently in human fetuses between 12 weeks and the neonatal stage. However, the histochemical technique used in this work was less specific for HA than the procedure used here. Tate et al. localized the molecule in the developing inner retinal layers of 20- to 22-week-old human embryos. Although the present study does not contradict these earlier findings, it does show that HA is already clearly detectable in the human internal retinal layers and in the vitreous at 6-weeks' gestation. It also shows that staining increases in these regions from the 8-week stage onward. These observations are in agreement with the results of Peterson et al., who described HA as appearing very early in the internal layers of the retinal epithelium in the macaque embryo, before invagination of the lens vesicle was complete. At comparable stages of development, staining of HA in the posterior vitreous is much greater in human than in mouse embryos. However, in both species, the equatorial part of the vitreous is more heavily stained than the posterior part. Comparison of the two species reveals similarities in the distribution of staining in the internal retinal layers and underlines the apparent simultaneity of staining in these retinal layers and in the vitreous. The exact function of HA in the vitreous is still unknown. In mesenchymal tissues, HA is thought to exert swelling pressure, to expand intercellular spaces and to inhibit cellular adhesions, or, depending on its concentration, to allow cellular aggregation of mesenchymal cells and to influence cellular differentiation. It could also play an important role in the early formation of epithelia during epithelial morphogenesis. In contrast to human vitreous, it may be that HA is a relatively minor component of the adult mouse posterior vitreous, as in the dog, rabbit, and guinea pig. Nevertheless, it appears that HA is universally necessary as a retinal mediator of early eye growth and that it is a permanent component of the zonular region of the eye. Further studies on the localization of this molecule in later stages of eye development, in different species, and in some particular experimental situations should better establish whether HA does modulate the axial expansion of the eye.

References

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