Missing Expression of pRb2/p130 in Human Retinoblastomas Is Associated with Reduced Apoptosis and Lesser Differentiation

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Purpose. Although the retinoblastoma gene (RB/p105) has been intensely investigated as a prototype suppressor gene in humans, mutational data on the Rb family member pRb2/p130 (p130) has only recently been reported. A protective role against apoptosis has been suggested for pRb/p105, both in vitro and in vivo. However, only limited information is available on the role of pRb2/p130 in controlling apoptosis. The purpose of this study was to determine the extent of a role of this gene in the neoplasms that give the Rb family its name.

Methods. Forty-two human retinoblastomas were retrospectively examined by immunohistochemical labeling of the Rb-related proteins and the results compared with cellular kinetic characteristics: the apoptotic index (AI) and the mitotic index (MI).

Results. The retinoblastomas that did not express p130 showed a significantly lower AI than those that expressed p130. This result was also supported by flow cytometry on a human Saos-2 cell line that was transiently transfected with RB2/p130. The $p130^-$ tumors displayed a lesser degree of differentiation than the $p130^+$ ones.

Conclusions. These observations give evidence that expression of p130 is inversely correlated with higher rates of apoptosis in human retinoblastomas and give an additional example of this regulator's role in cellular differentiation. (*Invest Ophthalmol Vis Sci.* 2002;43:3602–3608)

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Research in recent years has markedly advanced our knowledge of the functions of the retinoblastoma (Rb) tumor-suppressor family members and their products, pRb/p105, pRb2/p130 (p130), and p107, in healthy eukaryotic organisms and in neoplastic growth.[1] The Rb family is not only involved in the control of cell cycle progression and proliferation, but also participates in the regulation of differentiation and apoptosis. The retinoblastoma gene (RB/p105), the inactivation of which is closely related to many forms of neoplastic growth, has been intensely investigated as a prototypic tumor-suppressor gene.[2,3] Mutational data on the Rb family member p130, however, has only recently been reported, and preliminary data suggest that p130 may have some role in oncogenesis.[4]

A protective role against apoptosis has been well documented for pRb/p105, both in vitro[5–7] and in vivo.[8] However, little information is available on the role of p130 in the regulation of apoptosis.[9] This prompted us to ask whether p130, which was originally identified in the laboratory of a member of our group,[10] exerts a corresponding action in vivo. We retrospectively examined human retinoblastomas by immunohistochemical and Western blot analysis of the Rb-related proteins and compared the results with the cellular kinetic characteristics—the apoptotic index [AI] and the mitotic index [MI]—of the neoplasms that gave the Rb family of cell cycle regulators its name. Herein, we report that retinoblastomas that did not express p130 showed a significantly lower AI than the others, suggesting that p130 may have a proapoptotic effect. This result was also supported by flow cytometric analysis of the human Saos-2 cell line, which was transiently transfected with p130. We also report that the $p130^-$ tumors displayed a lesser degree of differentiation than the $p130^+$ ones.

Methods

Case Selection and Processing of Tissue for Histologic Evaluation

The paraffin blocks of a total of 42 pretreatment surgical specimens of ocular retinoblastomas were collected at the Institute of Pathologic Anatomy and Histology, University of Siena, Siena, Italy. Pertinent clinicopathologic data are summarized in Table 1. Tissues had been obtained at different hours of the day and were cut and fixed in a buffered 4% aqueous formaldehyde solution (pH 7.4). For conventional histology, 4-μm-thick sections were obtained from representative paraffin blocks and stained with hemalum and eosin, Giemsa, periodic acid-Schiff (PAS), Gomori silver impregnation, and Feulgen.

