Identification of a Novel Element in the Human Eye: The Inner Connective Tissue Layer of the Ciliary Body Characterized With Antibodies to the HNK-1 Epitope

Marita Uusitalo, Tero Kivelä, and Ahti Tarkkanen

Purpose. To characterize the nature and the developmental distribution of the HNK-1 epitope in the inner connective tissue layer of the human ciliary body, located between the ciliary epithelium and muscle with two monoclonal antibodies to the HNK-1 epitope common to many cell adhesion molecules.

Methods. Nine fetal (gestational age 13–40 wk) and 32 postnatal human eyes (age 3 mo to 78 yr) were studied by immunohistochemistry with monoclonal antibodies HNK-1 and VC1.1 to the HNK-1 epitope. Antibodies to cytoskeletal elements were used to characterize the cells in this region.

Results. The HNK-1-immunopositive cells appeared underneath the pigment epithelium of the pars plicata by the 20th gestational week, spread into the pars plana after the 28th week, and reached the ora serrata during the first year of life. The immunoreaction was constantly present in all adult eyes examined; they were sharply demarcated from the iris, ciliary muscle, and choroid. The HNK-1-positive subepithelial layer was not labeled with monoclonal antibodies V9 or Vim 3B4 to vimentin, monoclonal antibodies CAM 5.2 and CY-90 to cytokeratin 8 and 18, or monoclonal antibodies DE-U-10 and D33 to desmin in adult eyes, but was uniformly positive for vimentin in fetal eyes. The HNK-1 epitope was distributed along cell membranes or adjacent extracellular matrix of stromal cells.

Conclusion. The HNK-1-positive stromal region is a constant and conspicuous element of the human eye that may have a role in structurally stabilizing the ciliary body, perhaps in relation to accommodation or aqueous secretion. Invest Ophthalmol Vis Sci 1993;34:2372–2381.

The monoclonal antibody HNK-1 was raised against a membrane fraction of the HSB-2 human T-lymphoblastoid cell line and selected because it specifically labeled a subset of lymphocytes enriched in natural killer and killer cells.1 It recognizes a carbohydrate epitope subsequently demonstrated on a variety of extracellular matrix and integral membrane glycoproteins involved in cell adhesion, including myelin-associated glycoprotein,2,4 peripheral myelin glycoprotein P<sub>0</sub>,<sup>5</sup> neural cell adhesion molecule N-CAM,<sup>6</sup> L1 glycoprotein,<sup>6</sup> J1 and cytotactin related to tenascin,<sup>7,10</sup> and cytotactin-binding proteoglycan.<sup>10,11</sup> The epitope is present on migrating avian neural crest cells, so the molecules bearing it may have a role in cell-to-cell and cell-to-substratum interactions even during differentiation.<sup>12,15</sup>

In humans, the HNK-1 antibody reacts with large granular lymphocytes of peripheral blood,<sup>1,16</sup> myelin sheaths of central and peripheral nerves,<sup>17,18</sup> many neuronal, glial, neuroectodermal, and neuroendo-
HNK-1 Epitope

In the human eye, the HNK-1 epitope has been demonstrated to be present in the neuroretina, as well as in the ciliary and retinal pigment epithelial cells. In addition, using the monoclonal antibodies to the HNK-1 epitope positive immunostaining was found unexpectedly in the subepithelial connective tissue matrix of the ciliary body. The current study was undertaken to better determine the development and nature of this immunoreaction with two different monoclonal antibodies both recognizing the HNK-1 epitope.

METHODS

Histologic Specimens

A series of 41 formalin-fixed and paraffin-embedded human eyes representing different age groups was selected from the files of the Ophthalmic Pathology Laboratory, Department of Ophthalmology, Helsinki University Central Hospital. The eyes had been enucleated between 1975 and 1991. Eyes that had been irradiated or otherwise treated before enucleation were excluded and specimens with as normal as possible anterior segments were chosen.

Nine morphologically normal fetal eyes representing gestational weeks 13–40 were studied. One fetus came from a spontaneous abortion of an undetermined cause, two were aborted because of maternal rubella, and one each because of possible incontinentia pigmenti, lethal osteochondrodysplasia, and an unknown reason. One stillborn full-term fetus had an anomalous chromosome 22, one had Meckel’s syndrome, and in another an intrauterine infection was suspected. Eleven eyes represented children younger than 10 yr (range, 3 mo to 3 yr 9 mo). These included ten eyes with retinoblastoma and one eye from a child with an unknown metabolic disease. Finally, 21 eyes were from adults (range, 23–78 yr). Each 10-yr age group between 20 and 80 yr included up to six specimens. Eight of these eyes had been enucleated because of a posteriorly located malignant choroidal melanoma, seven because of an orbital tumor, and the remaining six were normal eye bank eyes.

