

N-Cadherin Expression in a Rat Model of Retinal Detachment and Reattachment

Hui-Jin Chen and Zhi-Zhong Ma

PURPOSE. To observe the changes in N-cadherin expression in the retina after experimental retinal detachment (RD) and reattachment in the rat and to explore the role N-cadherin might play after RD.

METHODS. Forty rat retinas were detached by transscleral injection of 1.4% sodium hyaluronate into the subretinal space. The eyes were enucleated at different time intervals ($n = 5$), followed by fixation, embedding, and sectioning. The differences in N-cadherin expression in the normal retina, detached retina, and spontaneously reattached retina were determined. Furthermore, an N-cadherin antagonist was injected in combination with 1.4% sodium hyaluronate into the subretinal space in another 10 eyes, in an attempt to demonstrate the role N-cadherin plays after RD.

RESULTS. N-cadherin was not expressed in the RPE layer of the normal rat retina. After RD, intense immunolabeling of N-cadherin was seen in the RPE cells, the photoreceptors, and the outer limiting membrane (OLM). An increasing number of cytokeratin (CK)-positive cells likely to be RPE cells was found attached to the outer surface of the detached neural retina. Where the retina was reattached, the N-cadherin immunolabeling rapidly decreased. In eyes treated with an N-cadherin antagonist, the retinas appeared thinner than that in eyes without treatment, and the photoreceptor nuclei showed significantly loss. Moreover, CK-positive cells attached to the outer surface of the detached retina were markedly fewer in number.

CONCLUSIONS. Increased expression of N-cadherin in the RPE cells, the photoreceptor cells, and the OLM of the retina after RD may contribute to RPE cell migration and photoreceptor survival. These changes could be reversed by retinal reattachment. (*Invest Ophthalmol Vis Sci.* 2007;48:1832-1838) DOI:10.1167/iovs.06-0928

Retinal detachment (RD) is a separation of the neurosensory retina from the retinal pigment epithelium (RPE). A series of events occur rapidly at the RPE-photoreceptor interface after RD.¹ Some of the changes are degenerative,² whereas others are proliferative.^{3,4} RD threatens visual function not only by inducing photoreceptor cell death, but also by the occurrence of secondary proliferative vitreoretinopathy (PVR). It has generally been accepted that chronic RD leads to PVR. However, the exact pathogenesis is not completely understood.^{5,6} The molecular alterations that drive the process from RD to PVR has been a subject of intensive investigation.

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A network of growth factors have been implicated in the development of PVR.⁷ Among these factors, platelet-derived growth factor (PDGF) has long been known as a potent chemotactic and mitogenic factor in RPE cells,⁸ a major constituent of the epiretinal membrane (ERM) in PVR. It has been suggested recently that the PDGF α receptor (α PDGFR) plays a pivotal role in PVR, since cell lines that do not express α PDGFR or express mutant α PDGFR gene have a poor potential for inducing PVR in a rabbit model.⁹⁻¹¹ Hepatocyte growth factor (HGF) is another growth factor that recently has been under intensive investigation. HGF is also known as scatter factor (SF), due to its ability to promote the dissociation or scattering of formed colonies of cultured epithelium.¹² It has been shown that human RPE cells respond to HGF/SF by epithelial-mesenchymal transformation and by cell migration.¹³ Also, tyrosine phosphorylation of HGF receptor (HGFR or c-Met) was demonstrated in a PVR model in the presence of IL-1.¹⁴ However, these observations are mainly dependent on the animal model of PVR, which was induced and accelerated by injection of either rabbit conjunctival fibroblasts or proinflammatory factors. Thus, these models are not suitable for investigation of the molecular events in the early phase of PVR. In 1975, Machemer and Laqua³ recognized that mitotically inactive RPE cells began to enter a phase of DNA synthesis 12 to 24 hours after RD. Therefore, they proposed that this animal model of RD could provide us with a good tool to investigate PVR. Thereafter, numerous studies demonstrated that RD could start RPE and glial proliferation. However, little is known about the molecular events that underlie other process such as RPE migration after RD.^{4,15-17} Besides, these studies did not show whether the events induced by RD could be reversed by retinal reattachment. In addition, it is proposed that it is the healing process of the retinal breaks that trigger the onset of PVR, because PVR occurs more often after rhegmatogenous RD than after exudative RD, even though the latter has more severe inflammation.¹⁸ It is not known whether the simple separation without retinal breaks would initiate the early stage of PVR. Based on these considerations, we fashioned an animal model of RD without retinal breaks.

