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[SHORT COMMUNICATION]

Cadherin Expression during Retinal Regeneration in the Adult Newt

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ABSTRACT—The present study used an immunohistochemical technique to examine the expression of cadherins in the regenerating retina of adult newts. After surgical removal of the neural retina, retinal pigment epithelial (RPE) cells proliferate into retinal precursor cells and regenerate a fully functional retina. At the beginning of retinal regeneration, retinal cells originating from RPE cells are undifferentiated precursor cells. Both E-cadherin and R-cadherin are expressed at the surface of these precursor cells. As regeneration proceeds, precursor cells differentiate into retinal neurons. R-cadherin is expressed at the surface of the differentiated neurons, but E-cadherin is lost to the differentiated neurons. The difference in expression pattern suggests that each cadherin plays distinctive roles in retinal regeneration. And our finding that E-cadherin is expressed transiently by undifferentiated precursor cells implies the importance of cell-cell adhesions by E-cadherin for differentiation.

INTRODUCTION

Newts have the ability to regenerate a functional retina after complete ablation of the original retina, even as adults. The retinal pigment epithelial (RPE) cells undergo depigmentation and proliferation after retinal ablation, producing all the retinal neurons and glia necessary to form a new functional retina (Hasegawa, 1958; Keefe, 1973). The processes of retinal regeneration, such as cell proliferation, differentiation during regeneration, and the cytoplasmic communication via gap junctions have been studied (Negishi *et al*., 1992; Cheon *et al*., 1998; Kaneko and Saito, 1992; Chiba and Saito, 2000). And Ortiz *et al.* (1992) showed that the components of extracellular matrix do change during regeneration, suggesting that these change play an important role (see Mitashov VI, 1996, 1997 for review). However, various aspects of regeneration process have not been investigated. It is important to elucidate the roles of cell-cell adhesions and to identify the adhesion molecules involved in regeneration, because cell-cell contacts play significant roles for cell fate determination in development (Austin *et al*., 1995). The cadherins are a family of cell surface, Ca^{2+}-dependent, homophilic adhesion molecules (Takeichi, 1990). They play important roles in the development of a variety of tissues including the brain (Jessell, 1988; Takeichi, 1988). Some cadherins are named according to where they are initially found; E-cadherin in epithelial cells, N-cadherin in neural epithelial cells, and R-cadherin in the retina (Nagafuchi *et al*., 1987; Hatta and Takeichi 1986; Inuzuka *et al*., 1991), yet these proteins have been shown to have a much broader tissue distribution (Geiger and Ayalon, 1992). In the developing chicken retina, N-cadherin is expressed in undifferentiated retinal cells during the early stages of development and some cadherins including R-cadherin are expressed by a specific subpopulation of differentiated retinal neurons at later stage (Matsunaga *et al*., 1988; Inuzuka *et al*., 1991; Wohrn *et al*., 1998), suggesting that cell-cell adhesions facilitated by cadherins are important for retinal development. Since cadherins have roles in embryonic retinal development, they should also be involved in the regeneration of the newt retina. In the present study, we examined the expression of cadherins in retinal regeneration and revealed that E-cadherin is expressed transiently at the surface of undifferentiated precursor cells.

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MATERIALS AND METHODS

Adult newts (*Cynops pyrrhogaster*) were anesthetized and the neural retina and lens were surgically removed as described previously (Kaneko and Saito, 1992). The subject animals were maintained at approximately 24°C and sacrificed at 20, 30, and 70 days after

surgery. The eyeballs were fixed in 10% formaldehyde in phosphate buffered saline (PBS) PH 7.4, embedded in paraffin, and cut into 10 μm sections. For immunostaining, the sections were mounted on gelatin-coated slides, deparaffined with xylene, treated with blocking solution (5% goat serum in PBS), and incubated with the following primary antibody overnight; monoclonal anti-human E-cadherin, monoclonal anti-mouse R-cadherin, monoclonal anti-mouse N-cadherin, and monoclonal anti-mouse synaptosomal-associated protein 25 (SNAP-25) (Transduction Laboratories, Lexington, KY, USA). Antibody binding was visualized by incubation with Alexa 488-labeled secondary antibody (Molecular Probes, Eugene, OR, USA). The preparations were examined using fluorescence microscopy (DMR; Leica Instruments, Germany).