Sections (5 μm thick) were cut from the specimens and mounted on chromium–gelatin-treated glass slides to ensure adherence (0.05 g potassium chromate(III)sulfate dodecahydrate and 0.5 g gelatin in 100 ml distilled water).

Normal retina was used as a positive internal control for antibodies to vimentin and cytokeratin 8 and 18 and ciliary and extraocular muscles as controls to antibodies to smooth muscle actin and desmin.

Immunohistochemical Staining

Sections from all specimens were immunostained using commercial versions (Vectorstain ABC Elite Kits for Mouse IgG and Rabbit IgG, Vector Laboratories, Burlingame, CA) of the avidin-biotinylated peroxidase method.

The primary rabbit polyclonal and mouse monoclonal antibodies (MAb) were used in the following optimal dilutions as determined by preliminary stainings: MAb HNK-1 (Leu-7), immunoglobulin M (Lot N1222, Becton Dickinson, San Jose, CA), 1:40, by definition reacting with the HNK-1 epitope; and MAB VC1.1, immunoglobulin M (Lot 071H4828, Sigma, St Louis, MO), 1:16 000, which detects the HNK-1 epitope but has a slightly different spectrum of reactivity with cell adhesion molecules bearing this epitope. To characterize the HNK-1 positive cells further, MAB V9, IgG1 (Lot 10H4808, Sigma) 1:600, and MAB Vim 3B4, IgG2a (Lot 12824420-03, Boehringer Mannheim, Germany), 1:20 against vimentin; polyclonal antibodies to vimentin (Lot 039, Euro-Diagnostics, BW Apeldoorn, The Netherlands), 1:75; MAB CY-90, IgG1, to cytokeratin polypeptide 18 (Lot 49F4815, Sigma), 1:2000; MAB CAM 5.2, IgG2a, mainly recognizing cytokeratin 8 (Lot N0435, Becton Dickinson), 1:100; MAB DE-U-10, IgG1 (Lot 117F4809, Sigma), 1:100 and MAB D33, IgG1 (Lot 011, Dakopatts, Glostrup, Denmark), 1:200 against desmin; and MAB IA4, IgG2a, to anti-α-smooth muscle actin (Lot 98F4808, Sigma), 1:4000 were also used.

In brief, the sections were routinely deparaffinized in xylene and hydrated in an ethanol series. Specimens immunostained with MAB V9 and Vim 3B4 to vimentin, MAB CAM 5.2 and CY-90 to cytokeratin, and MAB DE-U-10 to desmin were pretreated with 0.4% pepsin (2000 FIP-U/g; E. Merck, Darmstadt, Germany) in 0.01 N hydrochloric acid at 37°C for 10 min to reduce the background and to enhance the intensity of specific immunostaining. Pretreatment with pepsin did not significantly affect the positive immunoreaction with polyclonal antibodies to vimentin or MAB HNK-1 and VC1.1 recognizing the HNK-1 epitope, and it seemed to decrease the immunoreaction with MAB D33 to desmin.

Endogenous peroxidase activity was destroyed by treating the sections for 30 min in a solution containing 200 ml methanol and 3.2 ml 30% hydrogen peroxide (Perhydrol, E. Merck). Nonimmunologic binding of antibodies was blocked by incubation with normal serum (Vectorstain ABC Kits, horse serum for monoclo-
Immunohistochemistry of Fetal Eyes

RESULTS

Light Microscopy

In all specimens, the iris and ciliary body were structurally normal as examined by light microscopy. In some retinoblastoma eyes, clusters of tumor cells were seen in the anterior and posterior chamber, but the ciliary body was not invaded. The clinically estimated gestational age of the fetal eyes agreed with the developmental stage as determined by light microscopy.

Immunohistochemistry of Fetal Eyes

13–18 Weeks: By the 13th week, the condensation of the ciliary body had started, the first ciliary folds had formed, and the rim of the optic cup was advancing to form the iris (Fig. 1A). In the subepithelial region of the ciliary body, between the developing ciliary epithelia and muscle fibers, a relatively dense and homogenous population of morphologically undifferentiated cells was seen (Fig. 1A). No immunopositive cells were detected with either MAb HNK-1 (Fig. 1B) or VC1.1.

However, these antibodies reacted moderately with the inner layers and more weakly with the outer layers of the retina (Fig 1C). The developing ciliary muscle did not react with antibodies to DE-U-10 (Fig 1D) and D33 against desmin.

