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The Relationship Between Gap-Junctional Intercellular Communication and the Proliferative Activity of Retinal Pigment Epithelial Cells

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Purpose: To investigate the relationship between gap-junction mediated intercellular communication and the proliferative activity of retinal pigment epithelial cells in the retinal tear base.

Method: Retinal tears were created experimentally in white rabbits, and the changes of intercellular communication via gap junctions between retinal pigment epithelial cells in the retinal tear base were investigated using the dye-coupling method, which involves observing the spread of a fluorescent dye, Lucifer Yellow CH. In addition, the proliferative activity of these cells was investigated using an antibody for proliferating cell nuclear antigen and was compared with the changes in intercellular communication.

Results: Immediately after the creation of retinal tears, extensive spreading of Lucifer Yellow CH into adjacent cells was observed, which was markedly reduced 1 week later. *After 1 month*, the spreading of dye to adjacent cells was observed again. On the other hand, proliferative activity was enhanced at 1 week after retinal tear creation and was reduced again after 1 month.

Conclusions: It is known that tumor cells with enhanced proliferative activity show decreased intercellular communication via gap junctions. The present study suggests a possible relationship between intercellular communication and the proliferative activity of retinal pigment epithelial cells in the base of retinal tears. **Jpn J Ophthalmol 2000;44:250–256**

Key Words: Anti-proliferating cell nuclear antigen antibody, cell proliferation, dye-coupling method, intercellular communication, retinal pigment epithelial cell.

Introduction

The existence of gap junctions between the retinal pigment epithelial (RPE) cells is well known.[1] Gap junctions are organelles that allow a connection between the cytoplasm of adjacent cells. These are hydrophilic channels through which electric currents as well as small molecules such as sugars, amino acids, vitamins, and nucleotides can pass between cells, thus functioning as sites of intercellular communication.[2] However, the functional significance of intercellular communication via gap junctions has not been clarified for electrically inactive cells, although previous studies have shown that gap junctions are crucial for the regulation of cellular differentiation and proliferation.[2]

A fluorescent dye (Lucifer Yellow CH) is injected electrophoretically into a cell through a glass microelectrode, and the spread of the dye to adjacent cells is observed. Lucifer Yellow CH cannot pass through the cell membrane, but it moves through gap junctions. Accordingly, its spread can be regarded as an indicator of intercellular communication through

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gap junctions.[3] Accordingly, its spread, termed "dye-coupling," can be regarded as an indicator of intercellular communication through gap junctions.

Proliferating cell nuclear antigen (PCNA) is a polypeptide that specifically increases in the nucleus during the G1 and S phases of the cell cycle. It is considered to be an essential cofactor in the activation of DNA polymerase-δ during DNA replication.[4] Therefore, a PCNA-positive nucleus indicates that a cell is replicating DNA and is undergoing proliferation.

Accordingly, PCNA has been established as a reliable indicator of proliferative activity and its use for RPE cells has already been investigated.[5] At our department, we have been investigating the morphological and functional changes of RPE cells in experimentally created retinal tears.[6] In the present study, we observed the changes of intercellular communication between RPE cells via gap junctions using the dye-coupling method. We also investigated changes in the proliferative activity of RPE cells by immunostaining with an anti-PCNA antibody and comparing the results to assess the relationship between intercellular communication and cell proliferation.

Materials and Methods

Forty eyes were studied in 40 white rabbits weighing from 2 to 3 kg and without any abnormal ophthalmoscopic findings. As reported previously,[6] a retinal tear corresponding approximately to the diameter of the optic disc was created at the posterior pole of the eyeball by vitreous aspiration. Subsequently, 34 eyes of 34 animals without retinal detachment or hemorrhage were selected for further study. Immediately, 1 week, or 1 month after creating the retinal tear, animals were sacrificed by intravenous injection of a lethal dose of pentobarbital sodium (Nembutal®), and the eyeballs were enucleated. The enucleated eyeball was halved along the equator, and a chorioretinal section (15 mm × 15 mm) including the retinal tear was cut from the posterior half using a razor blade.

