Retinal Adhesiveness Is Weakened by Enzymatic Modification of the Interphotoreceptor Matrix In Vivo

Xiao-Ying Yao,* Gregory S. Hageman,† and Michael F. Marmor*

The role of interphotoreceptor matrix (IPM) constituents in mediating adhesion between the retina and retinal pigment epithelium (RPE) was investigated by injecting specific enzymes into rabbit eyes either intravitreally or subretinally. Retinal adhesiveness was measured by peeling the retina from the pigment epithelium 1–3 days later and observing the amount of adherent pigment. Effects of enzymes on the IPM were monitored by observation of peanut agglutinin (PNA) binding to cone matrix sheaths; retinal and RPE toxicity was excluded by electroretinography and histology. Three enzymes that degrade glycosaminoglycans or saccharides known to be constituents of the IPM (chondroitinase ABC, neuraminidase, and testicular hyaluronidase) both weakened adhesion and altered PNA binding, although the effects on the cone matrix sheaths were different for each enzyme. An enzyme specific for hyaluronic acid (Streptomyces-derived hyaluronidase), which has not been identified as a major IPM constituent, had no effect on either adhesion or PNA binding. The authors conclude that IPM-associated glycoconjugates participate in retinal adhesion, although their precise composition, interaction with IPM components, and relationship to other mechanisms of adhesion remain to be determined.


In the normal eye, the retina adheres firmly to the retinal pigment epithelium (RPE) although the mechanisms of adherence are not completely understood.1 Previous studies suggest that retinal adhesion depends on many factors, including metabolic transport processes,2,3 intraocular and osmotic pressures,4,5 physical interdigitation between the photoreceptors and RPE cells,6 environmental conditions,7 and the properties of the interphotoreceptor matrix (IPM).8,9

The IPM has been considered to have viscous adhesive properties,9 and recent studies suggest that regionalized constituents of the IPM, which are associated with cone photoreceptors,10–12 may participate in retinal adhesion.13–17 These “cone matrix sheaths” are firmly attached to both retina and RPE and become elongated when these layers are peeled apart; furthermore, disruption of the sheaths with xylosides (which interfere with proteoglycan synthesis) results in localized retinal detachments. There may also be other IPM constituents (eg, rod sheathing material) which participate in retinal adhesion.13

Cone matrix sheaths selectively bind the lectin peanut agglutinin (PNA)10,11 and are largely composed of aqueous-insoluble chondroitin sulfate-containing proteoglycan.12 Hyaluronic acid has not been identified as a major constituent of the IPM.18,19 In this study we injected enzymes that specifically degrade some of these molecules (chondroitinase [CA], neuraminidase [NA], and testicular [TH] and Streptomyces-derived hyaluronidases [SH]) into the vitreous or subretinal space of rabbits in vivo to evaluate their effect on retinal adhesiveness and IPM structure. We monitored the structure and function of the retina and RPE after these injections to exclude enzyme damage.

Materials and Methods

Animals

These experiments conform to the ARVO Resolution on the Use of Animals in Research and to the NIH “Guide for the Care and Use of Laboratory Animals” (NIH Publication 86-23). All studies were done on pigmented Dutch rabbits, anesthetized with xylazine (10 mg/kg), acepromazine maleate (1...
mg/kg), and ketamine (100 mg/kg). The eyes were enucleated before euthanasia to obtain tissue for the measurement of retinal adhesion or for cytochemistry.

**Reagents**

We used the following reagents: (1) NA, types V and X, from Clostridium perfringens (type X is a further purification by affinity chromatography of NA type VIII; Sigma, St. Louis, MO); (2) TH, type 1-S from bovine (Sigma); (3) SH, type IX (Sigma); (4) CA, protease-free from Proteus vulgaris (Seikagaku Kogyo, Tokyo, Japan); (5) Hanks' solution (Gibco, Grand Island, NY), for subretinal and some intravitreal injections and for associated peeling experiments; (6) phosphate-buffered saline (PBS), for intravitreal injections. The medium RPMI-1640 (Gibco) was used as the bath for peeling experiments after intravitreal injections. All enzyme doses are expressed as units.

**Enzyme Administration**

Enzymes were administered in two different ways. Intravitreal injection: in each rabbit, one eye was injected with enzyme (Table 1 for doses) dissolved in 30–50 μl of PBS or Hanks' solution (both at pH 7.4). The fellow eye was injected with 30–50 μl of PBS or Hanks' without enzyme as a control. Injections were made with a 30-G needle inserted just posterior to the limbus.