Adhesion molecules play an essential role in a variety of cellular events such as cell migration, proliferation, differentiation, and signal transmission through cell-cell or cell-extracellular matrix (ECM) contact.^{19,20} It has been determined that the major cells involved in PVR are RPE cells and glia.^{5,6} In the normal eye, the RPE cells comprise a monolayer of mitotically inactive cells. When isolated from the eye, RPE cells shift from the epithelioid to the fibroblastic morphotype and acquire invasive properties.^{21,22}

Cadherins are a superfamily of transmembrane glycoproteins that mediate calcium-dependent homophilic cell-cell adhesion.^{19,20} E-cadherin and N-cadherin are two classic members that have been most studied. As a rule, E-cadherin is expressed in epithelial cells, whereas N-cadherin is expressed in the neural cells and the mesenchymal cells like fibroblasts.²³ However, the human RPE cells differ from other epithelia in that they predominantly express N-cadherin rather than E-cadherin as a major component of the zonulae adherens by which the contiguous RPE cells are joined and the epithelial

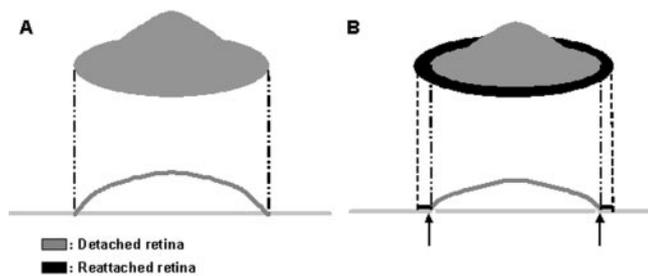


FIGURE 1. A schematic picture shows progressive retinal reattachment. Some time after the RD model was made, the detached area decreased in size (from A to B). Therefore, there was always a concentric area of reattached retina surrounding the RD region. In the section, consequently, the reattached retina lies contiguously to the two sides of the RD region. *Arrows*: conjunction points between the detached retina and the reattached retina.

phenotype is maintained.^{24,25} Therefore, N-cadherin serves as stable cell-cell adhesion molecules. However, N-cadherin has also been associated with cell motility and invasion. During morphogenesis, N-cadherin expression is associated with rearrangement and invasion of cells.^{26,27} N-cadherin may promote retinal neurite outgrowth and mediate retinal lamination.²⁸⁻³⁰ Moreover, it has been demonstrated in a variety of human epithelial cancers and melanomas that the loss of E-cadherin and switching to N-cadherin are linked with the metastasis of tumor cells.³¹⁻³⁴ It has been thought that N-cadherin enhances the invasiveness of the cancer cells by facilitating their attachment to N-cadherin-positive stromal cells.²³ Recent *in vitro* studies have shown that the invasion of collagen by RPE cells and retinoblastoma cells could be inhibited by a neutralizing antibody against N-cadherin,^{35,36} demonstrating that N-cadherin may promote RPE cells and retinoblastoma cells' migration on matrix. These apparently antithetic functions of N-cadherin have been thought to be associated with different contexts in which the RPE cells are placed. In the normal retina, N-cadherin is coupled to the cytoskeleton and serves cell-cell adhesion as an element of zonulae adherens, whereas in other situations such as in retinoblastoma, zonulae adherens are disassembled, and N-cadherin is released from the cytoskeleton and participates in cell invasion.^{36,37}

N-cadherin is differentially expressed in the neural retina among different species. In the chick retina,^{38,39} N-cadherin is limited to the outer limiting membrane (OLM); in the mouse retina,⁴⁰ N-cadherin is localized to the inner nuclear layer; and in the human retina,³⁶ N-cadherin is found in the inner and outer nuclear layer, as well as in the OLM. N-cadherin has been investigated in the normal human retina and in retinoblastoma, but the expression of N-cadherin and its functions in RD has not been investigated.

In the present study, in an attempt to demonstrate whether the separation of the neuroretina from RPE layer may induce changes in the expression of N-cadherin in RPE cells and the neural retina, we modified a conventional animal model of RD.⁴¹⁻⁴⁴ Furthermore, an N-cadherin antagonist, Ac-Ile-Asn-

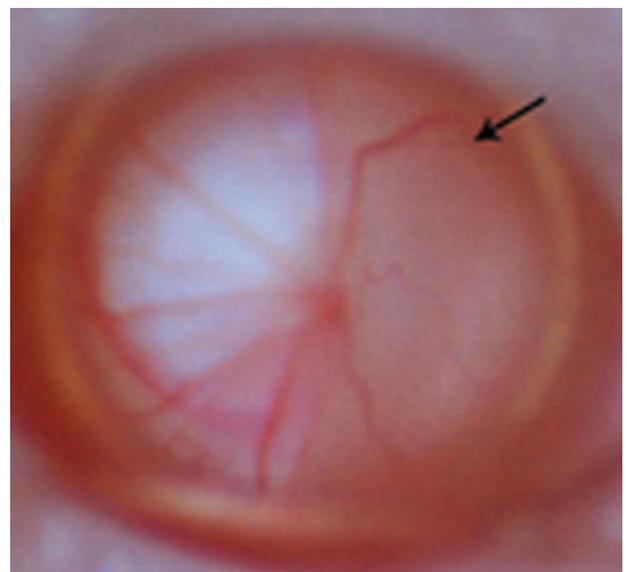


FIGURE 2. Fundus photograph shows half-side retinal detachment (*arrow*) without retinal breaks.