Assessment of Intercellular Communication

Twenty-five eyes of 25 rabbits were used for this study. The dye-coupling technique was done as follows: Lucifer Yellow CH (Sigma Chemical, St. Louis, MO, USA) was dissolved in 1 M LiCl to prepare a 2% solution, and glass microelectrodes made of glass capillary fibers (outside diameter: 1.2 mm; inside diameter: 0.8 mm) (Corning, New York, NY, USA) were filled with the solution. A microelectrode was attached to a micromanipulator and connected to a high input impedance amplifier (MEZ-8201; Nihon Koden, Tokyo) via silver/silver chloride electrodes. Using the micromanipulator, the electrode was advanced into a RPE cell in the center of the base of the retinal tear in the chorioretinal section, fixed with a pin for support and bathed in Ringer's solution. It was assumed that the electrode had entered the cell when a potential decrease of −30 to −40 mV was recorded, and injection of the dye was started by the application of load current. During dye application, which lasted for 3 minutes, the membrane potential was monitored. If a stable membrane potential could not be maintained, dye injection was assumed to have been unsuccessful. Sections containing RPE cells with successful dye injection were placed on glass slides, mounted in nonfluorescent glycerin, and observed under a fluorescence microscope with vertical illumination (BH2-RFK; Olympus, Tokyo). Photographs were taken using Kodak Tri-X pan 400 film. A cell with uniform fluorescence was regarded as positive, and the number of positive cells was counted in each section where dye injection had been successful.

Cell Proliferation

Nine eyes of 9 rabbits were used for this study. Chorioretinal sections were fixed in 3.7% formaldehyde for 1 minute and in 100% methanol for 10 minutes. After fixation, the sections were incubated with a mouse monoclonal antibody for PCNA at a dilution of 1:100 (Novacastra Laboratories, Newcastle, UK) as the primary antibody for 60 minutes at room temperature, and with a 1:30 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG-IgA-IgM (Southern Biotechnology Associates, Birmingham, AL, USA) as the secondary antibodies for 40 minutes at room temperature. After completion of incubation, the sections were immediately mounted in nonfluorescent glycerin and observed under a fluorescence microscope. Photographs were taken with Kodak Tri-X pan 400 film. In control sections, the primary antibody was replaced with the same amount of phosphate buffer solution.

Results

Assessment of Intercellular Communication

The number of eyes in which the glass microelectrodes could be held steady for 3 minutes and dye injection was successful as follows: 4 of 8 eyes examined immediately after the creation of retinal tears, 4 of 9 examined after 1 week, and 4 of 8 examined after 1 month. The success rate of dye injection at each