Cells within the subepithelial region of the ciliary body, most other stromal cells, the ciliary epithelial cells, and ciliary muscle fibers reacted with MAb V9, Vim 3B4 (Fig. 1E), as well as with the rabbit antisera against vimentin. In addition, a few cells underlying the pigmented ciliary epithelium were labeled with MAb CAM 5.2 mainly to CK 8, and with MAb CY-90 to CK 18 (Fig. 1F). The developing ciliary muscle fibers reacted with MAb 1A4 to α-smooth muscle actin and were sharply demarcated from the sclera. Toward the 18th week, they became sharply delimited also from the subepithelial region (Fig. 1G). Only extraocular muscles reacted with MAb DE-U-10 and D33 against desmin.

20–22 Weeks: By the 20th week, the number of ciliary processes had increased and their stroma had become more cellular (Fig. 2A). The pars plana region had not yet developed. In the subepithelial region of the ciliary body (Fig. 2B), a positive immunoreaction could be observed with MAb HNK-1 (Fig. 2, C and D) and VC1.1 focally near the base of the iris, immediately adjacent to the pigmented epithelium of the ciliary body. The ciliary epithelium and cells within the developing trabecular meshwork were likewise focally immunoreactive with both antibodies.

Concurrently, the ciliary muscle had become immunopositive with MAb DE-U-10 (Fig 2E) and D33 to desmin. The reaction pattern with MAb CY-90 and CAM 5.2 to cytokeratin, and MAb V9 and Vim 3B4 to vimentin was basically identical to that seen earlier. The cytokeratin-positive cells did not coincide with those reacting with MAb HNK-1 and VC1.1. All layers of the neuroretina, as well as the radial glial fibers were labeled with MAb HNK-1 (Fig 2F) and VC1.1.

28 Weeks: By the 28th week, the pars plana had formed. The immunoreaction with MAb HNK-1 (Fig. 3, A and B) and VC1.1 had become more extensive and extended throughout the subepithelial region of the pars plicata beneath the pigmented epithelium. Positive reaction was not seen in the developing pars plana (Fig. 3C). All antibodies to vimentin immunostained the ciliary muscle, the ciliary epithelia, and the subepithelial stromal cells of the ciliary body (Fig. 3D). Cells reacting with MAb 1A4 to α-smooth muscle ac-
FIGURE 1. The antigenic profile of the subepithelial region of the human ciliary body during the 13th (A–D) to 16th (E–G) gestational weeks. (A, hematoxylin-eosin, and B–G, immunoperoxidase staining). (A) The subepithelial region (se), the ciliary muscle (cm) and the iris root (ir) under the pigmented ciliary epithelium (double arrowheads), as well as the sclera (scl) appear densely cellular. The first ciliary folds (arrowhead) have developed. (B) None of these structures reacts with MAb HNK-1. Pigmented ciliary epithelium (double arrowhead) is visible due to its melanin content. (C) The outer (onb) and inner (inb) neuroblastic layers of the retina react with MAb HNK-1. (D) MAb DE-U-10 to desmin does not label any structures at this stage. Melanin is seen in the pigmented epithelium (double arrowhead). (E) The ciliary muscle (cm), subepithelial region (se), and the nonpigmented ciliary epithelium (arrowhead) react with MAb Vim 3B4 to vimentin. Melanin obscures positive reaction in pigmented ciliary epithelium (pe). (F) A number of cells in the subepithelial region (se) between the unlabeled ciliary muscle (cm) and pigmented epithelium (pe) react (double arrowhead) with MAb CY-90 to cytokeratin 18. Note labeling of the nonpigmented ciliary epithelium (arrowhead). (G) The ciliary muscle (cm) but not the nonpigmented epithelial epithelium (arrowhead) react strongly with MAb 1A4 to α-smooth muscle actin. (Original magnifications: A and B X260; C X420; and D–G X280)

which labeled the ciliary muscle, were not found in the subepithelial region (Fig. 3E). The staining pattern with other antibodies was unchanged.