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TABLE 1. Clinicopathologic Data and Descriptive Statistics of the Retinoblastomas Tested

	p130$^+$	p130$^-$
Age of patients (range in months)	6–120	12–120
Sex of patients		
Male	18	9
Female	10	5
Inheritance		
Familial	2	0
Sporadic	26	14
Laterality of tumors		
Unilateral	16	12
Bilateral	12	2
Optic nerve invasion		
Absent	17	9
Resected	9	5
Unresected	2	0
Optic or ocular coats invasion		
Absent	17	9
Into choroid	7	4
Into sclera	4	1
Survival		
Alive	14	20
Deceased	3	5
Differentiation		
Poorly differentiated	13	14
Well differentiated	15	0
MI ± SD (%)*	1.26 ± 0.75	1.16 ± 0.57
AI ± SD (%)*	3.26 ± 2.23	1.01 ± 0.86

$n = 42$.
* Refers to tumor cells.

Qualitative and Semiquantitative Assessment of Histology

Diagnoses, made independently by two experienced pathologists (CB and SL), were based on the World Health Organization's International Histologic Classification of Tumors of the Eye and its Adnexa.[11] The degree of differentiation was evaluated by test point analysis.[12] In particular, in each case 20 randomly chosen fields (28,000 μm^2/field), consisting of cellular areas of the diseased tissue without necrosis, were analyzed by using a projected grid.[13] The relative frequency of rosettes of the Flexner-Wintersteiner or Homer-Wright type and fleurettes (abortive elements of photoreceptor cells arranged in small groups resembling fleurs-de-lis) was registered and expressed as a percentage of differentiated retinoblastoma tissue.

Immunohistochemistry

We used an immunohistochemical detection system/HRP method (EnVision; Dako, Milan, Italy) to visualize immunohistochemical reaction products.[14] Antigen retrieval was achieved by the treatment of a deparaffinized section of 3 to 4 μm thickness with microwaves[15] and by pressure cooking in 1 mM EDTA (pH 8.0) for 5 minutes, followed by cooling at room temperature before incubation with the antibodies.[16] The monoclonal antibody anti-p130 was obtained from Transduction Laboratories (Lexington, KY) and was used at a dilution of 1:100 in Tris-buffered saline (TBS). We replaced the primary antibodies with normal mouse serum to obtain the negative control. Normal human tonsils served as the positive control.

Western Blot Analysis

To confirm the immunohistochemical results, we performed a Western blot analysis on three fresh samples of primary tumors that were shown by immunohistochemistry to be either positive or negative for p130. One normal retina sample, obtained from a patient who had undergone enucleation for painful absolute glaucoma, served as an experimental control. Fresh tissues were immediately frozen in liquid nitrogen. Whole tissue lysates were prepared by resuspending homogenized tissues in lysis buffer (50 mM Tris/HCl, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.1% Triton-X, 0.1 mM Na_3VO_4 plus fresh inhibitors). Cells were pelleted and then resuspended in lysis buffer. Total extracts (40 μg) were loaded and resolved on a 7% SDS-polyacrylamide gel. The gel was then transferred onto a nitrocellulose filter and checked by using 0.1% ponceau red. The anti-p130 (Transduction Laboratories) was used at a dilution of 1:500. The anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used according to the manufacturer's instructions.

Cell Counts

Cells exhibiting a positive immune reaction were counted in 20 randomly chosen high-power fields (HPF, 56,000 μm^2 each) of retinoblastoma tissue without necrotic areas. Results were expressed as a percentage of all neoplastic cells in the respective areas. A semiquantitative score of p130 staining intensity (i.e., the amount of reaction product per cell) was assessed as follows: 0, negative for nuclear staining in neoplastic cells; 1, weak nuclear staining in more than 50% neoplastic cells; 2, moderate nuclear staining in more than 50% neoplastic cells; and 3, strong nuclear staining in more than 50% neoplastic cells.[17]