Pro-Ile-Ser-Gly-Gln-NH₂ (INPISGQ or INP),⁴⁵ a linear peptide mimetic of a short sequence in the extracellular domain1 (ECD1) of N-cadherin, was used to elucidate the role N-cadherin might play after RD and its correlation with the onset of PVR.

METHODS

Retinal Detachments and Reattachments

Forty adult Sprague-Dawley (SD) rats of either gender (200-250 g; Vitalriver Laboratory Animal Equipment Co., Ltd., Beijing, China) were used in this study. The rats were anesthetized with an intraperitoneal injection of 1% pentobarbital sodium (45 mg/kg) and a topical anesthetic (Oxyben; Santen Pharmaceutical Co., Ltd., Osaka, Japan) was applied to the right eye. Pupils were dilated with a topically applied mixture of 0.5% tropicamide and 0.5% phenylephrine (Mydrin-P; Santen Pharmaceutical Co., Ltd.). Nasal retinas were detached in the right eyes of SD rats by transscleral injection of 1.4% sodium hyaluronate (Healon GV; Pharmacia and Upjohn Co., Kalamazoo, MI) into the subretinal space (SRS) with a 30-gauge needle (catalog no. 305128; BD PrecisionGlide; BD Biosciences, Franklin Lakes, NJ). Care was taken not to make a break in the detached retina. Animals in which intraocular hemorrhage developed were excluded. The left eyes of all the animals served as normal control eyes. Animals were killed at 0, 3, 12, and 24 hours and 3, 7, 14, and 28 days after surgery ($n = 5$). Hereafter, 0 hour refers to immediately after the induction of RD.

The injected sodium hyaluronate in the subretinal space spontaneously dissolved, and the detached area decreased in size with time. Figure 1 is a schematic representation of a concentric area of reattached retina surrounding the RD region. Our preliminary studies

TABLE 1. Antibodies

| Antibody | Company | Source | Dilution |
|--|-----------|-------------------|----------|
| Anti-N-cadherin | sc-8424* | Mouse, monoclonal | 1:100 |
| Anti-pan-cytokeratin | sc-15367* | Rabbit polyclonal | 1:100 |
| Fluorescein-conjugated goat anti-mouse IgG | ZF-0312† | Goat | 1:100 |
| TRITC-conjugated goat anti-rabbit IgG | ZF-0316† | Goat | 1:100 |

* Santa Cruz Biotechnology, Santa Cruz, CA.

† Zymed, South San Francisco, CA.

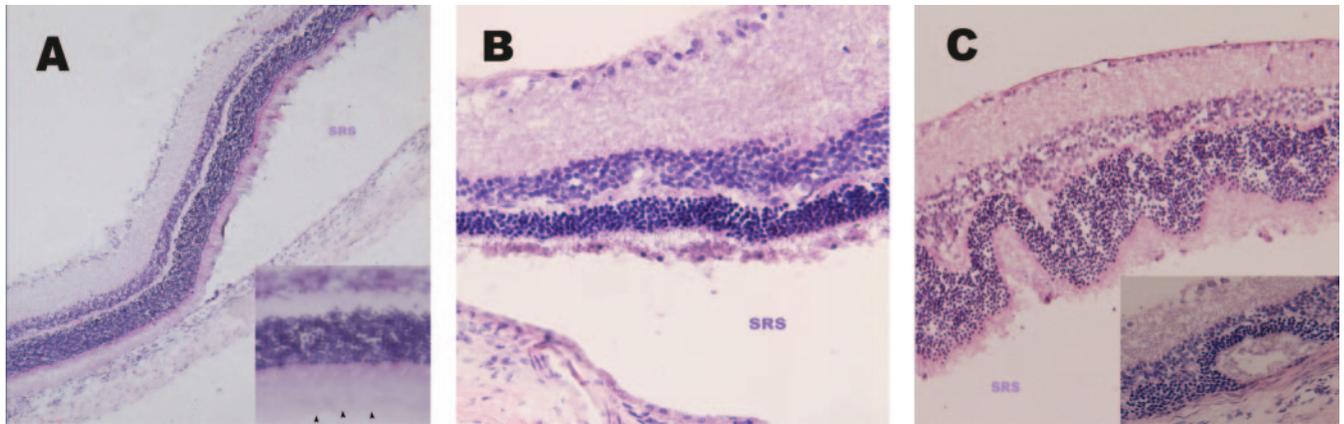


FIGURE 3. HE-stained sections show that more cells were attached to the outer surface of the detached retina at 28 days (**B**) compared with those at 1 day (**A**). Retinal folds were occasionally observed in the ONL of the detached retina (**C**). In the reattached retina, the ONL was frequently disorganized, forming a rosette pattern (**C**, *inset*). SRS, subretinal space. Original magnification: (**A**) $\times 100$; (**B**) $\times 400$; (**C**) $\times 200$.

showed that the time necessary for complete reattachment ranges from 30 to 50 days.

All procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Treatment with an N-cadherin Antagonist

Another 10 rats were used to explore further the function of N-cadherin during experimental RD. The N-cadherin antagonist Ac-Ile-Asn-Pro-Ile-Ser-Gly-Gln-NH₂ (INPISGQ or INP), which is a linear peptide mimetic of a short sequence in ECD1 of N-cadherin, was synthesized by Chinese Peptide Company (Hangzhou, China) with purity of 98.9%. INP functions as a highly specific and potent antagonist of N-cadherin with an IC₅₀ of $\sim 15 \mu\text{M}$.⁴⁵ The peptide was used at a final concentration of $15 \mu\text{M}$ to block N-cadherin's functions by subretinal injection of it in combination with 1.4% sodium hyaluronate. The animals were killed for 7 and 14 days after the surgery ($n = 5$). The

detached retinas of the same survival time without INP treatment served as the control.

Tissue Preparation and Histology

The animals were killed at different time intervals with an overdose of 1% pentobarbital sodium. The eyes were enucleated after cardiac perfusion of 4% paraformaldehyde, postfixed in 4% paraformaldehyde for 1 hour at room temperature, and submerged in phosphate-buffered saline (PBS; 1.0 M, pH 7.4) twice for 1 hour. The anterior segment of the eye and the vitreous were removed, and the remaining eye cups were immersed in 30% sucrose overnight at 4°C. The eye cups were then embedded in optimal cutting temperature (OCT) compound (Tissue Tek; Laboratory Tek, Naperville, IL), frozen in liquid nitrogen, and stored at -80°C . Five- to 8- μm -thick frozen sections were cut on a microtome (CM1900; Leica Microsystems, Deerfield, IL).

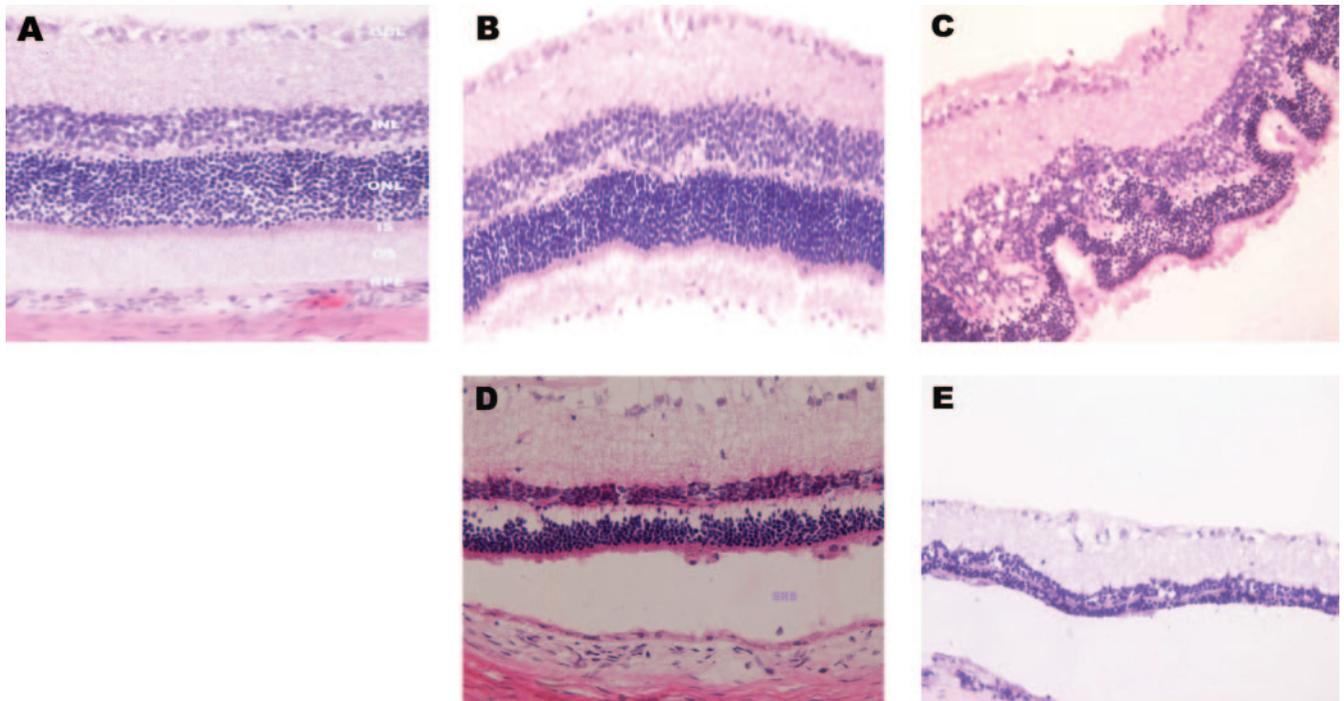


FIGURE 4. HE-stained sections show a normal control retina (**A**) and detached retinas at 7 (**B**, **D**) and 14 (**C**, **E**) days. The detached retinas treated with an N-cadherin antagonist (**D**, **E**) were markedly thinner than those without treatment (**B**, **C**). The loss of photoreceptor nuclei was also prominent (**D**, **E**). Abbreviations are as in Figure 3. Original magnification, $\times 400$.