RESULTS AND DISCUSSION

To reveal the expression of E-cadherin, R-cadherin, N-cadherin, and synaptosomal-associated protein 25 (SNAP-25) in the normal newt retina, immunohistochemistry was performed on sections of normal retina, and immunofluorescence results are shown in Fig. 1. The sections were also examined with difference interference contrast (DIC) image (Fig. 1A) to observe morphology and to identify cell type. To reveal the plexiform (synapse) layers, we used the antibody to SNAP-25, which is one of the nerve-terminal proteins and in the retina is expressed in the outer plexiform layer (OPL) and inner plexiform layer (IPL) (Catsicas *et al*., 1992). In newt retina, immunoreactivity was also observed in the IPL and OPL (Fig. 1D). Fig. 1(B) shows the retinal sections' immunoreactivity to anti-E-cadherin antibody. A faint fluorescence seen in the OPL and outer nuclear layer (ONL) was not the signal but autofluorescence (background), which was also seen in the control section (Fig. 1E). Therefore, no positive staining was detected for E-cadherin. On the other hand, R-cadherin was detected at the surface of cell bodies of all types of neurons in the nuclear layers and at the neurites in the plexiform layers (Fig. 1C).

Photographs in Fig. 2 show immunofluorescence results for regenerating retinas consisting of undifferentiated cells. Fig. 2A–C show the results for the regenerating retina 20 days after surgery. Line of cells 1–2 cells thick with non-pigmented cytoplasm was seen adjacent to the thin retinal pigment epithelial (RPE) cell layer. This regenerating retina corresponds to 'early'-regenerating retina, according to the definition of stages of regenerating retina by Cheon and Saito (1999). All these non-pigmented cells in this stage are proliferating (Negishi *et al*., 1992), suggesting that these cells are undifferentiated precursor cells. Both E-cadherin (Fig. 2A) and R-cadherin (Fig. 2B) were detected at the surface of these precursor cells, but SNAP-25 was not detected in these cells (Fig. 2C). Fig. 2D–G show the results for the regenerating retina 30 days after surgery. This regenerating retina consisted of cluster of elliptic cells 4–6 cells thick with no segregation into distinct layers observed (Fig. 2D), corresponding to 'intermediate II'-regenerating retina according to the definition of stages by Cheon and Saito (1999). Most of such elliptic cells in this stage are still proliferating (Negishi *et al*., 1992), suggesting that these cells are undifferentiated precursor cells. Both E-cadherin (Fig. 2E) and R-cadherin (Fig. 2F) were detected at the surface of these precursor cells, but SNAP-25 was not detected in these cells (Fig. 2G). Regenerating retinas at the stage between 'early' and 'intermediate II' stage were also investigated. These regenerating retinas consisted of cluster of precursor cells 3–4 cells thick, corresponding to 'intermediate I'-regenerating retina according to the definition of stages by Cheon and Saito (1999). Also in these retinas, both E-cadherin and R-cadherin were detected at the surface of the cells, but SNAP-25 was not detected (data not shown).

Photographs in Fig. 3 show immunofluorescence results for regenerating retina after retinal cells have differentiated and plexiform layers have developed (70 days after surgery). This regenerating retina corresponds to 'late'-regenerating