Subretinal injection: we injected the animals by methods for subretinal injection that have been described previously. In brief, a glass micropipette with tip diameter of 40–50 μm was passed through a scleral slit near the limbus and across the vitreous until the tip just penetrated the central retina. Air pressure through the micropipette forced 3–4 μl of Hanks' solution with or without enzyme into the subretinal space (Table 2 for doses), creating a small retinal detachment of approximately 3–4 mm in diameter. In each animal, enzyme dissolved in Hanks' solution was injected into one eye and Hanks' solution alone was injected into the fellow eye as a control.

**Table 1. Summary of intravitreal injections**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% Adhesiveness*</th>
<th>PNA binding†</th>
<th>ERG‡</th>
<th>Light microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>100</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Neuraminidase (Type V, 0.1 U/eye)</td>
<td>20</td>
<td>Diffuse</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Testicular hyaluronidase (Type I-S, 100 U/eye)</td>
<td>—</td>
<td>Distal cone sheath disruption</td>
<td>Normal</td>
<td>Normal</td>
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<tr>
<td>* Measured as the percentage of retina that retained adherent pigment after peeling; these are characteristic values obtained 1 or 3 days after intravitreal injection.</td>
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<tr>
<td>PNA binding† measured as the percentage of whole retina that retained adherent pigment after peeling; these are characteristic values obtained 1 or 3 days after subretinal injection.</td>
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<td>ERG‡ Electroretinograms (a-, b-, and c-waves).</td>
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<td>Light microscopy SEM§ Scanning electron microscopy of photoreceptors and RPE surfaces, away from direct site of enzyme injection.</td>
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**Table 2. Summary of subretinal injections**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% Adhesiveness*</th>
<th>PNA binding†</th>
<th>ERG‡</th>
<th>Light microscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>100</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Neuraminidase (Type X, 1.7 U/ml)</td>
<td>90</td>
<td>Diffuse</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Testicular hyaluronidase (Type I-S, 4200 U/ml)</td>
<td>90</td>
<td>Distal cone sheath disruption</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Protease-free chondroitinase ABC (0.02 U/ml)</td>
<td>—</td>
<td>—</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>(0.2 U/ml)</td>
<td>—</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Streptomyces hyaluronidase (0.2 U/ml)</td>
<td>—</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>* Measured as the percentage of whole retina that retained adherent pigment after peeling; these are characteristic values obtained 1 to 3 days after subretinal injection.</td>
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<td>† Peanut agglutinin binding to interphotoreceptor matrix.</td>
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<tr>
<td>‡ Electroretinograms (a-, b-, and c-waves).</td>
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<td>§ Scanning electron microscopy of photoreceptors and RPE surfaces, away from direct site of enzyme injection.</td>
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Electroretinograms

Direct-current electroretinograms (ERGs) were recorded between the cornea and a reference on the sclera, using Ag-AgCl electrodes, as described previously. Light stimulation was provided by a quartz bulb focused through a fiber optic light guide that terminated 1 cm from the eye and delivered 2000 lux to the cornea in 1-sec flashes. The ERGs were recorded from at least three eyes under each condition studied; baseline recordings were obtained before the enzymes were given, and then the measurements were repeated after the intravitreal or subretinal injections.

Light Microscopy and PNA Cytochemistry

Eyes were enucleated and rapidly cut open with a razor blade, and the eyecup (including attached retina) was fixed for 3 hr in freshly prepared 4% paraformaldehyde in 10 mM sodium cacodylate buffer, pH 7.4. The tissues were then transferred to 10 mM sodium cacodylate buffer for 12-18 hr and embedded in acrylamide. For light microscopy, sections were cut on a microtome and stained with hematoxylin and eosin. For cytochemistry, the tissue was sectioned on a cryostat at —20°C as described previously. To block nonspecific binding sites, the sections were exposed for 15 min to PBS containing 1 mM MgCl₂, 1 mM CaCl₂ (PBS/M/C), and 1% globulin-free bovine serum albumin. After rinsing in PBS/M/C, the sections were incubated in a solution of 5 µg/ml fluorescein-conjugated PNA in the same buffer, after which the sections were rinsed and observed subsequently by epifluorescence microscopy on an Olympus (Tokyo, Japan) Vanox light microscope. All images were recorded on Tri-X film (Eastman Kodak, Rochester, NY) at an exposure index of 800.