Jpn J Ophthalmol
Vol 44: 250–256, 2000

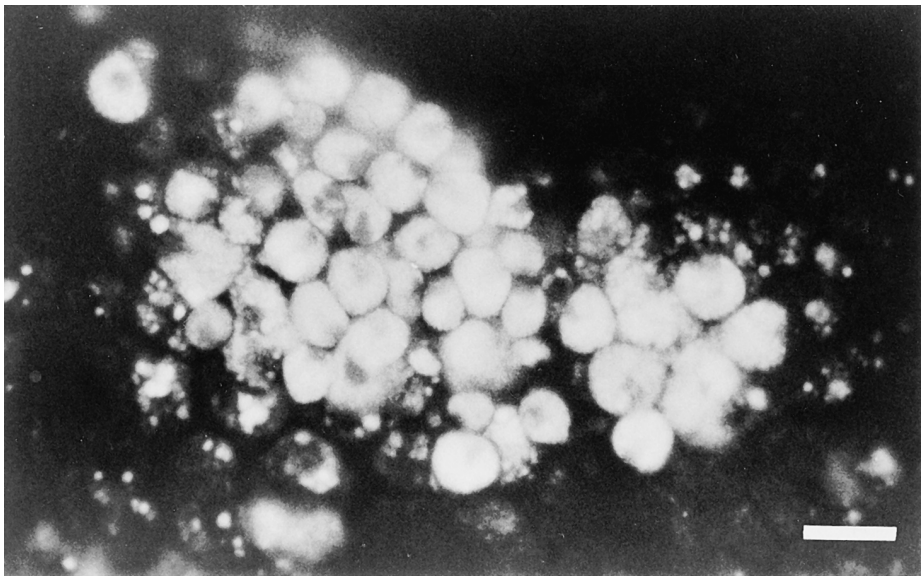

Figure 1. Immediately after creation of retinal tears. Note extensive spread of Lucifer Yellow CH into adjacent retinal pigment epithelial cells. Bar = 20 μm.

time was 50%, 44% and 50%, respectively, or 48% overall.

Immediately after the creation of retinal tears, extensive spread of dye into adjacent RPE cells was observed (Figure 1). Granular fluorescent staining was found in some surrounding cells, but these cells were not regarded as positive because dye can also flow through sites of membrane damage,[7] and there was a possibility that some RPE cells might have been damaged when the sensory retina was detached.

After 1 week, the spread of dye was clearly reduced; dye was detected in the cell injected with the microelectrode, but only slight spreading into adjacent cells was observed (Figure 2).

After 1 month, the spread of dye into adjacent RPE cells was observed again (Figure 3).

The number of positive cells at each time of measurement was compared using the Kruskal–Wallis test. The number of positive cells immediately, 1 week, and 1 month after creation of retinal tears was 33.8 ± 4.7,

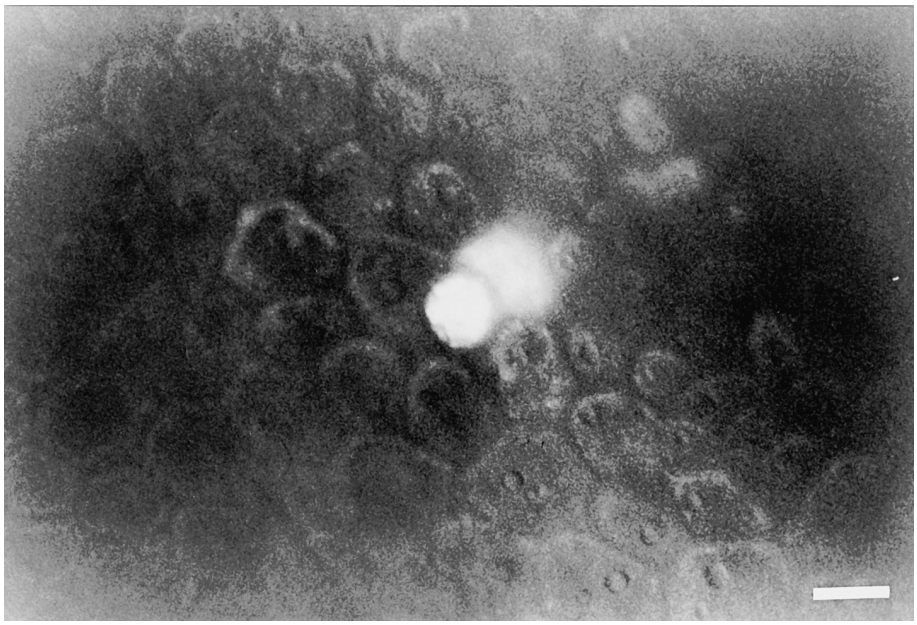

Figure 2. One week after creation of retinal tears. Spread of dye is markedly reduced. Bar = 20 μm.

