Apoptosis After Butyrate-Induced Differentiation in Retinoblastoma Cell Line Y-79

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Purpose: To study the fate of Y-79 human retinoblastoma cells after induction of differentiation.

Methods: Y-79 cells were cultured in a synthetic medium and were induced to neuronal differentiation by butyrate treatment. Neurofilaments, p53, and DNA-synthesizing nuclei labeled with 5-bromodeoxyuridine were immunostained, and apoptotic cells were labeled by in situ DNA nick end labeling (TUNEL). We combined these immunostaining and labeling methods to determine whether the cells expressed these markers at the same time. DNA fragmentation and p53 levels were also determined by electrophoresis.

Results: Y-79 cells proliferated in the synthetic medium. After butyrate treatment, they extended protrusions and increased neurofilament immunoreactivity. The differentiated features were striking on day 7. Thereafter, differentiated cells decreased and apoptotic cells increased. DNA synthesis was detected in the cells expressing immunoreactivity for neurofilaments or p53. At day 7, most of the cells with p53-positive nuclei were alive and neurofilament-positive. However, at day 10, the p53-positive cells were apoptotic and neurofilament-positive apoptotic cells accumulated.

Conclusions: We conclude that the Y-79 cells express p53 and undergo apoptosis after neuronal differentiation. There could be a p53-dependent apoptotic pathway in butyrate-induced differentiated Y-79 cells due to the inability to regulate cell cycling.

Key Words: Apoptosis, cell cycle, differentiation, p53, retinoblastoma.

Introduction

Retinoblastoma is a malignant tumor caused by a mutation in both alleles of the retinoblastoma-susceptibility gene, Rb.1,2 A p53 mutation is frequently found in many types of tumors, but it is rarely seen in human retinoblastoma cells.3 Retinoblastoma cells maintain their ability to differentiate, and in vivo in the tumor, they attempt to differentiate into specific retinal cell types.4–6

The Y-79 human retinoblastoma cell line, without a normal Rb gene,7 differentiates into several types of retinal cells in response to various substances.8–14 These earlier studies demonstrated that the Rb protein does not play a critical role in cell differentiation.

In retinoblastoma cells, p53 plays a key role in apoptosis induction as it does in other types of tumor cells. Immunoreactivity to p53 has been observed at the site where terminal deoxynucleotidyl transferase-mediated deoxyUTP nick end labeling (TUNEL)-positive cells were present in the tumor mass in vivo.15 Increased levels of p53 expression induced by forced expression of p5315 or gamma irradiation16 induced apoptosis in retinoblastoma cell lines.

In addition to inducing apoptosis, p53 plays a role in cell cycle control. The function of Rb is related to both roles of p53. Functional Rb protein inhibits the apoptosis induced by the overexpression of p5317 and by irradiation18 with increased levels of p53. The function of Rb is linked to p53 degradation.19 Expression of p53 induced by DNA damage and differentiation20–22 stops cell cycling at the entrance of the S phase through induction of cyclin-dependent kinase inhibitor, p21WAF1, which blocks phosphorylation of the Rb protein.23

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In normal development, terminal differentiation in neurons is thought to be closely related to cessation of proliferation. Although there have been several reports on the differentiation of retinoblastoma cells into putative neurons, the fate of the differentiated cells has not been determined. In the present study, we examined the fate of Y-79 retinoblastoma cells after the induction of neuronal differentiation. We shall show a loss of cell cycle control and the induction of apoptosis after the neuronal differentiation.

Materials and Methods

Cell Culture

Y-79 cells were maintained as stock cultures in Eagle’s MEM with Earle’s salt (GIBCO, Grand Island, NY, USA) supplemented with 50 IU/mL penicillin, 50 μg/mL streptomycin (ICN Biomedicals, Mesa, CA, USA), and 10% fetal bovine serum (FBS; Inter-gen, Purchase, NY, USA) (MEM-10% FBS).

To induce differentiation, the cells were cultured in a modified synthetic medium. The cells were collected, washed with serum-free MEM, and suspended in MEM containing a G5 supplement (GIBCO) (MEM-G5). The cell suspension was plated on poly-D-lysine (Boehringer Mannheim, GmbH, Germany) coated tissue culture vessels with 10 μg/mL laminin (Biomedical Technologies, Stoughton, MA, USA) added into MEM-G5. After 3 days, the medium was replaced with MEM-G5 containing 1 mM sodium butyrate as an inducer of neuronal differentiation, and the cells were maintained in the butyrate-containing medium thereafter. The cultures were maintained in a humidified atmosphere of 5% carbon dioxide and 95% air at 37°C.

