Effects on Retinal Adhesion of Temperature, Cyclic AMP, Cytochalasin, and Enzymes

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We have studied factors that contribute to retinal adhesion, by measuring directly the force required to peel rabbit retina from the retinal pigment epithelium (RPE). Exposing the tissue to either testicular hyaluronidase or neuraminidase, or acidifying the medium to pH 6.0, reduced the peeling force within 2 min. Cooling the tissue to 25°C or 4°C increased the peeling force to such a degree that retinal separation occurred by rupture of the RPE cells rather than widening of the subretinal space. Inhibition of the cytoplasmic contractile system with cytochalasin had no significant effect on peeling force, but dibutyryl cAMP (which decreases fluid absorption by the RPE) weakened adhesion. These results suggest that retinal adhesion involves a number of interrelated physical and metabolic factors. Invest Ophthalmol Vis Sci 29:910-914, 1988

The subretinal space, between sensory retina and retinal pigment epithelium (RPE) contains an interphotoreceptor matrix (IPM), composed of glycosaminoglycans (GAGs) and proteins. The viscous properties of this matrix may contribute to adhesion between retina and RPE, although a variety of other mechanisms appear to be involved, including interdigitation between photoreceptor outer segments and RPE microvilli, tamponade by the vitreous gel, hydrostatic and osmotic forces on the retina, and active ionic transport across the RPE which moves fluid out of the subretinal space.

The relative importance of these various factors has not been established. We undertook these in vitro rabbit experiments to evaluate conditions that have selective influence on metabolic transport, subcellular structures or IPM, in an effort to clarify their role in maintaining retinal adhesion.

Materials and Methods

Dutch rabbits weighing 1.0–1.5 kg were used, in a manner that conformed to the ARVO Resolution on the Use of Animals in Research. Animals were initially sedated with 1 mg/kg acepromazine, followed by 50 mg/kg ketamine and 5 mg/kg xylazine intramuscularly. Retrobulbar anesthesia with 2% xylocaine was sometimes added as needed.

We monitored adhesiveness by measuring the force required to peel retina from the RPE in vitro, as described previously. In brief, the posterior segment of an enucleated eye was placed immediately in a Petri dish containing physiologic RPMI solution and a long, 5 mm wide strip was cut from the inferior retina, parallel to the visual streak. Each strip was fixed to a small platform, and the inferior end of the retina was glued with cyanoacrylate to a transducer that measured force as the retina was peeled off at a speed of 24 mm/min.

Experimental agents were either added to the bathing solutions or injected intravitreally before enucleation. Reagents used include: testicular hyaluronidase (type 1-s, from bovine testes; Sigma Chemical Co., St. Louis, MO), neuraminidase (type V, from Clostridium perfringens; Sigma), dibutyryl cAMP (1 mM; Sigma), dibutyryl cGMP (1 mM; Sigma), isobutyl-methylxanthine (IBMX) (0.5 mM; Sigma), cytochalasin B (from Helminthosporium dematioides; Sigma), cytochalasin D (from Zygosporium masonii; Sigma) and dimethylsulfoxide (DMSO; Sigma). To make cytochalasin solutions, 1 mg of cytochalasin was dissolved in 0.8 ml of DMSO and diluted to a volume of 5 ml with Hanks' solution.

Since adhesion falls steadily after death, we always made comparisons between measurements obtained at the same time after death. To minimize variation, we tried as much as possible to compare the effects of changing conditions while peeling the same piece of tissue, or to use the second eyeball from the same animal as control. We excluded experiments in which adhesive force under control conditions did not fall within a normal range.
Results

Matrix-Degrading Enzymes

Testicular hyaluronidase (150 NF units/ml) was added to the peeling bath at 37°C, titrated with 1 N HCl to pH 6.0 to approach the optimal pH for enzyme activity. Neuraminidase (2 × 10^-2 units/ml) was used under the same conditions. (To rule out a pH effect, we used pH 6.0 RPMI as a control solution). As shown in Figure 1, both enzymes weakened adhesion. The mean values between enzyme-treated eyes and control eyes were significantly different (P < 0.01) at every point.

Temperature and pH

We have shown previously that retinal adhesion declines monotonically with time at 37°C and the decline is faster near pH 6.0 than at pH 7.4. Figure 2 shows the effect of lower temperatures on adhesive force at two different pH levels. The peeling force was measured two or three times in the same piece of tissue, 1–1.5 min after changing the temperature of the bathing solution.

Room temperature (25°C) reversed the postmortem decline in adhesion and increased the adhesive force rapidly and reversibly at pH 7.4. The adhesion was strong enough that clean separation between retina and RPE could not be obtained and RPE pigment remained adherent to the retina as the layers peeled apart. At pH 6.0, a condition which is detrimental to adhesion, 25°C temperature failed to increase the adhesive force.

Cooling even further to 4°C at pH 7.4 made the adhesive force so strong that the retina tore or the cyanoacrylate glue failed, and an accurate measurement of peeling force could not be obtained. The 4°C temperature also increased adhesion at pH 6.0, but to a lesser degree than at pH 7.4.
Cytochalasin

Cytoplasmic microfilaments can participate in cell-cell or cell-substratum adhesion and actin filaments have been observed in the RPE microvilli. To study whether microfilaments affect interdigitation with photoreceptor outer segments and adhesion, we used cytochalasin B and D to disrupt filament polymerization. Peeling forces were measured 1-2 hr after intravitreal injection of 0.05-0.1 ml cytochalasin solution, causing about 20 µM of final concentration in the rabbit vitreous. As shown in Figure 3, both cytochalasin B and D caused a very slight decrease in peeling force but the differences were not statistically significant (P > 0.1), compared to control eyes which had received intravitreal injections of 0.05-0.1 ml Hanks' solution with 16% DMSO. We also peeled retinas in the presence of 10 µM cytochalasin B in the bathing solution used after enucleation; the adhesive force was unchanged from normal.