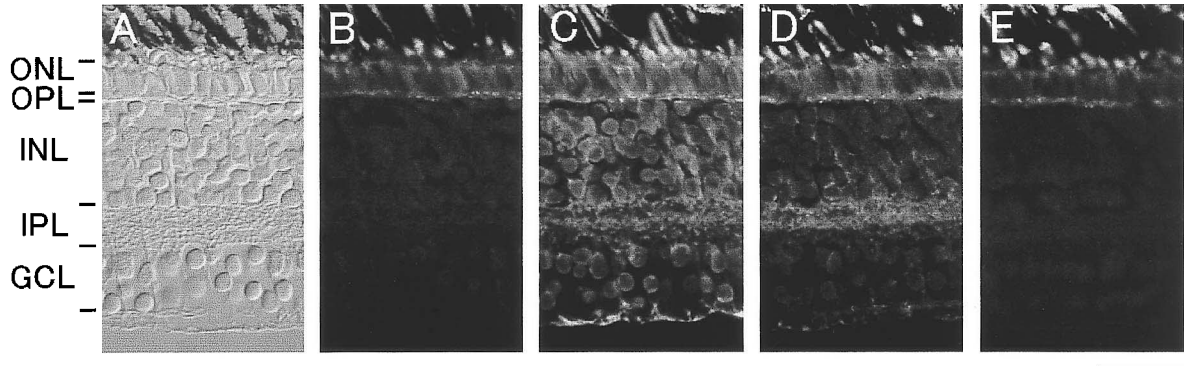

Fig. 1. Immunohistochemistry for E-cadherin (B), R-cadherin (C), and synaptosomal-associated protein 25 (SNAP-25) (D) in normal newt retina. (A) Difference interference contrast (DIC) photomicrograph of the same field as (B). (B) Immunoreactivity to anti-E-cadherin antibody. A faint fluorescence seen in the outer nuclear layer (ONL) and outer plexiform layer (OPL) was not the signal but autofluorescence (background), which was also seen in control section shown in (E). Therefore, immunoreactivity for E-cadherin is not observed. (C) Immunoreactivity to anti-R-cadherin antibody is observed in the plexiform layers and at the surface of all types of neurons in the nuclear layers. (D) Immunoreactivity to anti-SNAP-25 antibody is observed in the plexiform layers. (E) Negative control with no primary antibody. All of the sections were obtained from the same eyeball. Abbreviations: ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar: 50 μm.