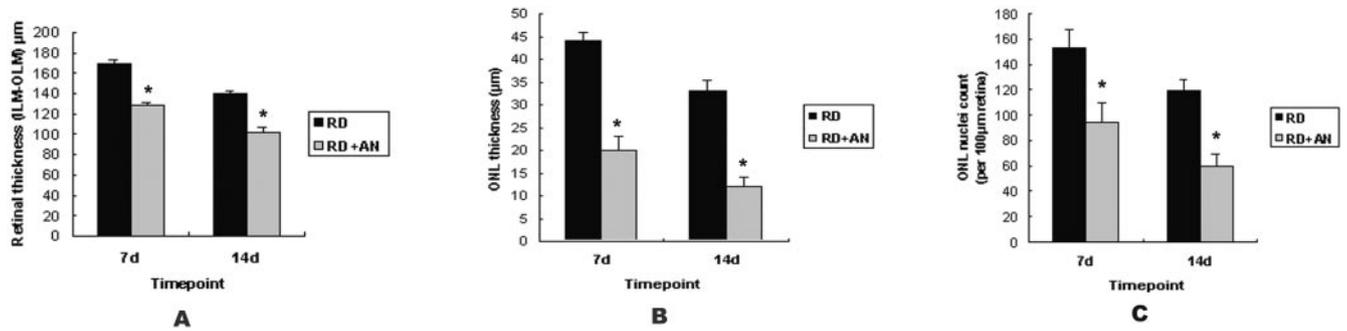


FIGURE 5. The thickness of the retina from the ILM to the OLM (A) as well as the ONL (B) were both significantly reduced in the retina treated with N-cadherin blockade. The number of photoreceptor nuclei counted per 100 μm was also dramatically decreased in the treated retina (C). * $P < 0.001$, $n = 5$. RD+AN, retinal detachment+ antagonist.

For histologic examination, sections were stained with hematoxylin and eosin (HE).

Immunofluorescence

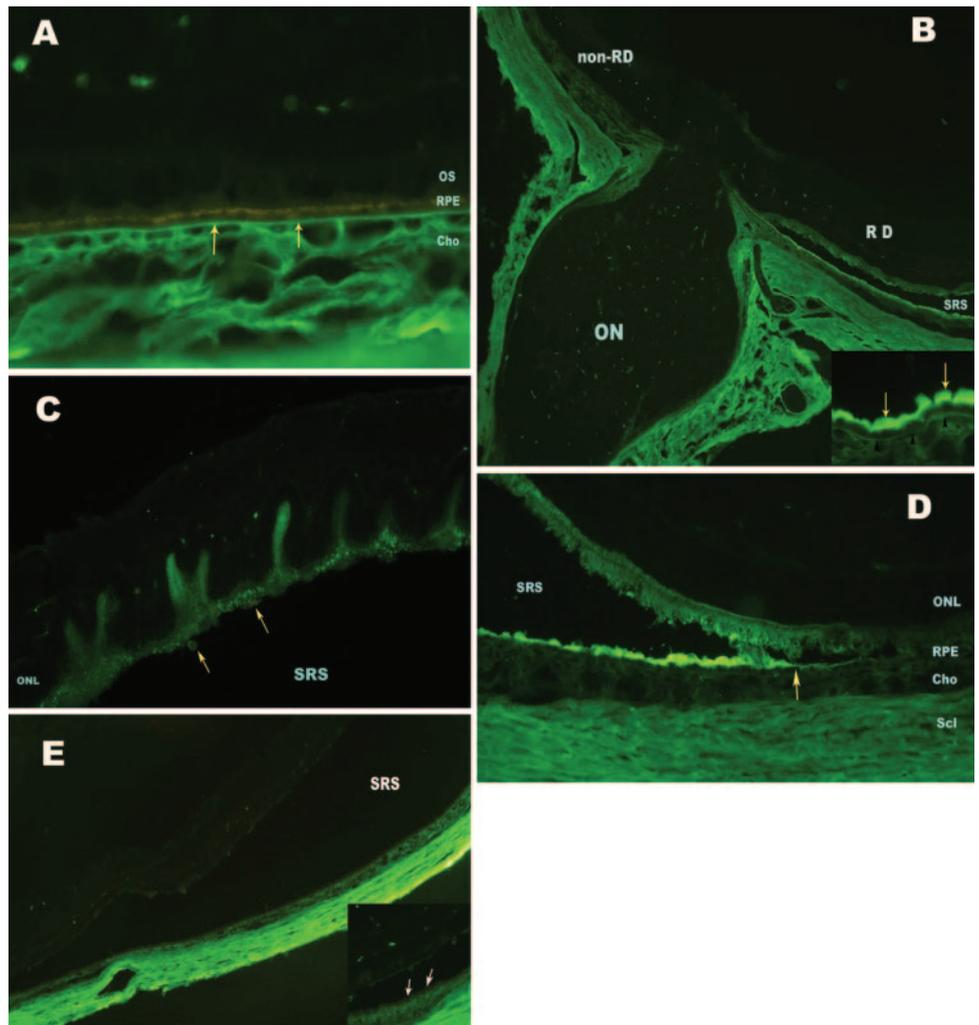
All antibodies used in the study are listed in Table 1. All antibodies were diluted in PBS with 0.1% Tween-20 and 0.5% bovine serum albumin (BSA), pH7.4. Slides were blocked, incubated with primary antibodies overnight at 4°C, and then washed in PBS. Fluorescein-conjugated or rhodamine-conjugated secondary antibodies were applied for 45 minutes at room temperature. After another PBS wash,