Measurement of Retinal Adhesion

The degree of adherence of RPE pigment to the retina after peeling from the RPE in vitro was used as a quantitative index of adhesiveness; this technique has been shown previously to be a reliable indicator of the degree of adhesion. In brief, eyes were enucleated rapidly, eyecups were prepared, and the tissue was cut into strips that included the areas of interest (eg, experimental detachments). The retina was peeled manually from the RPE under Hanks’ or RPMI solution at 37°C. The time from cutting the optic nerve to peeling the retinas was less than 48 sec, so that all observations were made within 1 min; speed is important since retinal adhesiveness weakens rapidly after enucleation. The strength of retinal adhesion was estimated by measuring the area of retina that retained adherent RPE pigment using an International Business Machines (Boca Raton, FL) computer video image analysis system (100%, firm adhesion; 0%, weak adhesion).

Scanning Electron Microscopy

After peeling, pieces of retina and RPE-sclera were pinned to sheets of wax to expose the apical surfaces of the RPE and neural retina. The tissues were fixed at room temperature for 30 min and overnight at 4°C with 1.25% glutaraldehyde and 1.0% paraformaldehyde in 0.072 mM sodium cacodylate buffer, pH 7.4. The tissues were dehydrated in a graded series of ethyl alcohol solutions, critical point dried, sputter coated with gold, and examined using an ISI-40 scanning electron microscope (SEM).

Results

Enzyme Effects on Retinal Adhesion and IPM Cytochemistry

Intravitreal injection of enzymes: Table 1 summarizes the experiments and lists the doses of enzymes that were used under each condition. Examination of the fundi ophthalmoscopically before enucleation showed no abnormalities, but fine retinal folds, indicating low retinal adhesion, were observed after opening some of the NA- and TH-treated eyes. These two enzymes caused a dramatic decrease in retinal adhesiveness, whereas SH had no effect (Fig. 1).

The effects on adhesion caused by NA and TH correlated with changes in PNA-binding in the IPM (Fig. 2). In normal eyes, or control eyes that were injected with Hanks’ solution, PNA bound intensely to the cone matrix sheaths, with no binding elsewhere in the IPM (Fig. 2A). However, in NA-treated eyes
Fig. 2. Fluorescence light micrographs depicting binding of PNA to rabbit retinas in the region between the RPE and outer plexiform layer (OPL). (A) In the normal eye, PNA bound intensely and specifically to cone matrix sheaths (arrows), which are domains of IPM associated with cone outer segments and ellipsoids. (B) After intravitreal injection of neuraminidase, PNA bound homogenously throughout the interphotoreceptor matrix, and the binding in the region of cones appeared to be diminished (note gaps indicated by arrows). A similar pattern of binding was observed after subretinal injection of neuraminidase. (C) After intravitreal injection of testicular hyaluronidase, cone matrix sheaths were disrupted, especially at their apices (asterisks), and shallow separations between the photoreceptors and RPE were commonly observed.

(Fig. 2B), PNA binding was observed throughout the IPM, except for gaps (arrows) which may represent sites of cone matrix sheaths or cone inner segments. In eyes treated with TH (Fig. 2C), the cone matrix sheaths bound PNA but were disrupted at their distal tips, and a shallow separation was observed between photoreceptors and RPE. The IPM of eyes given SH showed no changes and appeared normal.

Subretinal injection of enzymes: The purpose of these experiments was to determine whether enzymes would degrade specific IPM constituents and weaken adhesion at locations distant from the site of injection. Table 2 summarizes the doses of enzyme used under each condition. Examination of the fundi before enucleation showed no ophthalmoscopic abnormalities except at the site of the injection, but fine folds were observed over broad areas of retina after opening some of the eyes treated with NA, TH, and CA. Retinas treated with these enzymes were noted to peel off the RPE with unusual ease, consistent with the quantitative measures of reduced adhesion. In fact, some NA- and TH-treated retinas detached spontaneously when placed into Hanks' solution for peeling.