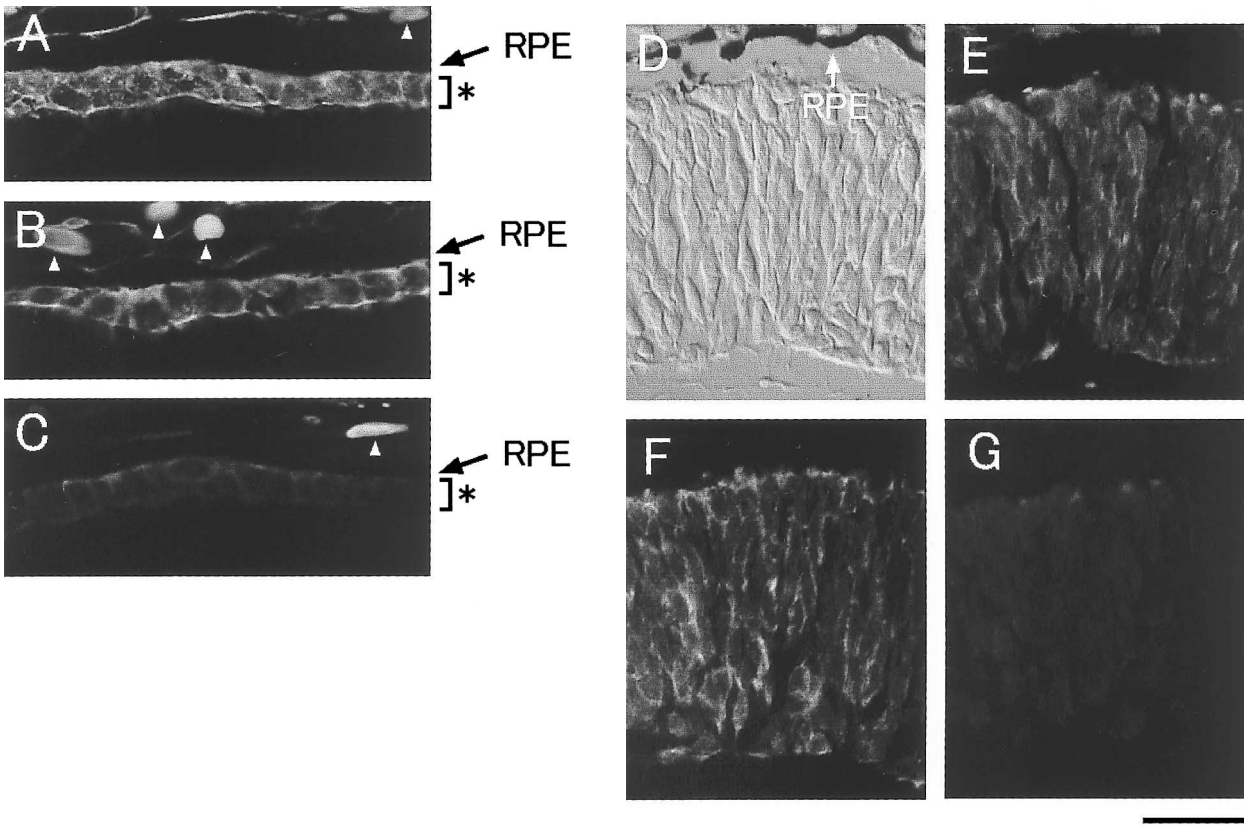

Fig. 2. Immunohistochemistry for E-cadherin (A and E), R-cadherin (B and F), and SNAP-25 (C and G) in regenerating retina 20 days after surgery (A–C), and in regenerating retina 30 days after surgery (D–G). (A–C) The results for regenerating retina 20 days after surgery. Line of cells 1-2 cells thick with non-pigmented cytoplasm (asterisks) was seen adjacent to the thin retinal pigment epithelial (RPE) cell layer. Fluorescence seen in the outside of the retinas (arrowhead) was not the signal but autofluorescence (background) of blood corpuscles. (A) Immunoreactivity to anti-E-cadherin antibody is observed at the surface of cells. (B) Immunoreactivity to anti-R-cadherin antibody is observed at the surface of cells. (C) Immunoreactivity to anti-SNAP-25 antibody is not observed. These sections were obtained from the same eyeball. (D–G) The results for regenerating retina 30 days after surgery. (D) Difference interference contrast (DIC) photomicrograph of the same field as (E). This regenerating retina consists of cluster of elliptic cells 4-6 cells thick. Retinal pigment epithelial (RPE) cell layer was artificially detached from prospective neural layer. (E) Immunoreactivity to anti-E-cadherin antibody is observed at the surface of cells. (F) Immunoreactivity to anti-R-cadherin antibody is observed at the surface of cells. (G) Immunoreactivity to anti-SNAP-25 antibody is not observed. These sections were obtained from the same eyeball. Scale bar: 50 μm.

retina, according to the definition of stages of regenerating retina by Cheon and Saito (1999). Fig. 3A, C, and E show the results of immunohistochemistry at the central region of this regenerating retina. In the central region, two distinct plexiform layers (OPL and IPL) were observed morphologically and SNAP-25 was detected in the plexiform layers (Fig. 3E). In this stage of regeneration, cell type-specific proteins such as opsin are expressed (Negishi *et al*., 1992) suggesting that retinal cells have differentiated. R-cadherin was detected at the surface of cell bodies of all types of neurons in the nuclear layers and at the neurites in the plexiform layers (Fig. 3C), but E-cadherin was not detected in these differentiated cells (Fig. 3A). Fig. 3B, D, and F show the results of immunohistochemistry at the peripheral region of the same regenerating retina. In the peripheral region, a cluster of cells, which had no plexiform layers, was observed (white asterisk). Such cells in the peripheral region are still proliferating (Negishi *et al*., 1992), suggesting that these cells are undifferentiated precursor cells. Not only R-cadherin (Fig. 3D) but also E-cadherin (Fig. 3B) was detected at the surface of these precursor cells, while SNAP-25 was not detected in these cells (Fig. 3F). However, in the region where the plexiform layers developed, SNAP-25 was detected in the plexiform layers (Fig. 3F). As far as investigated, at any stage of regeneration E-cadherin was never detected in the same region of retina where SNAP-25 was detected, suggesting that E-cadherin is not expressed by differentiated cells.

In the present study, E-cadherin was detected in undifferentiated precursor cells, but not detected in differentiated cells, suggesting that E-cadherin is expressed by undifferentiated precursor cells before cells differentiate and lost as the cells differentiate. On the other hand, R-cadherin was detected both in precursor cells and in differentiated cells, suggesting that R-cadherin continues to be expressed during regeneration. The difference in expression pattern suggests that each cadherin plays distinctive roles in retinal regeneration. And transient expression of E-cadherin before differentiation implies its essential roles for differentiation, because some