slides were coverslipped with glycerol. All sections were viewed and photographed on a microscope (BX5 -1WL; Olympus, Melville, NY).

Quantification and Statistical Analysis

Three different regions from each eye were analyzed. Data are presented as the mean \pm SD. At each survival time, cytokeratin (CK)-positive cells attached to the outer surface of the retina were counted per 100 μm of the retina on immunofluorescence images. Statistical significance was assessed with analysis of variance (one-way ANOVA) followed by SLD multiple comparisons test. The width from the inter-

FIGURE 6. Immunofluorescence photographs in the normal eye of SD rat (A). N-cadherin was strongly expressed in the sclera and Bruch’s membrane (A, arrows), weakly expressed in the INL and GCL, but not expressed in the RPE. The yellow fluorescence was the autofluorescence inside the RPE cells. After the retina was detached (B), N-cadherin was present in the RPE, the photoreceptors, and the OLM. N-cadherin immunolabeling was intense at the top of the RPE cells (B, arrows, inset); arrowheads show Bruch’s membrane. Note that the clumps of cells attached to the photoreceptors were also N-cadherin positive (C, arrows). In an intriguing finding, N-cadherin labeling stopped abruptly at the junction of the detached retina and reattached retina (D, arrow). After the retina was treated with an N-cadherin antagonist (E), N-cadherin immunoreactivity was not observed at the top of the RPE cells (E, arrow, inset). OS, outer segment; RPE, retinal pigment epithelium; Cho, choroid; SRS, subretinal space; ON, optic nerve; RD, retinal detachment; non-RD, non-retinal detachment; ONL, outer nuclear layer; Scl, sclera. Original magnification: (A) $\times 400$; (B, E) $\times 100$; (C, D) $\times 200$.



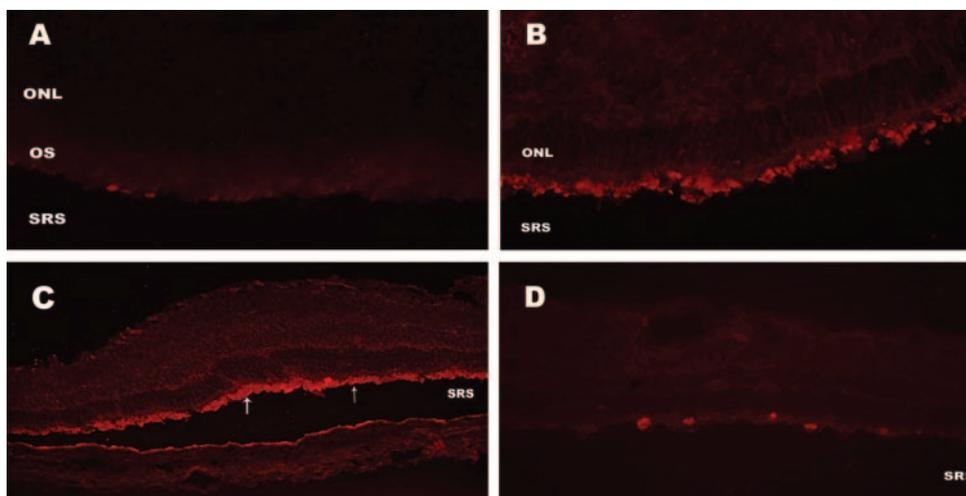


FIGURE 7. In the retina detached for 1 day (A), immunofluorescence staining with antibodies to CK showed few cells attached to the outer surface of the detached retina. More cells were accumulated at 7 (B) and 28 (C) days after the detachment. In the retina treated with an N-cadherin antagonist, CK-positive cells were dramatically decreased (D). ONL, outer nuclear layer; OS, outer segment; SRS, subretinal space. Original magnification: (A, B, D) $\times 400$; (C) $\times 200$.

nal limiting membrane (ILM) to the outer limiting membrane (OLM) and the thickness of the outer nuclear layer (ONL) were measured with an optical reticule on hematoxylin and eosin (HE)-stained sections. Counts of nuclei in the ONL per 100 μm of the retina were also made on HE-stained sections. No measurements or analyses were performed in the regions with retinal folds. A two-sample *t*-test was used to compare the retinas with and without INP treatment. $P < 0.05$ was considered statistically significant.