Eyes were enucleated 1–3 days after the injection of enzymes, and the retinas were peeled to measure adhesion. The degree of pigment adherence to retinal whole-mounts is shown in Figure 3. The Hanks' injected control eyes had firm pigment adherence to the retina in all regions of the fundus except at the site of subretinal injection (Fig. 3A). However, after injection with NA and TH, pigment adherence decreased progressively over 3 days in an expanding zone around the site of injection (Fig. 3B). Figure 4 shows graphically that, by 3 days after injection, the overall adherence of the retina was reduced markedly by NA and TH, moderately reduced by the high dose of CA, and was not affected by the low dose of CA or by SH.

These enzyme-induced changes in pigment adherence correlated closely with changes in PNA binding to the cone sheaths. In regions distant from the site of direct enzyme injection, where NA weakened adhesiveness, PNA bound homogenously throughout the IPM (identical to results obtained after intravitreal injection, Fig. 2B). The PNA binding was disturbed (Fig. 2C) in the regions where TH weakened adhesiveness. Protease-free CA had no recognizable effect on the cone-associated binding of PNA at the low dose (which did not affect adhesiveness), but the high dose (which weakened adhesion) eliminated PNA binding to the cone matrix sheaths (Fig. 5). The SH had no effect on PNA binding.

Enzyme Effects on Function and Structure of the Retina and RPE

ERGs: These were obtained 1–3 days after intravitreal and 3 days after subretinal injection of NA, TH, and CA to determine whether these enzymes were damaging to retinal or RPE cells. We observed no significant changes in a-waves, b-waves, or c-waves relative to control recordings made in the same eye before injection. Not only amplitudes (Fig. 6) but also waveforms and implicit times were stable.
Fig. 3. Peeled whole-mount retinas after subretinal injection of various enzymes: the areas of strong adhesion show RPE pigment that stuck to the retina during peeling, whereas the areas of weak adhesion are clear. (A, left) Three days after injection of control (Hanks') solution; adhesion is normal (strong) except at the injection site. (B, below) One, 2, and 3 days after testicular hyaluronidase, showing a progressive increase in the area of weakened adhesion (the injection sites are shown by dashed lines). A similar progression of adhesive loss was observed after neuraminidase.

**Light microscopy and SEM:** We were concerned that the enzymes might be disrupting cellular structures other than IPM, thereby weakening adhesion. For this reason, all of the tissue samples obtained for PNA cytochemistry after intravitreal or subretinal enzyme injection were also examined by light microscopy. With the exception of the subretinal injection site itself, the photoreceptors, RPE, and inner retina appeared morphologically normal. In addition, the peeled photoreceptor and RPE surfaces were examined by SEM in selected eyes (Fig. 7). Eyes were enucleated 3 days after subretinal injection of NA (eight eyes), high-dose CA (four eyes), and TH (three eyes) in the doses shown in Table 2. The enzyme-treated tissues were peeled within 1 min after enucleation, since adhesion was weak and we wished to avoid possible postmortem changes. Control (normal) tissue samples were peeled 4–5 min after enucleation so that adhesion would be comparably weak. The outer segment and RPE microvillous morphology was identical in the enzyme-treated eyes and the controls; we could detect no shearing, swelling, or fragmentation of either the outer segments or the RPE.

**Discussion**

Investigators have speculated for many years about the role of IPM constituents in retinal adhesion. The IPM was first thought to function as a viscous “glue,” but later experiments on retinal adhesion suggested that metabolic and other factors might be equally or more important than IPM viscosity. Furthermore, in vitro experiments with matrix-degrading enzymes showed variable effects on retinal adhesion, possibly because the enzymes had little time to penetrate or act. Recent biochemical and structural studies have shown that the IPM is not an amorphous material but that it has a complex physi-
Fig. 5. Fluorescence light micrographs depicting PNA binding in the regions of the photoreceptors at a distance of 2 to 5 mm from the site of subretinal injection of protease-free chondroitinase ABC. (A) In eyes given a low dose of enzyme, the cone matrix sheaths showed normal binding of PNA (see Fig. 2A). (B) In eyes given a high dose of enzyme, we observed no binding of PNA to the cone matrix sheaths.

Fig. 6. Effect of enzymes on the ERG a-, b-, and c-waves, after intravitreal (left) and subretinal (right) injections.