Immunocytochemical and TUNEL Staining

Cultures were fixed with neutralized formalin for 10 minutes and washed with calcium- and magnesium-free Dulbecco’s phosphate buffer saline (DPBS). For immunocytochemical localization of neurofilaments, the cultures were incubated with anti-human neurofilament antibody, 2F11 (DAKO, Glostrup, Denmark), which reacts with neurofilament L and S, and stained with avidin biotin peroxidase system (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA, USA) with 3,3′-diaminobenzidine (DAB) as a chromogen. Nuclei were counterstained with contrast green solution (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). The immunopositive cells were counted on randomly selected photographed fields.

For the detection of apoptotic cells, we performed the TUNEL reaction with fluorescein-conjugated dUTP as substrate (in situ Cell Death Detection Kit; Boehringer Mannheim). TUNEL-reacted samples were treated with peroxidase-conjugated anti-fluorescein antibody and DAB. Nuclei were counterstained.

To double stain for neurofilaments and 5-bromodeoxyuridine (BrdU), the culture was labeled with 10 μM BrdU (Sigma, St. Louis, MO, USA) for 1 hour before fixation. Neurofilaments were stained with an inert polymer coupled to horseradish peroxidase and anti-neurofilament antibody 2F11 (EPOS, DAKO) with 3-amino-9-ethyl-carbazole (AEC) as a chromogen. After the immunostaining for neurofilaments, the culture was treated in 2 N hydrochloric acid to denature DNA and neutralized with 0.1 M borate buffer pH 9.0. After blocking with unlabeled anti-mouse IgG antibody (Vector Laboratories), the culture was reacted with anti-BrdU antibody (IU4; Caltag Laboratories, Burlingame, CA, USA), biotinylated anti-mouse IgG antibody and rhodamine avidin D (Vector Laboratories). Cultures were also double-immunostained for p53 and BrdU. The BrdU-labeled culture was reacted with anti-human p53 antibody, PAb 1801 (Cymbus Bioscience, Southampton, NJ, USA), Envision peroxidase system (DAKO) and AEC. Then the cultures were immunostained for BrdU as described above.

To triple stain for p53, TUNEL and neurofilaments, p53 was stained with anti-human p53 antibody PAb 1801 using Envision peroxidase system and AEC. Then the TUNEL method was performed using fluorescein conjugated deoxyUTP as substrate. Samples were blocked with unlabeled anti-mouse IgG antibody and the neurofilaments were immunostained with 2F11 anti-neurofilament antibody, biotinylated anti-mouse IgG, and rhodamine avidin D.

For negative controls of immunostaining, mouse IgG1 against Aspergillus niger oxidase (DAKO) was used instead of primary antibody in each immunostaining procedure and no staining was detected. For negative controls for the TUNEL method, terminal deoxytransferase was omitted from the reaction mixture, and no fluorescence or DAB was localized in the nuclei.

Samples were observed and photographed with an inverted microscope with fluorescence and bright field illumination.

Detection of DNA Fragmentation by Agarose Gel Electrophoresis

Culture media containing floating cells were transferred to a tube and the cells were collected by cen-
trifugation. Attached cells were lysed in 500 μL of 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA) (TE buffer), 100 mM sodium chloride and 1% sodium dodecyl sulfate (SDS) and transferred into the tube containing the pellet of the floating cells. The lysate was digested by 0.5 mg/mL proteinase K (Boehringer Mannheim) for 40 minutes at 50°C. The lysate was extracted in phenol-chloroform-isoamyl alcohol (25:24:1) and then in chloroform-isoamyl alcohol (24:1). Nucleic acid was precipitated from the aqueous phase with 1 mM sodium acetate (pH 5.2) and 2-propanol, washed with 70% ethanol and dried. The precipitate was dissolved in 50 μL of TE buffer and digested with ribonuclease A (Boehringer Mannheim). After measuring the absorbance at 260 nm, the same amount from each sample was loaded onto agarose gel for electrophoresis. DNA was observed with the fluorescence of ethidium bromide.

**SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis**

Floating and attached cells were collected, washed with DPBS, and dissolved with lysis buffer containing 50 mM Tris-HCl (pH 8.0), 120 mM sodium chloride, 0.5% SDS, 1 mg/mL pefabloc SC, 0.5 mg/mL EDTA, 10 μg/mL leupeptin, 10 μg/mL pepstain, and 1 μg/mL aprotinin (Boehringer Mannheim). An aliquot was taken for protein content determination by BCA protein assay reagent (Pierce, Rockford, IL, USA). Equal quantities of protein from the samples were loaded on Laemli’s discontinuous SDS-polyacrylamide gel. After electrophoresis, the proteins were blotted onto polyester supported nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA) and reacted with the anti-p53 antibody. Immunoreactive bands were detected on X-ray film by chemiluminescence reaction (Western Light; Tropix, Bedford, MA, USA) and densitometrically analyzed with the NIH Image software. After chemiluminescence detection, the membrane was stained with amidoblack to confirm the quantity of protein in each lane.

**Results**

**Proliferation and Morphology**

The cells in the stock culture maintained on uncoated culture vessels in MEM-10% FBS were globular in shape, formed aggregates, and attached loosely to the culture substrate. After the cells were plated on poly-D-lysine-coated vessels, they adhered more tightly to the coated surface, maintained their globular shape and proliferated exponentially in MEM-10% FBS (Figure 1).

The cell density was not determined after day 7 because the culture vessels could not hold enough medium to sustain the proliferation of the cells. When the cells were transferred to new vessels at a decreased cell density, they continued to proliferate at approximately the same rate.

The cells that were plated on poly-D-lysine-coated vessels in the synthetic medium continued to proliferate, but more slowly than in serum-supplemented stock culture.

After the addition of butyrate on day 3, the proliferation rate decreased (Figure 1), and the cells flattened on the substrate and extended protrusions from the cell bodies, as reported previously. The morphological changes were most notable on about day 7.

**Immunocytochemistry for Neurofilament and DNA Synthesis**

In the serum-supplemented culture or in the synthetic medium before the addition of butyrate, the dividing cells expressed strong immunoreactivity for neurofilaments, while most of the other cells showed only faint immunoreactivity for neurofilaments in the cytoplasm (Figure 2A).

At day 3, approximately 10% of cells, including dividing cells, showed immunoreactivity in the cytoplasm, but most did not possess protrusions. Positivity for neurofilaments in the cytoplasm and the protrusions of the cells was most evident on day 7.
Figure 2B) when 35% of cells harbored immunostained protrusions and/or strong immunoreactivity in the cytoplasm. Thereafter, the cells showing immunopositive protrusions or cytoplasm decreased and globular cells increased (Figures 2C and D). The percentages of immunopositive cells were 18% and 8% at days 10 and 14, respectively. The morphological changes and immunocytochemical staining for neurofilaments demonstrated that the cells differentiated into putative neurons after the addition of butyrate.

To determine whether the differentiated cells synthesized DNA, we labeled the culture with BrdU for 1 hour before fixation on day 7 and double-stained for neurofilaments and BrdU (Figure 3).

None of the chromosomes in any of the dividing cells were stained with anti-BrdU antibody, which indicated that the BrdU-labeled cells had not divided during the labeling time of 1 hour. The labeled cells were expected to stay in the S or G2 phase of the cell cycle. BrdU-positive nuclei were found in the cells with neurofilament-positive cytoplasm and protrusions demonstrating that differentiated cells synthesized DNA.

Detection of Apoptosis

The TUNEL-positive nuclei were rarely found in cells in the stock culture. Following the induction of differentiation by butyrate, the number of cells with TUNEL-positive nuclei increased significantly during the later culture periods (Figure 4).

Apoptotic cells did not adhere to the substrate as tightly as living cells and were more easily lost by the repeated washing steps of the TUNEL procedure. In order to examine the degree of apoptosis in the total cell population, we collected floating and attached cells, purified the DNA and performed agarose gel electrophoresis of the DNA (Figure 5).

No ladder pattern was observed in the DNA of the cells in stock culture, but DNA laddering, a characteristic of apoptosis, became detectable after induction of differentiation by butyrate. The intensity of the laddering pattern of DNA increased toward the end of the culture period.

