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Expression of p27(KIP1) and Cell Proliferation in Human Retina and Retinoblastoma

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Abstract. *Background: Retinoblastoma is a rare cancer of the eye, in which biallelic inactivation of the retinoblastoma gene is a hallmark. Although retinoblastoma protein (Rb) and p27(KIP1) block the cell cycle transition from G1- to S-phase, the interaction has not been confirmed in vivo. The aim of this study was to examine the correlation between the expression of p27(KIP1) and cell proliferation in human retina and retinoblastoma. Materials and Methods: Human retinoblastoma, surgically removed, was fixed by 4% paraformaldehyde. Then, paraffin-embedded tissue sections were examined using immunohistochemistry with anti-p27(KIP1) and anti-proliferating cell nuclear antigen (PCNA) antibodies. Results: Retinoblastoma tissue was adjacent to the normal retina in which tumor cells with homogeneous nuclei proliferated and it was impossible to identify the layer structure of the inner nuclear layer (INL) and the outer nuclear layer (ONL). In normal retina, PCNA-positive nuclei were not observed, whereas nuclear immunoreactivity for PCNA was detected in a variety of tumor cells. Many p27(KIP1)-positive nuclei were detected in INL and ONL, while p27(KIP1) immunoreactivity was not detected in retinoblastoma cells. Conclusion: The correlation between disappearance of p27(KIP1) and induction of proliferation activity suggests that functional loss of Rb leads to down-regulation of p27(KIP1) and uncontrolled retinal cell proliferation.*

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Retinoblastoma, a rare cancer of the eye that develops predominantly in children under four years of age, is the phenotype of hereditary cancers in humans. Biallelic inactivation of the retinoblastoma gene (RB-1) is a hallmark in hereditary and sporadic retinoblastomas (1, 2). The best-characterized biological activity of the retinoblastoma protein (Rb) is blocking the cell cycle transition from G1- to S-phase (2).

Cell cycle progression is controlled by a series of kinase complexes, composed of cyclins and cyclin-dependent kinases (CDKs) (3). The regulatory mechanism includes the action of CDK inhibitors (CKIs) (4, 5). It was recently shown that cyclin/CDK activity decreased in Rb-deficient condition (6). p27(KIP1) is one of the CKIs and elimination of p27(KIP1) during the late G1-phase is required for cell cycle progression from the G1- to S-phase in various cell lines (7-10). Forced expression of p27(KIP1) blocks cell cycle progression during the G1-phase, whereas targeted p27(KIP1) mRNA antisense vectors increase the fraction of cells in the S-phase. Although p27(KIP1) mRNA levels are constant during the cell cycle, in mitogen-stimulated cells the p27(KIP1) protein undergoes rapid degradation by the ubiquitin-proteasome pathway, and this proteolysis is dramatically reduced in resting cells (11). We have demonstrated that p27(KIP1) is involved in the proliferation of cells in the retina (12-15). Although it has been demonstrated that p27(KIP1) supported Rb's function to induce cell proliferation block and senescence (16), and that Rb inhibits p27(KIP1) ubiquitination and causes G1 arrest (17), the interaction between Rb and p27(KIP1) has not been confirmed *in vivo*.

In this study, the expression of p27(KIP1) in human retina and retinoblastoma was examined. In addition, the proliferating cell nuclear antigen (PCNA), one of the essential replication factors, which appeared at late G1-phase and reached at a peak level in S-phase of the cell cycle (18, 19) was analyzed.

Materials and Methods

Operative specimen. A one-year-old Japanese girl was diagnosed with retinoblastoma of the left eye clinically and underwent surgical resection in the Hokkaido University Hospital, Sapporo, Japan. She had not received preoperative radio- and/or chemotherapy. Slides prepared from the retinoblastoma were washed in phosphate-buffered saline (PBS), fixed in ice-cold 4% paraformaldehyde in 0.1 M borate buffer (pH 9.5) for 2 h and processed for paraffin sectioning.