Full-term: In the full-term infant, the iris and ciliary body were almost mature morphologically. The immunoreaction with MAb HNK-1 and VC1.1 involved the entire subepithelial region of the pars plicata and reached the stroma of the ciliary processes. It also continued underneath the pigmented ciliary epithelium into the pars plana, but did not yet reach the ora
FIGURE 2. The antigenic profile of the subepithelial region of the human ciliary body by the 20th gestational week. (A and B, hematoxylin-eosin, and C–F, immunoperoxidase staining). (A) The subepithelial region (se) is more loosely arranged and morphologically similar to the stroma of the iris (ir). For localization, note the major arterial circle (arrowhead in A, C, E). (B) By a higher magnification, the cells in the subepithelial region resemble fibroblasts with cytoplasmic processes. (C) The first focal positive immunoreaction with MAb HNK-1 (double arrowhead) is present in the subepithelial region close to the base of the iris (ir) just beneath the pigmented epithelium. (D) The immunoreaction with MAb HNK-1 surrounds a population of stromal cells and their processes. (E) The ciliary muscle (cm) reacts faintly with MAb DE-U-10 to desmin. No label is seen in the subepithelial region (se). (F) MAb HNK-1 labels all retinal layers, except the photoreceptor cell layer (double arrowhead). Onh, outer and inb, inner neuroblastic layer; opl, outer and ipl, inner plexiform layer; and gel, ganglion cell layer. (Original magnifications: A, C, E, and F ×240; B and D ×600)

serrata. No change was seen in the staining pattern with other antibodies tested.

Immunohistochemical Staining of Postnatal Eyes
In all infant and adult eyes studied, the entire subepithelial region of the ciliary body, between the pigmented epithelium and the ciliary muscle, reacted with MAb HNK-1 and VC1.1 (Fig. 4). The immunoreactive layer was thick in the pars plicata and thinner over the pars plana, as was the light microscopically observable subepithelial stromal tissue. The positive reaction continued as far as the ora serrata, where it very abruptly ended in all eyes studied (Fig. 4, A and B). No immu-
FIGURE 3. The antigenic profile of the subepithelial region of the human ciliary body by the 28th gestational week (immunoperoxidase staining). (A) MAb HNK-1 labels (double arrowhead) the subepithelial layer (se) of the pars plicata (pli), but the developing pars plana (pla) and iris (ir) remain negative. (B) The positive immunoreaction with MAb HNK-1 outlines stromal cells with cytoplasmic processes in the pars plicata (pli). (C) The subepithelial tissue (se, double arrowheads) and the ciliary muscle (cm) at the pars plana (pla) do not react with MAb HNK-1. (D) Ciliary muscle (cm) and the subepithelial region (se) react with MAb Vim 3B4 to vimentin. Note positive label in the nonpigmented epithelium (arrowhead). (E) The ciliary muscle (cm) reacts with MAb 1A4 to α-smooth muscle actin, but the subepithelial region (se) and nonpigmented epithelium (arrowhead) remain negative. (Original magnifications: A X160; B X420; C X560; D X210; and E X280)
FIGURE 4. The antigenic profile of the subepithelial region of the human ciliary body in postnatal eyes (A, hematoxylin-eosin; B–H, immunoperoxidase staining). (A) The subepithelial layer of the ciliary body (se) merges relatively imperceptibly with that of the choroid (ch) at the ora serrata. Ret, retina. (B) The strong immunoreaction of the subepithelial region (se) with MAb HNK-1 sharply ends at the ora serrata. The choroid (ch) is devoid of any immunoreaction, although some pigmented melanocytes can be seen, and the retina (ret) reacts positively. (C) The subepithelial stroma (se) of the pars plicata reacts uniformly with MAb HNK-1, but the stroma of the iris (ir) does not (double arrowhead). Round pigmented cells (arrowheads) within the subepithelial region are visible because of their melanin content. (D) MAb HNK-1 outlines coarse fibers seemingly related to processes of stellate cells, as well as a more fine meshwork of filaments. (E) The immunostaining pattern with MAb VCL1 is qualitatively identical, but somewhat less extensive. (F) MAb HNK-1 reveals a stromal cell (double arrowhead) with long, relatively thick cytoplasmic processes (arrowheads). (G) The entire subepithelial region (se) but not the ciliary muscle (cm) reacts with MAb HNK-1. (H) Instead, MAb Vim 5B4 to vimentin reveals only a few cell processes within the subepithelial region (se), whereas the ciliary muscle (cm) is uniformly labeled. (Ages: A–C, 4 yr; D–F, 7 mo; and G–H, 30 yr. Original magnifications: A–C X160; D–E X360, F X850; and G–H X320.)
for vimentin and contained only a few immunopositive fibers belonging to melanocytes (Fig. 4, G and H). In the adult eyes, only single stromal cells reacted with MAb CAM5.2 and CY-90 to cytokeratin.

DISCUSSION

The histology, ultrastructure, and physiology of the ciliary epithelium and muscle have been thoroughly investigated, but only a few remarks have been made about the stromal tissue filling the space between them, and enmeshing individual bundles of ciliary muscle. By electron microscopy, fibroblasts, melanocytes and lymphocytes, as well as a few mast cells and macrophages have been identified within this region, together with blood vessels, collagen bundles, and nerves. Indeed, the stroma of the ciliary body has been regarded as a nondescript tissue, and no specific functional role has been assigned to it.