Immunodetection of p53 and DNA Synthesis

We determined the level of p53 protein by Western blot analysis (Figure 6).
Densitometric scanning showed that the immunoreactivity for p53 was increased on day 3 and reached the first peak on day 7 when most cells showed differentiated features. The immunoreactivity for p53 then decreased on day 10 and increased again on day 14. The decrease of immunoreactivity on day 10 was detected in three independent experiments.

We assumed that p53 regulated the cell cycle only partially because the Y-79 retinoblastoma cell line lacks normal Rb function. To determine whether the cells synthesized DNA while expressing p53 in their nuclei, double immunostaining for p53 and BrdU was performed on the culture labeled with BrdU for 1 hour before fixation. Some cells expressed immunoreactivity to both p53 and BrdU in the same nuclei (Figures 7A and B).

These results indicated that p53 failed to stop cell cycling at the S phase entrance in these cells, and these cells synthesized DNA even though they were expressing p53 in their nuclei.

**Triple staining for p53, Neurofilament, and TUNEL**

Butyrate induced differentiation, increase of p53 expression, and later apoptosis of the differentiated cells. To determine whether the differentiated cells expressed p53 and underwent apoptosis, we stained for p53 and neurofilaments immunocytochemically, and labeled the apoptotic nuclei by the TUNEL method.

In serum-supplemented stock cultures and cultures on day 3, there were no cells harboring both p53 immunoreactivity and TUNEL stain in the same nuclei (data not shown). The percentage of p53- and TUNEL-positive cells to the total population of cells, and the percentage of neurofilament-positive cells to the cells in each fraction are shown in Table 1. Cells harboring p53 immunoreactivity and TUNEL stain were first detected on day 7, although the ratio was low (Table 1). There were more cells with p53-immunoreactive nuclei than TUNEL-positive cells (Table 1, Figures 8A and B).

The cells with p53 immunoreactive nuclei often showed intense neurofilament immunoreactivity (Table 1, Figure 8C). The TUNEL-positive cells without p53 immunoreactivity were smaller than the other cells, had no protrusions, and were usually neurofilament-negative (Figure 8C).
On day 10, the percentage of neurofilament-positive cells to the cells in p53-positive fraction with or without TUNEL positivity did not change, but the main population of p53-positive fraction changed from TUNEL-negative on day 7 to TUNEL-positive on day 10. The TUNEL-positive fraction without p53 positivity also increased and the neurofilament-positive cells accumulated in this fraction (Table 1). Intense neurofilament staining was often observed in the middle-sized cells with positivity for p53 and TUNEL (Figures 8D–F).

**Table 1. Percentage of p53- and/or TUNEL-Positive Cells to Total Cell Population**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Day 7 (n = 1344)</th>
<th>Day 10 (n = 1360)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53 (+) TUNEL (-)</td>
<td>14.1 (87.9)*</td>
<td>3.0 (85.4)*</td>
</tr>
<tr>
<td>p53 (+) TUNEL (+)</td>
<td>1.6 (85.7)*</td>
<td>8.3 (87.6)*</td>
</tr>
<tr>
<td>p53 (-) TUNEL (+)</td>
<td>6.8 (31.5)*</td>
<td>27.6 (76.1)*</td>
</tr>
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*Values in parentheses are percentages of neurofilament-positive cells to cells in each fraction.

**Discussion**

Under our culture conditions, Y-79 cells changed their morphology to neuron-like cells with protrusions and increased neurofilament immunoreactivity in the synthetic medium after the addition of butyrate. With time, the number of differentiated cells decreased and apoptotic cells increased. However, the total cell number continued to increase during the culture period. There are two possible explanations for this apparent discrepancy. First, there are two types of cells; one type differentiated into the neuronal cells and then underwent apoptosis, and the other type continued to proliferate in the undifferentiated state without responding to butyrate. Second, part of the Y-79 cells which had once differentiated returned to the undifferentiated state and continued to proliferate instead of undergoing apoptosis. Although the switching between cell death and cell proliferation pathways is of great interest, we have focused on the cell death pathway in this study.