Immunohistochemistry. The sections were dewaxed and rehydrated, rinsed twice in PBS, incubated with normal goat serum and then with a 1:1000 dilution of mouse monoclonal anti-p27(KIP1) antibody (C-19, sc-528, Santa Cruz Biotech, CA, USA) or anti-PCNA antibody (1:1000; Zymed, South San Francisco, CA, USA). Binding of the primary antibody was visualized by fluorescence microscopy using FITC-conjugated goat anti-mouse IgG (Jackson Immuno Research Laboratories, West Grove, PA, USA) at a dilution of 1:200. To examine the specificity of immunostaining, the primary antibody was replaced with Tris-buffered saline. Control slides were invariably negative for immunostaining. In addition, as a negative control for anti-p27(KIP1) immunohistochemistry, the anti-p27(KIP1) antibody was preincubated with 1 μg/ml of the blocking peptide for competition studies (sc-528P; Santa Cruz Biotech).

Results

At low magnification, the retinoblastoma showed an exophytic growth pattern. Retinoblastoma tissue was adjacent to the normal retina, in which tumor cells with homogeneous nuclei proliferated (Figure 1 a,b). Large or small irregular calcification was formed in the tumor tissues. The cellular distribution of the PCNA and p27(KIP1) was examined using immunohistochemistry in human retinoblastoma tissue. In normal retina, PCNA-positive nuclei were not observed (Figure 1c). In contrast, nuclear immunoreactivity for PCNA was detected in a variety of tumor cells (Figure 1c). Many p27(KIP1)-positive nuclei were noted in normal retina, whereas p27(KIP1) immunoreactivity was not detected in retinoblastoma cells (Figure 1d). In a negative control to test the specificity of the antibody used, the anti-p27(KIP1) antibody was completely blocked by incubation with the p27(KIP1) blocking peptide immunogen (Figure 1e).

At high magnification, the inner nuclear layer (INL) and outer nuclear layer (ONL) were confirmed in normal retina (Figure 2a). In retinoblastoma adjacent to the normal retina (Figure 2a, arrow), the number of atypical cells increased, and it was impossible to identify the layer structure of the INL and ONL. A variety of rosette-forming tumor cells were located in the tumor tissues. No immunoreactivity of PCNA was detected in normal retinal cells, whereas nuclear immunoreacitivity was noted in many retinoblastoma cells (Figure 2b). Many p27(KIP1)-positive nuclei were noted in INL and ONL in the normal part of the retina (Figure 2c). In contrast, no p27(KIP1)-immunopositive retinoblastoma cells were detected.

Discussion

The proliferation of human retinal cells ceases after 10 week's gestation during normal development (20). In this study, hematoxylin and eosin staining showed that human retinoblastoma tissue was adjacent to the normal retina, and it was impossible to identify the layer structure of the INL and the ONL in retinoblastoma. PCNA appeared at late G1-phase and reached a peak level in the S-phase of the cell cycle (18,19). On immunohistochemical examination, no PCNA-positive nuclei were observed in the retina, but they were detected in retinoblastoma tissue, indicating that retinoblastoma cells are in the S-phase (18,19). The elimination of p27(KIP1) during the late G1-phase is required for cell cycle progression from the G1- to S-phase (7-10). In this study, many p27(KIP1)-positive nuclei were noted in the INL and ONL of the human retina, but were not detected in retinoblastoma cells. These results suggest that the disappearance of p27(KIP1) correlates with proliferation activity in the normal retina and retinoblastoma.

Most clinical phenotypes of retinoblastoma can be explained by the double mutational inactivation of RB1 (1), the prototype of the tumor suppressor gene that controls cell cycle progression (2). In the non-hereditary form of the disease, both inactivating events occur during the somatic development of retinal cells and result in the relatively late onset of a single tumor in one eye. Recently, it was reported that p27(KIP1) supported Rb's function to induce proliferation block and senescence (16). It was also reported that Rb inhibits p27(KIP1) ubiquitination and causes G1 arrest (17). Taken together, the correlation between the disappearance of p27(KIP1) and proliferation activity suggests that functional loss of Rb leads to down-regulation of p27(KIP1) and uncontrolled retinal cell proliferation. The mechanism in regulation of p27(KIP1) degradation and cell proliferation might contribute to a novel therapeutic molecular targeting for human retinoblastoma.