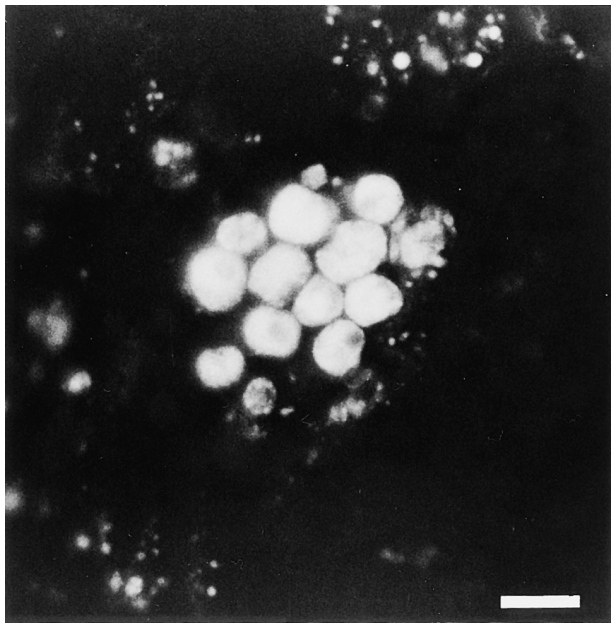

Figure 3. One month after creation of retinal tears. Spread of dye into neighboring cells is observed again. Bar = 20 μm.

2.3 ± 0.9, and 17.5 ± 4.1 (mean ± standard error of the mean), respectively, with a significant decrease after 1 week as compared to immediately after retinal tear creation ($P < .05$). The number of positive cells increased after 1 month as compared with after 1 week, but it was lower than immediately after retinal tear creation ($P < .05$). The success rate of dye injection and the number of positive cells at each examination time are compared in Table 1.

PCNA

No PCNA-positive RPE cells were found in the base of the retinal tears of the 3 eyes enucleated immediately after tear creation (Figure 4).

In the 3 eyes enucleated after 1 week, PCNA-positive RPE cells covered almost the entire base of the retinal tear. Many cells of the PCNA-positive cells had 2 or more nuclei (Figure 5).

PCNA-positive RPE cells were also observed in the 3 eyes enucleated after 1 month. However, the number of PCNA-positive cells was markedly reduced compared to that at 1 week (Figure 6) and there were no longer any multinucleated cells. In all 3 eyes, PCNA-positive cells were present in the center of the base of the retinal tear; 2 eyes also had positive cells at the tear edge. Almost no PCNA-positive cells were found in the intermediate zone between center and edge.

In control eyes, no positive cells were found immediately, 1 week, or 1 month after retinal tear creation.

Discussion

The present study indicated that RPE cells in the base of experimentally created retinal tears showed decreased intercellular communication from 1 week after retinal tear creation, with recovery after 1 month. Conversely, the proliferative activity of RPE cells was enhanced 1 week after creation of retinal tears and was reduced after 1 month.

Studies on changes of intercellular communication via gap junctions have been conducted in connection with the process of carcinogenesis. In 1966, Lowenstein demonstrated, by the use of electrophysiological methods, that intercellular communication was reduced in tumor cells. Subsequent studies have shown that communication between tumor and normal cells is reduced, but not that between tumor cells.[8,9] It was also reported that inhibition of tumor cell proliferation and normalization of morphology occurred when tumor cells were brought into contract with normal cells and intercellular communication between them was established.[10] Based on these results, Yamasaki et al advanced the hypothesis that intercellular communication via gap junctions inhib-

Table 1. Success Rate of Dye-Coupling and Number of Positive Retinal Pigment Epithelial (RPE) Cells per Dye Injection (Mean ± SEM)

	Success Rate of Dye-Coupling	Number of Positive RPE Cells per Dye Injection (Mean ± SEM)
Immediately after tear creation	50% (4/8)	33.8 ± 4.7
1 week after tear creation	44% (4/9)	2.3 ± 0.9
1 month after tear creation	50% (4/8)	17.5 ± 4.1