On day 7, the cells showed the most differentiated morphology and highest p53 immunoreactivity, the p53 immunoreactivity was mainly observed in the living neurofilament-positive cells. The co-localization of p53 immunoreactivity and TUNEL stain in the cells indicated that these cells had undergone apoptosis expressing p53. Many of the TUNEL-positive cells without p53 immunoreactivity did not show intense neurofilament immunoreactivity. We do not know whether these apoptotic cells on day 7 had died while undergoing differentiation, or had differentiated and lost immunoreactivity to neurofilaments before apoptosis. The low number of neurofilament-positive cells in the TUNEL-positive cells suggests that many differentiated cells survived without undergoing apoptosis at this time.

On day 10, on the other hand, neurofilament-positive cells with TUNEL-positive nuclei increased. The presence of TUNEL-positive cells harboring neurofilament immunoreactivity suggests that apoptosis occurred in the differentiated cells. This can explain the decrease of differentiated cells at the later culture periods. Although neurofilament positivity in p53-positive cells with or without TUNEL stain did not change, the number of the cells positive for both p53 and TUNEL increased. Cells with intense neurofila-
ment positivity often expressed both p53 immunoreactivity and TUNEL positivity, indicating that apoptosis expressed p53. These results suggest that the differentiated cells expressed p53, underwent apoptosis expressing p53 and then lost p53 immunoreactivity.

The necessity for p53 expression for the induction of apoptosis in the central nervous system (CNS) is controversial. For example, the CNS of p53 null zygotic mice develops normally, suggesting that p53 was not necessary for apoptosis in the developmental processes of the CNS. In addition, p53-independent apoptosis is induced in neurons by some treatment that does not directly damage DNA or chromosomes. Butyrate does induce apoptosis at a higher concentration than the concentration that induces differentiation in the Y-79 cell line without increasing levels of p53. Butyrate has been shown to induce apoptosis even in p53-deficient fibroblasts. However, p53 is capable of inducing apoptosis in neuronal cells of the CNS under pathological conditions which induce DNA damage or excitotoxicity. Overexpression of p53 itself induces apoptosis in neurons.

Although there are reports that butyrate diminishes p53 expression, the levels of p53 were increased by the addition of butyrate in our study. The TUNEL-positive cells with p53 immunoreactivity appeared after the addition of butyrate. Most of these cells were immunopositive for neurofilaments. They increased and were accompanied by an increase of apoptotic cells on day 10. Even though there could be p53-independent apoptotic pathways in the Y-79 cells induced by butyrate, increased levels of p53 followed by apoptosis and the coincidence of p53 immunopositivity and TUNEL positivity in the same cells imply the presence of a p53-dependent pathway in the cells after differentiation.

In human retinoblastoma, cells differentiate into different types of retinal cells in vivo and in vitro. Thus, defects of the Rb gene do not completely abolish the ability to differentiate, although Rb plays a role in cell differentiation in other neurons. Substances that induce neuronal differentiation also decrease proliferative activities after neuronal induction by up-regulating Rb function. The factors that regulate Rb function by phosphorylation, such as cyclin-dependent kinase and p21 WAF also influence neuronal differentiation. In our culture conditions, Y-79 cells could not survive in a differentiated state and so underwent apoptosis. Rb function might be required for survival in the differentiated state after neuronal induction of Y-79 cells.

Persistence of DNA synthesis seems to inhibit neuronal differentiation or reduce the stability of differentiated neuronal cells. Forced DNA synthesis or up-regulation of factors that lead cells into S phase induces apoptosis in various cells including neuronal cells. Lack of Rb function in Y-79 cells suggests an unscheduled S phase entry because of the failure of cell cycle control related to the Rb gene. In our study, some cells synthesized DNA when they were morphologically differentiated expressing intense neurofilament immunoreactivity. Moreover, some cells synthesized DNA even when they were expressing p53 immunoreactivity in their nuclei. These cells were thought to be undergoing unscheduled DNA synthesis. If this unscheduled DNA synthesis induces p53-triggered apoptosis instead of cell proliferation, it would explain the decrease of differentiated cells at later culture times.

Stimulation of neuronal differentiation of Y-79 cells is correlated with the increase of p53 activity. The increased levels of p53 and the inability to halt DNA synthesis may induce p53-triggered apoptosis in the cells lacking normal Rb function. Although introduction of the Rb1 gene itself failed to reverse the malignant phenotype of Y-79 in the undifferentiated state, how this gene affects cell differentiation remains unresolved. Reintroduction of a functional cell cycle control system may revive the stability of neuronal differentiation in retinoblastoma cells.

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