The HNK-1 Epitope and the Elastic Fiber System of the Human Ciliary Body

An Immunoelectron Microscopic Study

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Purpose. To localize at the electron microscopic level the cell adhesion–related HNK-1 carbohydrate epitope in the inner connective tissue layer (ICTL) of the human ciliary body.

Methods. Seven specimens representing the pars plicata (age range, 12 to 86 years) and three specimens representing the pars plana (age range, 29 to 71 years) of the ciliary body were sampled at death from eight normal human eyes. Three additional specimens from the pars plicata were taken from three eyes with exfoliation syndrome (age range, 78 to 81 years). All specimens were embedded in LR white resin and studied by postembedding immunogold labeling using two monoclonal antibodies (mAbs) HNK-1 and NC-1 in detecting the HNK-1 epitope.

Results. In the ICTL of the pars plicata and the pars plana, mAbs HNK-1 and NC-1 constantly bound to the surface of fibroblast-like cells present in the subepithelial connective tissue matrix. The immunoreaction was localized along the cell membrane, both around the cell body and around its long, slender cytoplasmic processes. Long microfibrillar bundles, which consisted of ~10-nm thick microfibrils in close association with these subepithelial matrix cells and elastic fibers, were also labeled. The periphery of elastic fibers was likewise immunolabeled for the HNK-1 epitope. The reaction pattern was essentially the same, regardless of age and presence or absence of exfoliation syndrome.

Conclusions. At the ultrastructural level, the HNK-1 epitope in the ICTL is a common denominator to the subepithelial matrix cells, microfibrillar bundles, and elastic fibers. This suggests that the subepithelial matrix cells secrete this epitope, and that molecules bearing it may be involved in joining these connective tissue elements that structurally stabilize the ciliary body. Invest Ophthalmol Vis Sci. 1997; 38:1426-1433.
FIGURE 1. Light microscopic immunohistochemistry (A) and routine electron microscopy (B, C) of the ICTL of the human ciliary body. (A) Immunoreaction with monoclonal antibody HNK-1 to the HNK-1 epitope distinctly defines the ICTL against the ciliary muscle and the base (arrow) of the iris. (B) A slender subepithelial matrix cell, which resembles a fibroblast, is situated in the ICTL within a collagenous matrix (subject's age, 40 years). (C) A microfibrillar bundle (arrow), which consists of ~10-nm thick microfibrils, is seen in close association with a subepithelial matrix cell (subject's age, 46 years; ict = inner connective tissue layer; cm = ciliary muscle; ir = iris; mc = matrix cell; c = collagen fibers. Original magnification, (A) $\times$120, (B) $\times$7100, (C) $\times$16,400.
and the molecules bearing it in the ICTL are still unknown. It is also impossible to assign definitely the immunoreaction to the cell membrane, to the extracellular matrix, or to both, either by light microscopy or from available ultrastructural data. 12,15 This study was performed to determine at the electron microscopic level where the cell adhesion-related HNK-1 epitope in the ICTL of the human ciliary body is situated, to gain insights into its possible physiologic function.

MATERIALS AND METHODS

Specimens

Specimens representing the pars plicata of the human ciliary body were obtained at the time of autopsy from seven eyes (age range, 12 to 86 years; five men, two women) and specimens representing the pars plana from three eyes (age range, 29 to 71 years, one man and 2 women), respectively. Three additional specimens of the pars plicata were taken from three eyes with exfoliation syndrome, but no history or morphologic evidence of glaucoma (age range, 78 to 81 years, 1 man and 2 women). All specimens came from eyes used as donors for corneal transplantation. The corresponding patients had no other known ocular diseases, and their deaths had resulted from major injuries, cerebral vascular accidents, or metastatic cancer.

Corneal specimens taken from two eyes (ages, 43 and 88 years; one man, one woman) were studied as negative control subjects for the HNK-1 epitope. The ciliary epithelium was used as an internal positive control subject for the HNK-1 epitope. 14,16 Routinely processed specimens embedded in Epon and contrasted with uranyl acetate–lead citrate were used for comparative purposes, owing to their better preserved ultrastructure. The tenets of the Declaration of Helsinki were followed. Experiments were conducted according to the guidelines of the local ethical committee.