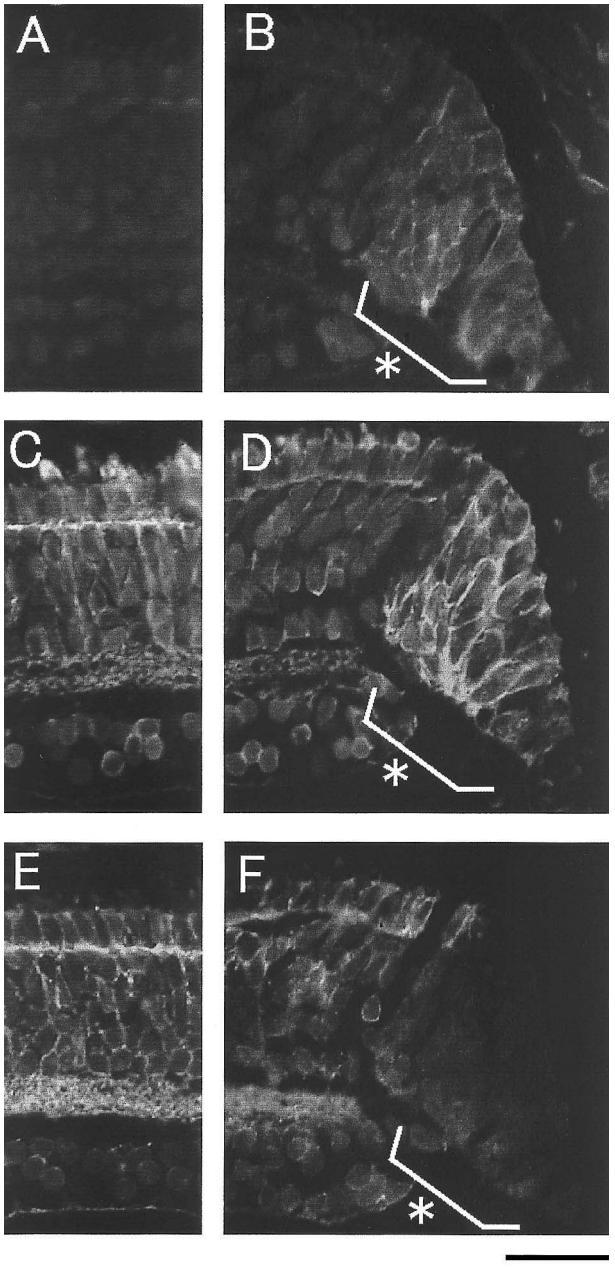

Fig. 3. Immunohistochemistry for E-cadherin (A and B), R-cadherin (C and D), and SNAP-25 (E and F) in the central region (A, C, and E) and in the peripheral region (B, D, and F) of regenerating retina 70 days after surgery. In the central region of the retina two distinct plexiform layers are observed morphologically, but in the peripheral region a cluster of undifferentiated precursor cells is observed (white asterisk). (A) Immunoreactivity to anti-E-cadherin antibody is not observed. (B) Immunoreactivity to anti-E-cadherin antibody is observed at the surface of the precursor cells (white asterisk). (C) Immunoreactivity to anti-R-cadherin antibody is observed in the plexiform layers and at the surface of all types of neurons in the nuclear layers. (D) Immunoreactivity to anti-R-cadherin antibody is observed at the surface of the precursor cells (white asterisk). (E) Immunoreactivity to anti-SNAP-25 antibody is observed in the plexiform layers. (F) Immunoreactivity to anti-SNAP-25 antibody is not observed in the precursor cells (white asterisk). However, in the region where the plexiform layers have developed, immunoreactivity is observed in the plexiform layers. All of the sections were obtained from the same eyeball. Scale bar: 50 μm.

signaling pathways for cell fate determination, such as the Notch signaling pathway, is mediated by cell-cell contacts (Austin *et al*., 1995; Dorsky *et al*., 1997). As well, this transience of expression would enable us to use E-cadherin as a marker of the precursor cells during regeneration. We also investigated expression of N-cadherin. N-cadherin was not detected in any neural cells of regenerating and normal retina, but it was detected on the lens epithelial cells (data not shown). The binding of anti N-cadherin antibody to the lens epithelial cells supports the possibility that N-cadherin is not expressed in the neural retina, and dispels the possibility of N-cadherin being present but undetected. In the developing chicken retina, N-cadherin is expressed in the neural retina from the beginning of its formation, thereafter gradually diminished from most part of the retina (Matsunaga *et al*., 1988; Inuzuka *et al*., 1991). And when N-cadherin function was blocked by incubation with antibody to N-cadherin, the retina tended to dissociate and could not be maintained (Matsunaga *et al*., 1988). Although the types of expressed cadherins differ between in the developing retina and in the regenerating retina, these facts suggest that cadherins play essential roles at retinal morphogenesis and that during regeneration E-cadherin substitutes for N-cadherin.

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