RESULTS

RD Model in Rats

Nasal retinal (one half to one third) detachments were created in the right eyes of the animals (Fig. 2).

Retinal Morphology

Frozen sections stained with HE showed separation between the neural retina and the RPE layer (Fig. 3). When the retina was freshly detached, there were only a few cells attached to the detached photoreceptors (Fig. 3A), but with time, more and more cells accumulated around the photoreceptors of the detached neural retina (Fig. 3B). In the detached retina, folds were occasionally observed in the ONL (Fig. 3C). In the reattached retina adjacent to the detached area, the ONL was frequently disorganized with rosette formation and thinned (Fig. 3C, inset).

In the retina treated with INP, the thickness of the retina markedly decreased after detachment (Figs. 4D, 4E, 5A; $P < 0.001$), the thickness of the ONL (Fig. 5B) and the photoreceptor nuclei count (Fig. 5C) also decreased dramatically compared with the retina without INP treatment ($P < 0.001$). Folds were rarely observed in the ONL (Figs. 4D, 4E).

N-cadherin Expression in the Rat Retina

In the normal retina, N-cadherin was strongly expressed in the Bruch's membrane, weakly expressed in the inner nuclear layer (INL) and the ganglion cell layer (GCL), and not expressed in the RPE (Fig. 6A). In the detached retina, N-cadherin was intensely localized in the RPE, the photoreceptors, and the OLM (Fig. 6B). The cells attached to the photoreceptors were N-cadherin-positive as well (Figs. 6C). The N-cadherin labeling was initially detected in the RPE layer at 12 hours after detachment, and persisted as long as the retina was detached. In the reattached retina adjacent to the detached area, dramatic changes were observed at the junctional point between the two areas (Fig. 6D). Once the neural retina had adhered to the RPE, the immunoreactivity of N-cadherin decreased rapidly, approaching the level of the normally attached retina.

In the retina treated with INP, N-cadherin was still positive in the basement membrane of the RPE cell, whereas the immunolabeling in the cell bodies disappeared (Fig. 6E).

Cytokeratin-Positive Cells

The cells attached to the outer surface of the detached photoreceptors were CK-positive (Figs. 7A–C), and there was a steady rise in the number of the cells where the retina remained detached (Fig. 8A; $P < 0.001$).

In the retina treated with INP, very few CK-positive cells were seen on the detached retina (Fig. 7D) by comparison with the retina without INP treatment (Fig. 8B, $P < 0.001$).

DISCUSSION

PVR is a complication that results from chronic retinal detachment.^{5,6} Clinically, PVR frequently develops as RD persists, and once the retina is successfully reattached, PVR may not occur.

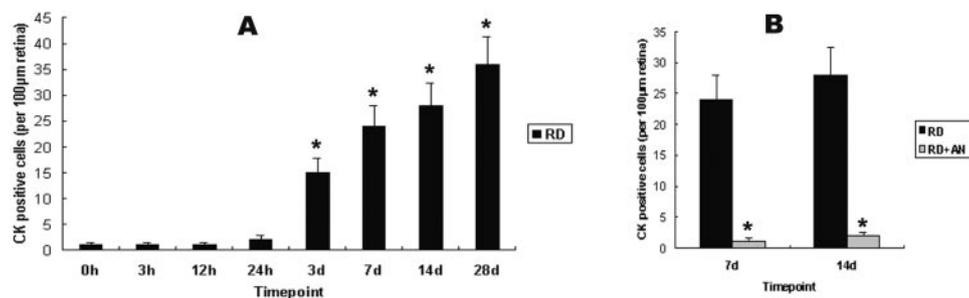


FIGURE 8. (A) The number of CK-positive cells increased in dependence on the detachment duration, compared with 0 hours ($*P < 0.001$; $n = 5$). (B) The number of cells was significantly reduced in the retina treated with N-cadherin. $*P < 0.001$, $n = 5$.

Tseng et al.⁴⁶ reported in a prospective study that chronic, large RD was significantly associated with increased prevalence and severity of PVR. Previous studies also demonstrated that experimental RD may trigger RPE and glial proliferation.^{4,15-17} However, the pathobiology of PVR in an experimental model of RD has not been defined.

Adhesion molecules play a key role in cell migration and proliferation, and are actively involved in the process of PVR.²² N-cadherin is a transmembrane glycoprotein that mediates homophilic cell-cell adhesion.^{19,20,24} In the normal human retina, the contiguous RPE cells are joined by a series of intercellular junctions, and N-cadherin acts as a major element of the zonula adherens.^{21,25,35} However, *in vitro* studies showed that N-cadherin may promote RPE cell invasion of collagen.³⁵ In summary, in physiological circumstances, N-cadherin contributes to RPE cell stability, whereas in some pathologic circumstances, N-cadherin may facilitate RPE cell motility and migration.