Antibodies

Two mouse monoclonal antibodies, mAb HNK-1 (Leu-7; IgM, Becton Dickinson, San Jose, CA, diluted 1:100) 17 and mAb NC-1 (CD57; IgM, Immunotech, Marseille, France; diluted 1:100) 18 were used to detect the HNK-1 epitope. A secondary goat antiserum, detecting both IgG and IgM, that was conjugated to 10 nm colloidal gold particles (Bio Cell, Cardiff, UK; diluted 1:30) was used as a secondary antibody. An unrelated mouse IgM mAb to CK 14 (CK B1; Sigma, St. Louis, MO; diluted 1:100) was used as a control to exclude nonspecific binding of IgM antibodies.

Immunoelectron Microscopy

The specimens were fixed within 45 minutes to 8 hours after death in a freshly made solution of 4% (wt/vol) paraformaldehyde and 0.1% (vol/vol) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 hours at 4°C. After being rinsed in the same buffer containing 3.7% (wt/vol) saccharose, the specimens were sequentially dehydrated in 30%, 50%, and 70% ethanol (vol/vol) at -20°C, and embedded in LR white resin (medium grade) for 24 hours at 45°C. Ultrathin sections were cut and mounted on uncoated nickel grids.

The grids were rehydrated in drops of TRIS–buffered saline (TBS, pH 7.4) and then floated on drops of TBS containing 0.05 M glycine. Nonspecific binding of antibodies was blocked by floating the grids in TBS containing 0.5% (wt/vol) ovalbumin (Sigma) and 0.5% (vol/vol) teleost fish gelatin (Sigma) between each incubation step. The grids were first incubated on drops of the primary antibody diluted in TBS containing 0.5% (wt/vol) ovalbumin (Sigma) and 0.5% (vol/vol) teleost fish gelatin (Sigma) overnight at 4°C. They were then incubated on drops of the gold-conjugated secondary goat antiserum IgG + IgM antibody diluted in TBS containing ovalbumin and fish gelatin for 1 hour at room temperature. Finally, the grids were rinsed in distilled water, stained with uranyl acetate for...
FIGURE 3. Binding to other elements of the human ciliary body of mAb NC-1 against the HNK-1 epitope, seen by immunoelectron microscopy. (A) Gold particles are found at the periphery of elastic fibers in the ICTL. The elastin core is not labeled (subject’s age, 12 years). (B) No immunolabeling is seen in collagen fibers in the ICTL (subject’s age, 12 years). (C) Gold particles are seen within the basement membrane of the endothelial cell of a blood vessel in the ICTL. A red blood cell is present in the vessel lumen (subject’s age, 75 years). (D) The basement membrane of the pigmented ciliary epithelium is immunostained for the HNK-1 epitope (subject’s age, 12 years; e = elastic fiber; c = collagen fibers; bm = basement membrane; ec = endothelial cell; rbc = red blood cell; pe = pigmented epithelium. Original; magnifications: (A) X42,100; (B) X41,400; (C) X45,300; (D) X40,100).

RESULTS

The ICTL was defined as the stromal layer between the ciliary epithelia and the ciliary muscle. By light microscopy, immunoreaction for the HNK-1 epitope sharply defines the ICTL against the ciliary epithelia, the ciliary muscle, the iris (Fig. 1A), and the choroid.