CAMP

CAMP has been found to have an inhibitory effect on retina-to-choroid fluid movement across the RPE. We measured the peeling force from tissue exposed two or three times sequentially to bathing solutions, with or without 1 mM dibutyryl cAMP and 0.5 mM IBMX; control experiments compared solutions with or without 0.5 mM IBMX alone. After incubation for 1 to 2 min in each solution, a portion of the tissue strip was peeled and the bath was changed. Adding or removing IBMX alone had no effect on adhesive force, but dibutyryl cAMP in the bath decreased the peeling force reversibly (Fig. 4). The average force in dibutyryl cAMP solution relative to control solution was 66.5 ± 4.4% (mean ± SEM).

We also injected 0.05 ml Hanks' solution containing 1.5 µM dibutyryl cAMP (but no IBMX) into the mid-vitreous of five eyes, 0.5 to 2 hr before enucleation. Fundus examination was normal, but after enucleation and removal of vitreous we found localized failure of retinal adhesion at the posterior pole (Fig. 5A). The injections were made slowly and carefully, but to exclude the possibility that the effect resulted from the jet stream during injection, or from the dibutyryl moiety, we did the same procedure in nine eyes with Hanks' solution alone and in four eyes with Hanks' solution containing 1.5 µM dibutyryl cGMP. Neither produced any retinal loosening after injection (Fig. 5B). Finally, we studied the peeling force in eyes enucleated 1.5 hr after intravitreal injection with dibutyryl cAMP (three eyes), dibutyryl cGMP (seven eyes) or normal saline (three eyes). No IBMX was given. The peeling force, relative to the saline controls, was nearly 50% lower after cAMP (P < 0.05) but was insignificantly lower (P > 0.1) after cGMP.

Discussion

Hyaluronidase and neuraminidase degrade chondroitin sulfate proteoglycan and sialoglycoconjugates, respectively, which are major components of IPM. (Hyaluronic acid is also degraded by hyaluronidase but is not a major IPM component.) Our results show that these enzymes weaken adhesion, which suggests that IPM plays a role in normal retina-RPE adhesion. However, the mechanism by
which IPM contributes to adhesion remains to be demonstrated, and may involve more than one matrix property, such as viscosity and the action of cell-adhesion molecules. Furthermore, hyaluronidase also liquefies the vitreous and could in theory affect adhesion by altering vitreous support or tamponade effects. In previous experiments, we failed to show an effect from hyaluronidase. This discrepancy might relate to the pH that was used, since hyaluronidase is most effective at pH 4.5–5.5; we used pH 6.0 for the present experiments, but pH 7.4 in our earlier study.

Our finding that temperature reversibly alters retinal adhesivity is consistent with other experiments that showed temperature-sensitive variation in the amount of RPE pigment adherent to separated retina. Low temperature could increase adhesiveness in several ways. Low temperature may increase the viscosity of the IPM, an effect which might be rapidly reversible. It could slow down postmortem degradative processes, but this effect would not be reversible. Temperature might reversibly affect certain properties of the cellular membrane that have been postulated to influence adhesion. Finally, low temperature will inhibit general metabolic processes. Inhibition of RPE transport would seem likely to weaken adhesion in the long run, but we have found in other experiments with ouabain that short-term swelling of the interdigitating photoreceptors and RPE microvilli, as a result of sodium pump inhibition, can increase adhesiveness quite markediy. The mechanisms by which low pH not only weakens adhesion but diminishes the recovery of peeling force by cold temperature, remain to be shown.

The apical microvilli of the RPE are known to contain actin filaments, which may participate in cell-substratum adhesion. Iodoacetate, which decreases chick RPE-substratum adhesiveness through the disassembly of microfilament bundles has been reported to decrease sensory retina-RPE adhesion. However, blocking microfilament function in the present study with cytochalasins, either by intravitreal injection or by intra-bath addition, failed to show a significant effect on the retinal peeling force. Cytochalasin B can affect glucose transport in addition to actin structure, but the results with cytochalasin B were identical with those from cytochalasin D. It is possible that our incubation time was not long enough for cytochalasins to act, or that microfilaments are not a major component of adhesion in this species. We used dibutyryl cAMP rather than cAMP in these experiments since it enters cells more easily and is less susceptible to phosphodiesterase. We have shown previously that metabolic factors contribute to retinal adhesion, possibly through the enhancement of active fluid transport of subretinal fluid. RPE transport processes can be inhibited by hypoxia, metabolic inhibitors and cAMP, and our results with cAMP might be explained by this same mechanism. The gross loosening of the retina after intravitreal injection of cAMP (Fig. 5) is especially suggestive of a shift in the direction of fluid movement across the RPE. These effects cannot be a result of either IBMX or the dibutyryl moiety since neither IBMX nor dibutyryl cGMP were effective. The lack of an opposite cGMP effect is also intriguing insofar as cGMP often has actions that oppose cAMP, and cGMP does act on the RPE to enhance fluid transport.
Although our experiments implicate IPM and metabolic activity in adhesion, they neither rule out other factors, nor demonstrate that one single factor is dominant or independent of the others. For example, the physical characteristics of IPM might be dependent upon fluid transport by the RPE. It appears that retinal adhesion is maintained by a combination of metabolic and physical forces which are undoubtedly interrelated in the living state.

**Key words:** retinal pigment epithelium, retinal adhesion, cytochalasin, cyclic AMP, interphotoreceptor matrix

**References**