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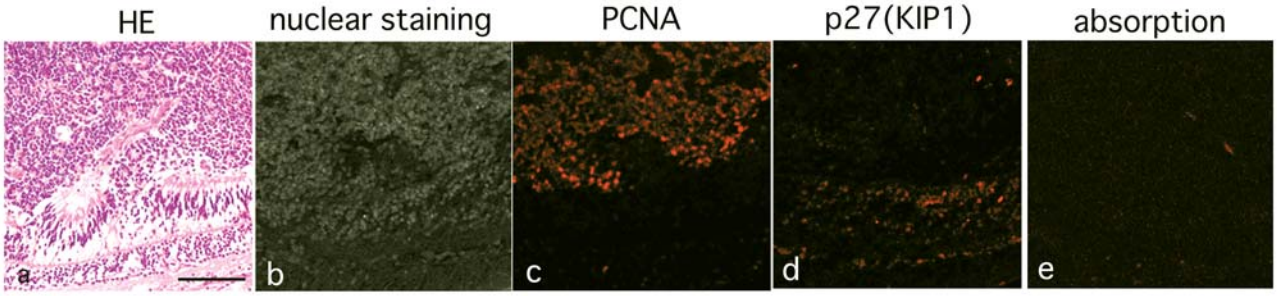

Figure 1. *Hematoxylin and eosin staining (a), YO-pro-1 nuclear staining (b), immunodetection of proliferating cell nuclear antigen (PCNA) (c) and p27(KIP1) (d) in human retinoblastoma. Retinoblastoma tissue was adjacent to the normal retina (a). Tumor cells with homogeneous nuclei proliferated (b). PCNA-positive nuclei were not observed in the normal retina, whereas nuclear immunoreactivity for PCNA was detected in retinoblastoma cells (c). Many p27(KIP1)-positive nuclei were noted in the normal retina, but were absent in retinoblastoma cells (d). In a negative control to test the specificity of the antibody used, the p27(KIP1) immunoreactivity was completely blocked by incubation with the p27(KIP1) blocking peptide immunogen (e). Bar indicates 50 μm.*

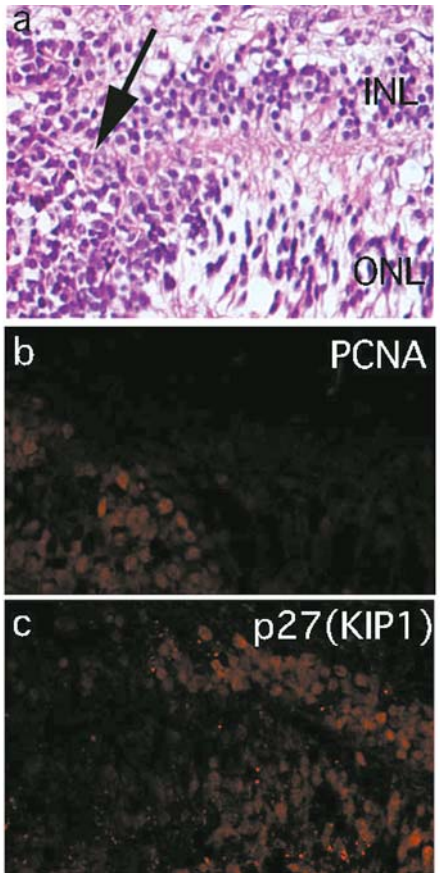

Figure 2. *Hematoxylin and eosin staining (a), immunodetection of proliferating cell nuclear antigen (PCNA) (b) and p27(KIP1) (c) in normal human retina adjacent to retinoblastoma tissue at high magnification. The inner nuclear layer (INL) and outer nuclear layer (ONL) were confirmed in the normal retina. The number of atypical cells increased and the layer structure of the INL and ONL was broken in retinoblastoma (a, arrow) as compared to the normal retina (a). No immunoreactivity of PCNA was detected in normal retinal cells, whereas nuclear immunoreactivity was noted in a variety of retinoblastoma cells (b). Many p27(KIP1)-positive nuclei were noted in the INL and ONL in the normal retina (c). In retinoblastoma cells, p27(KIP1) immunoreactivity was not detected.*

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