Electron Microscopy

By transmission electron microscopy, the ICTL contained numerous melanocytes, macrophages, blood vessels, and ciliary nerves embedded in a matrix of collagen and elastic fibers. Collagen was present throughout the ICTL. Elastic fibers were more prominent in the outer portion of the ICTL near the ciliary muscle than in the inner portion under the ciliary epithelium and in the pars plana than in the pars plicata.
In addition, cells that ultrastructurally resembled fibroblasts (Figs. 1B, 1C) and were characterized by long slender cytoplasmic processes that extended from the cell body were always present. These cells were surrounded by a fragmentary basement membrane, and they corresponded in location to the subepithelial matrix cells identified in the ICTL by light microscopic immunohistochemistry. Most of them were located in the inner portion of the ICTL of the pars plicata. Finally, long microfibrillar bundles that consisted of ~10-nm thick microfibrils were present in the ICTL (Fig. 1C). These bundles were often found immediately adjacent to the subepithelial matrix cells and to elastic fibers. Similar bundles were identified close to blood vessels and adjacent to the basement membrane of the pigmented ciliary epithelium.

**Immunoelectron Microscopy Using Antibodies to the HNK-1 Epitope**

Monoclonal antibodies HNK-1 and NC-1 to the HNK-1 epitope constantly bound along the surface of the subepithelial matrix cells in the ICTL of the pars plicata and the pars plana (Figs. 2A, 2B). The gold particles were situated along the cell membrane in regions where fragmentary basement membrane was present. The labeling highlighted microfibrils that inserted on the cell surface (Figs. 2A, 2B). Similar immunoreaction was present both around the cell bodies and along their slender cell processes. Some subepithelial matrix cells were surrounded by a particularly heavy immunolabeling (Fig. 2A), whereas others were only focally immunostained. Gold particles were never present in their cytoplasm or in the nucleus (Figs. 2A, 2B). No immunostaining was seen when the primary antibody was omitted or substituted with an unrelated IgM mAb CK B1 (Fig. 2C).

In addition to the periphery of the subepithelial matrix cells, the microfibrillar bundles were consistently labeled with mAb HNK-1 and NC-1 (Figs. 2D, 2E, 2F). Gold particles were regularly present on these bundles in all specimens studied. Additionally, gold particles were present along elastic fibers coinciding with the mantle of microfibrils (Figs. 2E, 2F, 3A) that surrounded the unstained amorphous elastin core. Monoclonal antibodies HNK-1 and NC-1 did not bind to the collagenous matrix (Fig. 3B), melanocytes, and macrophages. However, some gold particles bound to the region of the basement membrane of the endothelial cells of blood vessels in the ICTL (Fig. 3C). Many gold particles also coincided with the basement membrane of both the nonpigmented and the pigmented (Fig. 3D) ciliary epithelium in all specimens studied. Additional cytoplasmic labeling was present in the pigmented, but not in the nonpigmented, ciliary epithelial cells. No immunoreaction was seen in the two corneal specimens studied as control subjects.

The immunoelectron microscopic findings were basically identical regardless of the age of the subject. However, with increasing age less subepithelial matrix cells were seen, and they were more focally immunostained with mAbs HNK-1 and NC-1. Instead, the immunoreaction associated with the microfibrillar bundles, the mantle of elastic fibers, and the basement membrane of the ciliary epithelia remained identical. No qualitative difference in the immunoreaction pattern could be detected in the ICTL of eyes with exfoliation syndrome compared with that of normal eyes, except that exfoliation deposits on the nonpigmented ciliary epithelium were labeled with mAbs HNK-1 and NC-1.

**DISCUSSION**

By light microscopy the HNK-1 epitope is associated with stellate cells that are restricted to the ICTL of the ciliary body and are designated subepithelial matrix cells. By immunoelectron microscopy the immunoreaction was localized to the surface of these cells, both around their cell bodies and along their long slender cytoplasmic processes. These stellate cells resembled fibroblasts in ultrastructure. Like fibroblasts of the uveal tract, they had patches of basement membrane on their surface. However, the subepithelial matrix cells differ from fibroblasts present among the ciliary muscle fibers, in the iris, and in the choroid because of their antigenic profile. The former are selectively identified by antibodies to the HNK-1 epitope. They also have failed to react to vimentin, the intermediate filament usually present in fibroblasts. Therefore the subepithelial matrix cells form a distinct cell population in the ciliary body stroma. So far we lack the method to identify the subepithelial matrix cells in species other than humans, because the HNK-1 epitope could not be detected in the ICTL of the studied animal species with the available antibodies.