Establishment of MI and AI

Mitotic figures and apoptotic cells or bodies can be recognized more easily in semithin resin-embedded sections than in paraffin-embedded sections.[18] We therefore re-embedded, in each case, part of the retinoblastoma tissue into Epon-Araldite resin (Monojet, Kildare, Ireland) and stained 1-μm sections with Azur A. First, running means ± SD of the number of intact neoplastic cells per HPF were registered in 20 randomly chosen HPFs, using an oil immersion objective (100×). We then assessed the MI and the AI by registering the numbers of neoplastic cells in mitotic and apoptotic cells or bodies in each HPF of an entire section (often >100 HPF) to obtain the MI and AI per case. With this approach, we obtained an AI per case that corresponded well with the percentage of cells exhibiting in situ end labeling (ISEL) of DNA to show strand breaks,[19] with intra- and interobserver reproducibility of counts at more than 95%.[20]

Plasmids

The construct consisting of the full-length RB2/p130 was cloned into a plasmid (pCDNA3-HA Tag; Invitrogen, San Diego, CA), containing the hemoagglutinin (HA) repeats and a c-*myc* nuclear localization signal (NLS). To construct pCDNA3, the Rb2-HANLS plasmid was cut with *Eco*RV and *Xba*I, and the full-length RB2/p130 (1–3486) was inserted.

Cell Lines

The human osteosarcoma Saos-2 cell line, which does not express p53 and contains a nonfunctional form of the retinoblastoma gene product pRb/p105, was used for transfection with p130. The Saos-2 cell line was grown in DMEM supplemented with 10% heat-inactivated FBS, 1% L-glutamine, 1% penicillin-streptavidin at 37°C at 5% CO_2. For the overexpression of p130, cells were transiently transfected with 10 μg pCDNA3-Rb2-HA Tag or with 10 μg pCDNA3 as a negative control, with a calcium phosphate kit (Invitrogen), according to the manufacturer's recommendations. The experiment was performed in triplicate and repeated three times. Transfection efficiency was checked by Western blot analysis.

For morphologic observations, pellets of Saos-2 cells overexpressing p130, Saos-2 cells containing the empty vector, and the untransfected Saos-2 cells, as controls, were fixed in 10% formaldehyde, embedded in paraffin, and analyzed by the ISEL method to detect apoptosis.[20]

Flow Cytometry

Saos-2 cells overexpressing p130, Saos-2 cells containing the empty vector, and the untransfected Saos-2 cells, as controls, were used for flow cytometry. Both attached and supernatant cells were collected and used for the assay to maintain a comparable cell number in all samples. Supravital exposure to propidium iodide (PI) of unfixed cells was applied to detect apoptosis.[21] Briefly, 48 hours from transfection nonpermeabilized cells were incubated during the last 30 minutes with 50 μg/mL PI, in medium. Cells were washed twice in PBS to remove PI and then harvested by trypsinization. Cells were pelleted by centrifugation, washed with PBS, and analyzed by flow cytometry (FACStar Plus; BD Biosciences, Mountain View, CA) equipped with an argon ion laser tuned at 488 nm. The filter used was a 650-nm band pass. In addition, at each experiment samples were subjected to forward scatter-side scatter (FSC/SSC) analysis (data not shown). It is well known that, because of cell shrinkage, apoptotic cells generally have decreased FSC. For this reason, FSC usually represents a gross indicator of morphologic changes that occur in cells that are undergoing apoptosis.

Statistical Analyses

Descriptive statistics, including frequency counts for categorical variables, interval ranges for survival time and mean ± SD for quantitative variables, were calculated separately for the two groups: $p130^-$ and $p130^+$ retinoblastomas. The equality of $p130^+$ and $p130^-$ group means of MIs and AIs and the percentage of differentiated retinoblastoma tissues was tested simultaneously by the multivariate Hotelling T^2 test, transforming the statistics to an equivalent F.[22] A univariate F test was also performed to evaluate the separate effect of single variables.

A 95% tolerance ellipse of the $p130^-$ group for pairs of variables was calculated and plotted. Pearson and Spearman correlation analyses (respectively, correlation coefficient r and ρ) were performed between quantitative variables. An associated F test was used to evaluate the significance of the square correlation coefficient r^2. When a significant probability of error of $P < 0.05$ was found, the regression lines were computed as well.