Jpn J Ophthalmol
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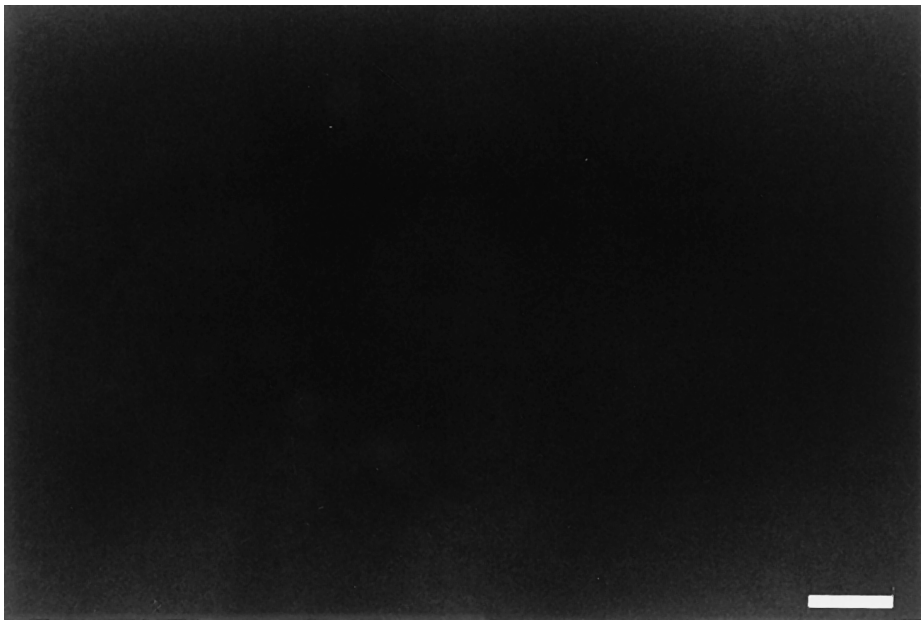

Figure 4. Immediately after creation of retinal tears. No proliferating cell nuclear antigen-positive retinal pigment epithelial cells are found in the base of retinal tear. Bar = 50 μm.

its tumor gene expression[11] and this hypothesis has become better established today.

Studies by Yasuda et al on the relationship between proliferation of RPE cells and gap junctions are well known.[12] In their experiments, the RPE cells that were cultured in the presence of phenylthiourea and hyaluronidase showed active proliferation, and then lost their differentiated characteristics and entered a state of dedifferentiation. Under certain conditions, these cells are reported to show characteristics normally specific to lens cells. These RPE cells appear morphologically normal, but no dye coupling was observed between them. The highly proliferative and dedifferentiated RPE cells have lost mutual communication via gap junctions and are independent of each other.

Figure 5. One week after creation of retinal tears. Proliferating cell nuclear antigen (PCNA)-positive retinal pigment epithelial (RPE) cells cover entire base of retinal tear. Many PCNA-positive RPE cells are multinucleated. Bar = 50 μm.