The structures most prominently labeled with antibodies to the HNK-1 epitope in the ICTL were numerous microfibrillar bundles. These consisted of ~10-nm thick microfibrils. Such microfibrillar bundles have previously been reported to be part of the elastic fiber system of the ciliary body, and they have been termed immature elastin, isolated bundles of tubular microfibrils without elastin, or oxytalan fibers. By immunoelectron microscopy, the HNK-1 epitope links these bundles to the subepithelial matrix cells.

Elastic fibers consist of an amorphous elastin core within a mantle of microfibrils. This sheath of elastic microfibrils was immunolabeled for the HNK-1 epitope throughout the ICTL, whereas no immunoreaction was present in the amorphous elastin core. These immunolabeled microfibrillar bundles and elastic fibers correspond excellently to the long, coarse fibers...
Microfibrillar bundles were seen in close association not only with subepithelial matrix cells and elastic fibers, but also with the basement membrane of the pigmented ciliary epithelium and the basement membrane of the endothelial cells of blood vessels. A similar arrangement has been reported previously in the bovine ciliary body.\(^22\) The HNK-1 epitope was to some extent present in all of these structures. This elastic fiber system in the ciliary body stroma thus seems to form an interconnecting structure in which the HNK-1 epitope is involved (Fig. 4).

The elastic fiber system of the ICTL has been reported to extend from the ciliary body to the choroid, beyond the level of the ora serrata.\(^23\) Morphologically similar elastic and oxytalan fibers are also found among ciliary muscle fibers.\(^22\) In contrast, the HNK-1 epitope is strictly limited to the ICTL. The immunoreaction for the HNK-1 epitope ends sharply at the ora serrata, at the base of the iris, and at the border of the ciliary muscle.\(^11\) This strictly limited localization of the cell adhesion–related HNK-1 epitope to the elastic fiber system of the ICTL is most conspicuous and probably must be attributed to the presence of a specific cell type, the subepithelial matrix cell. Fibroblasts in the ciliary body have been shown to synthesize elastin at least in chick embryos.\(^24\) Conversely, fibroblasts derived from human skin have been able to synthesize the HNK-1 epitope in cell culture.\(^25\) It is thus reasonable to suggest that the microfibrillar bundles, elastic fibers, and the HNK-1 epitope associated with them in the ICTL are all synthesized by the subepithelial matrix cells that ultrastructurally resemble fibroblasts.

With the subject’s increasing age the subepithelial matrix cells were more focally labeled for the HNK-1 epitope. The epitope persisted unaltered in other ocular cell types. By light microscopy, the immunoreaction pattern of the HNK-1 epitope in the ICTL was found to change to a somewhat more granular one in eyes with exfoliation syndrome.\(^13\) The reason for this was not apparent by immunoelectron microscopy. However, only three eyes with exfoliation syndrome were studied.

In conclusion, the HNK-1 carbohydrate epitope is a common denominator of the subepithelial matrix cells and the elastic fiber system of the ICTL of the human ciliary body. Because the HNK-1 epitope has been associated with cell-to-cell and cell-to-extracellular matrix adhesion,\(^9,10,26\) one might suggest that it is involved in maintenance of normal tissue organization, architecture or integrity in the ciliary body. It might aid in connecting its various structural elements to provide stability for accommodation or secretion of aqueous humor. In this regard, no change has been detected in the HNK-1 immunoreactivity of the ICTL in pseudophakic eyes.\(^27\) However, the zonules in pseudophakic eyes remain intact and the elastic fiber system may still be involved in transmitting the mechanical forces of the ciliary muscle to them.

**Key Words**

CD57, connective tissue, elastic fibers, exfoliation syndrome, fibroblast, inner connective tissue layer, leu-7, microfibrils, subepithelial matrix cell
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References