Our results would be suspect if the enzymes did not cross the retina after intravitreal administration or diffuse within the subretinal space after subretinal administration. However, our lectin cytochemical results indicate clearly that the enzymes not only reached the IPM but affected specific components of it. Our results would also be suspect if the enzymes damaged either the photoreceptors or the RPE, both of which may control other aspects of adhesion. However, light microscopy and SEM showed that the morphology of the outer segments and RPE microvilli was normal in areas where retinal adhesion was decreased, and ERG signals involving both retina and RPE remained normal after enzyme treatment. It is interesting that the c-wave was unaffected by enzyme treatment, since this response depends upon potassium concentration changes in the subretinal space and is sensitive not only to RPE cellular damage but to widening of the subretinal space. Our data indicate that even though enzymes weakened adhesion, spontaneous separation did not occur (at least within the time frame of these experiments). This is consistent with evidence that both active and passive forces work continually to transport subretinal fluid across the RPE independent of retinal adhesiveness.

We found that doses of protease-free CA that weak-
Fig. 7. Scanning electron micrographs of the photoreceptor (left) and RPE (right) surfaces 3 days after subretinal injection of neuraminidase. The enzyme-treated samples were taken roughly 1 mm outside the site of enzyme injection. The surface morphologic findings are normal even though adhesion in this area was sharply reduced. Similar results were obtained after testicular hyaluronidase and chondroitinase ABC. Magnification bar: 1 μm.

ened adhesion caused concomitant disruption and loss of PNA binding in the cone matrix sheaths. Since this enzyme degrades chondroitin sulfate,25 these results suggest that IPM molecules containing chondroitin sulfate participate in retinal adhesion. These data are also consistent with recent evidence that the cone matrix sheaths form a significant structural and molecular bond between the photoreceptors and RPE microvilli.13-17 However, PNA binds to the cone matrix sheaths selectively, and we cannot judge from PNA binding to what degree rod matrix material (that also contains chondroitin sulfate) may be affected or involved with retinal adhesion.

The NA cleaves sialic acid residues from oligosaccharides, and sialic acid-containing glycoconjugates have been identified in the IPM of a number of species including the rabbit.21-26,29 Our finding that NA-treated eyes showed PNA binding throughout the IPM is consistent with the observations of others28-30 and suggests that the removal of sialic acid residues exposes new PNA binding sites throughout the IPM. Since NA weakened retinal adhesion, these sialic acid residues may be involved in the maintenance of adhesion; however, other effects of NA that are not revealed by our cytochemistry could also be relevant to adhesion.

The SH served as a type of control, insofar as hyaluronic acid, which it degrades specifically,31,32 has not been identified as a major constituent of the IPM.18,19 The fact that this enzyme had no effect on either retinal adhesion or PNA binding in the IPM argues against the possibility that the enzymes were producing nonspecific effects.

The TH is not as specific as the other three enzymes; it degrades chondroitin sulfates33 but may also digest protein because the routine commercial preparation is often contaminated with protease (judging by casein hydrolysis assays; Hageman, unpublished data). Nevertheless, the fact that adhesion was weakened simultaneously with cytochemical alteration of the IPM, in the absence of ERG or histologic changes, supports our general conclusions that intravitreal and subretinal enzymes reach the IPM, and that the IPM is involved with adhesion.

Our previous studies4-7 suggested that retinal adhesion is a multifactorial process that depends upon several complementary physical and physiologic systems. This current work does not contradict the earlier findings but indicates that IPM is one of the systems involved. The role of IPM in adhesion may be purely structural (involving bonding and/or viscosity effects) or may involve interaction with ionic and metabolic factors. For example, dehydration of the subretinal space by active RPE fluid transport2,3 could enhance the firmness, viscosity, or bonding properties of the IPM, as could nonmetabolic factors such as temperature, pH, and calcium concentration.2,22 Our cytochemical data show that reduced adhesion from enzyme treatment correlated with changes in PNA binding to the cone matrix sheaths in the IPM, but different patterns of PNA binding were observed for each enzyme. It is possible that PNA binding does not reveal all, or even the most critical, of the IPM components which participate in retinal adhesion.

These cautions notwithstanding, our experiments show clearly that IPM constituents are involved in retinal adhesion and that its adhesive properties can be modified in vivo. Since retina with weakened adhesiveness will be at greater-than-normal risk to de-
tach under the influence of traction or pathologic fluid movements, the IPM may be a site to consider with respect to the mechanisms and management of clinical detachments.

Key words: cone matrix sheaths, interphotoreceptor matrix, peanut agglutinin, retinal adhesion, retinal pigment epithelium

References