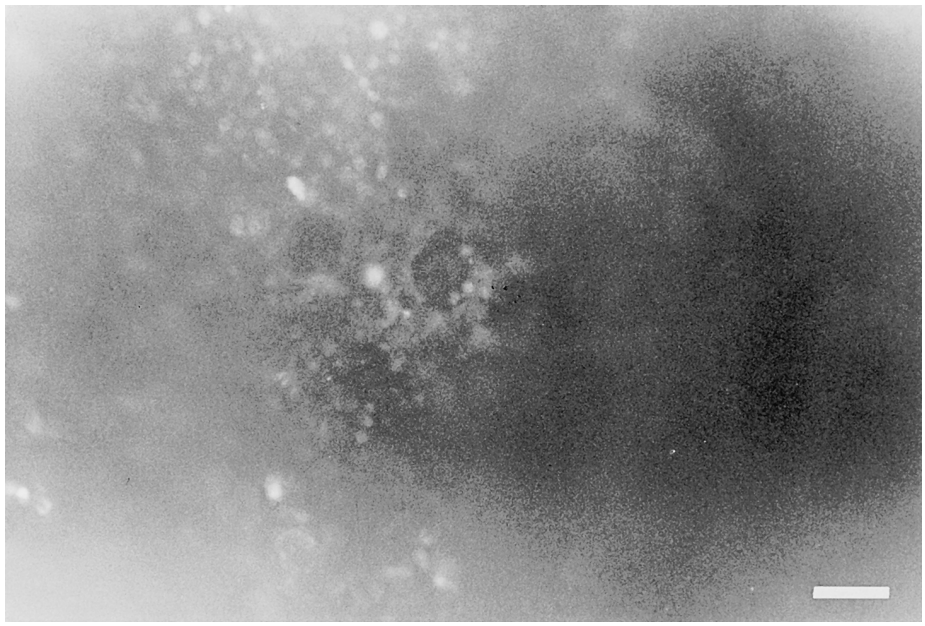

Figure 6. One month after creation of retinal tears. Proliferating cell nuclear antigen-positive retinal pigment epithelial cells are also observed but markedly reduced compared to that at 1 week. Bar = 50 μm.

A number of other reports have indicated that tumor cells or cultured RPE cells with enhanced proliferative activity show reduced intercellular communication via gap junctions. Moriwaki et al[13] studied the proliferation of RPE cells in the base of retinal tears using 5-bromodeoxyuridine (BrDU) and reported that the cells incorporated BrDU and synthesized DNA. DNA synthesis was observed 3 days after retinal tear creation, increased thereafter, and was maintained until at least 2 months later. Thus, RPE cells in the base of retinal tears seem to be highly proliferative. In the present study, the proliferative activity of the RPE cells in retinal tears was assessed using PCNA expression. The cells were found to be PCNA-positive, which supported an increase of proliferative activity as shown by Moriwaki et al. The number of PCNA-positive cells changed in a way that indicated variation of proliferative activity with time. Although the exact reason why proliferative activity changes with time is unknown, it is possible that such activity increases in the process of wound healing caused by cell injury during retinal tear formation and decreases when injured RPE cells are replaced by new cells. Comparison between proliferative activity and the changes of intercellular communication via gap junctions detected by the dye-coupling method revealed that intercellular communication was reduced during the period of enhanced proliferation and stabilized during the period of reduced proliferative activity. These findings correspond well with the earlier opinions concerning the relationship between proliferation of tumor cells or cultured RPE cells and intercellular communication. In RPE cells, there also seems to be a relationship between intercellular communication via gap junctions and proliferative activity.

A direct correlation between the two processes would need to be proved by double staining using dye-coupling and PCNA. In addition, differences of intercellular communication by cells at various sites within the base of the retinal tear were insufficiently investigated in our study. However, double staining and observation through repeated insertion of the microelectrode into different parts of the same section was not possible, because sections have to be observed by fluorescence microscopy without delay to avoid artifacts at damaged sites[7] and because Lucifer Yellow CH fades rapidly.[3] Another problem with our experiments was that no comparison could be made between untreated cells and the cells in the base of retinal tears, because the cells had to be exposed in order to confirm exact injection of the electrode or to allow efficient reaction with the PCNA antibody. We hope to conduct further investigations using improved techniques, eg, shorter treatment times.

Very little has been elucidated about the mechanism that controls intercellular communication via gap junctions. It is known that gap junctions open and close through the binding of calcium ions to

Jpn J Ophthalmol
Vol 44: 250–256, 2000

calmodulin, a calcium ion receptor present in the junctions,[14] and that the hydrogen ion concentration plays a role in changes of intercellular communication between the horizontal cells of fish.[15] Not many reports are available concerning RPE cells, but intercellular communication via gap junctions is known to play an important role in the control of differentiation and proliferation. Further investigation of this issue is required.

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