In our experiment, the animal model of RD was created by transscleral injection of 1.4% sodium hyaluronate into the subretinal space. Animals that developed choroidal hemorrhage were excluded because the serum of the extravasated blood contained chemoattractants to RPE cells.⁴⁷ Retinal breaks were not made in this experimental model because our investigation focused on the induced changes in N-cadherin expression in the separation between the neural retina and the RPE, and any additional injury was avoided. As in Figure 1, there was invariably a concentric area of reattached retina surrounding the detached area. Consequently, contiguous regions of detached and reattached retina could be observed in one histologic section and provided reattached controls for the detached retina because the experimental conditions were comparable.

Our results revealed that normal rat RPE did not express N-cadherin (Fig. 6A), although normal human RPE expresses N-cadherin abundantly.^{25,35} These differences may be a species variation. Alternatively, the level of N-cadherin expression in normal rat RPE may not be detected by our immunoreactive technique. However, we observed that the expression of N-cadherin was dramatically elevated in the RPE cells in response to RD (Fig. 6B). In contrast, after retinal reattachment, the immunolabeling decreased rapidly, approaching the level of the normally attached retina (Fig. 6D). The prominent difference in the expression of N-cadherin between the detached retina and the reattached retina suggests that RD may stimulate RPE cells to produce adhesion molecules related to cell proliferation and migration, and this change may be reversed by retinal reattachment. This observation is in agreement with the hypothesis that there seems to be contact inhibition between the RPE and the photoreceptors.⁵ When these two layers of cells are separated, the RPE cells should be activated. Conversely, the apposition of the two cells is a prerequisite to the quiescence of the RPE cells. More interesting, an increasing number of CK-positive cells likely to be RPE cells were attached to the outer surface of the detached retina (Fig. 7, Fig. 8A). Some of the RPE cells may have detached from Bruch's membrane and adhered to the photoreceptors at the time of experimental separation. However, we hypothesized that RPE cell migration is a major factor contributing to the increasing number of these cells, although the stabilization of the detachment by sodium hyaluronate may also promote RPE migration along the outer surface of the neural retina. It is noteworthy that these displaced RPE cells and the photoreceptors to which the cells adhered were both N-cadherin positive (Fig. 6C), and when the N-cadherin was blocked, the number of the displaced cells was markedly decreased (Figs. 7D, 8B). Taken together, our observations led to the suggestion that N-cadherin plays a crucial role in RPE cell migration by N-cadherin-mediated RPE-photoreceptor adhesion.

The upregulation of N-cadherin was prominent in the photoreceptors and in the OLM after RD (Figs. 6B, 6C). This alteration could have several implications. First, N-cadherin could prevent photoreceptor cell apoptosis. This is supported by the observation that the thickness of the retina and the ONL were dramatically decreased after N-cadherin inhibition (Figs. 4, 5). Other investigators have reported that N-cadherin-mediated cell contact could prevent apoptosis and promote cell survival.^{23,48,49} The survival of the photoreceptors after RD might be achieved by N-cadherin-mediated interphotoreceptor contact and possible signal transmission pathways. Moreover, N-cadherin could cause the neighboring photoreceptors to adhere to each other and result in U-shaped folds in the ONL (Figs. 3C). When N-cadherin was blocked, ONL folds did not form (Figs. 4D, 4E). This intriguing phenomenon indicates that adhesion molecules may also be involved in the formation of retinal folds that frequently result from contraction of a proliferative membrane. Finally, N-cadherin may facilitate retinal reattachment, because both the RPE cells and the photoreceptors of the detached area express N-cadherin simultaneously (Fig. 6B). Normally, adhesion between the RPE and the neural retina is mediated by interphotoreceptor matrix (IPM.) and by neural cell adhesion molecule (NCAM).¹⁵ However, N-cadherin may form a "cell-adhesion zipper" effect between the two layers of cells after RD.

Previous studies of the animal model of RD and reattachment focused on the apoptosis or regeneration of the photoreceptors,⁴¹⁻⁴⁴ which are directly associated with the visual outcome of the patient with RD. However, PVR may also be devastating to visual rehabilitation after RD. Our present study provided observations on the molecular alterations that were involved in the development of PVR after RD. Adhesion molecules are crucial for cell proliferation and migration, and would play a key role in the development of PVR.²² N-cadherin is an important adhesion molecule found in the retina.^{25,29,35} To our knowledge, we are the first to explore N-cadherin expression in RD and retinal reattachment. In this study, the N-cadherin-mediated cell adhesion played a role in RPE cell migration and photoreceptor survival, and these changes were reversed when the retina reattached. Timely surgical intervention to reattach the retina may be the most effective way to prevent PVR in patients with RD.

Acknowledgments

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