Interestingly, although the subepithelial stroma of the ciliary body merged almost imperceptibly with that of the choroid and iris when studied by light microscopy, a conspicuous immunopositive layer was noticed just beneath the ciliary epithelium with MAb HNK-1. At the light microscopic level, it was impossible to definitely assign the positive reaction to either of these. Indeed, the stroma of the ciliary body has been identified by any known immunohistochemical or ultrastructural methods.

The HNK-1 epitope has, indeed, been identified both on glycoproteins of cell membranes and extracellular matrix components. In specimens from older adults, in which the stroma of the ciliary body is largely hyalinized, the immunoreaction was definitely too extensive to be solely attributable to membrane staining of the relatively scarce stromal cells. Because it is unlikely that the ciliary epithelium would be capable of maintaining this rather extensive immunoreaction by secretion, we postulate that it may be formed by a hitherto unidentified cell population, the subepithelial matrix cells, which are resident in the inner connective tissue layer of the human ciliary body.

Although the stromal cells of the ciliary body have usually been believed to be fibroblasts, which generically express the vimentin type of intermediate filament, the stromal cells that seemed to react with MAb HNK-1 and VC1.1 were not labeled with antibodies to vimentin in the adult eyes studied, nor were they immunopositive for cytokeratins or desmin. However, in all fetal eyes studied, an obvious overlap of cells reacting for the HNK-1 epitope and vimentin was seen in the inner connective tissue layer.

The appearance of the HNK-1 epitope in the ciliary body logically followed its morphologic development. The epitope was first observed in the inner connective tissue layer by the 20th gestational week, definitely later than the ciliary processes started their development during the 12th week. Likewise, although the pars plana starts to form during the 24th gestational week, it was already well developed when the HNK-1 immunoreaction appeared within this region. The development of the ciliary muscle is also well under way at the time of appearance of the HNK-1 epitope, because the muscle fibers could be identified by the 12th gestational week by electron microscopy and they reacted with antibodies to α-smooth muscle actin during the 13th week. However, expression of the HNK-1 epitope coincided with the appearance of a positive immunoreaction for desmin within the ciliary muscle and thus, possibly, its biochemical maturation.

The embryonic origin of the ciliary muscle and stroma remains somewhat disputed. Developmentally, the ciliary epithelia derive from the optic vesicle, whereas stromal and muscle cells of the ciliary body, the corneal endothelial and trabecular cells, and part of the corneal and scleral keratocytes are now thought to develop from cranial neural crest, by analogy to birds and reptiles. Although the HNK-1 epitope has been suggested to be a marker of neuroectodermal cells, many of the above elements did not react with MAb HNK-1 and VC1.1, and the immunoreaction in the inner connective tissue layer is unlikely to be simply a reflection of its supposed neuroectodermal ancestry. The retinal pigment epithelium has been assigned a central role in the development of the retina and the choriocapillaris. That the positive HNK-1 immunoreaction first appeared adjacent to the pig-
mented ciliary epithelium is consistent with the theory that the latter induces and organizes its development.

Our findings strongly suggest that the inner connective tissue layer of the ciliary body, as identified by antibodies to the HNK-1 carbohydrate epitope, is a distinct, previously unrecognized element of the human eye. Its physiologic functions, if any, remain speculative. Lack of desmin and α-smooth muscle actin in this area would seem to preclude a contractile function. This layer might transmit the force of the ciliary muscle to the zonular fibers and play a mechanical role in accommodation. It might also physically stabilize the ciliary processes and zonular attachment region.

Both of these functions might be related to the fact that the HNK-1 epitope is common to many cell adhesion molecules. Alternatively, its close apposition to the ciliary epithelium might denote a supportive role in the secretion of aqueous humor. These important questions remain to be answered after further studies.

Key words
HNK-1 carbohydrate epitope, ciliary body, embryonic development, cell adhesion, humans

Acknowledgments
The authors thank Mrs. Marjatta Koikkalainen, Mrs. Sirkka Elomaa, and Mrs. Pirkko Yliharju for their expert technical assistance.

References
22. Bunn PA Jr, Linnoila I, Minna JD, Carney D, Gazdar AF. Small cell lung cancer, endocrine cells of the fetal
bronchus, and other neuroendocrine cells express the Leu-7 antigenic determinant present on natural killer cells. Blood. 1985;65:764–768.