Results

Qualitative and Semiquantitative Observations

The retinoblastomas studied exhibited a considerable amount of regional, probably ischemic, necrosis. This well-known phenomenon led us to restrict the cell counts that were clearly outside the disintegrated areas. With rare exceptions, mitotic figures and apoptotic cells and bodies were easily recognized by light microscopy of Azur A–stained semithin sections. The immunohistochemistry analysis revealed that, in 14 cases, no retinoblastoma cells expressed p130 ($p130^-$ group). In all these cases, endothelial cells and fibroblasts showed a nuclear stain for p130 and served as an inner positive control. In the remaining cases ($n = 28$) the expression of p130 in retinoblastoma cells was variable, ranging from 5% to 90% ($p130^+$ group median: 40%; mean: 44.96%) The intensity of p130 immunostaining correlated significantly with the percentages of positive cells per case (Spearman $\rho = 0953$; $P < 0001$) and the degree of tumor differentiation ($\rho = 0659$; $P < 0001$). Examples of the histology and immunohistochemical staining of retinoblastomas are shown in Figure 1.

Expression of pRb2/p130 in Normal Retina and Primary Retinoblastoma

To validate the results of immunohistochemistry, we performed Western blot analysis in samples of primary tumors that were negative and positive for immunohistochemical detection of p130. Figure 2 shows that the expression of p130 was strongly decreased in the primary retinoblastoma that was shown to be negative for p130 by immunohistochemistry compared with expression in tumors that were shown to be positive for p130 and in the normal retina.

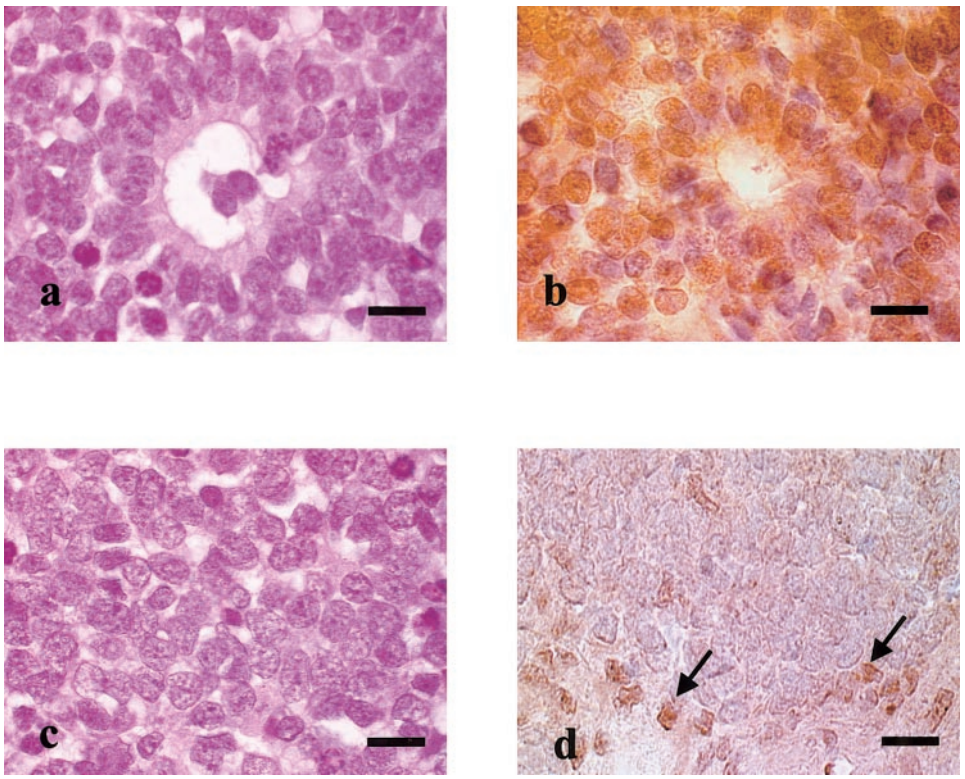

Figure 1. Examples of histology (**a**, **c**; hemalum-eosin) and immunostaining (**b**, **d**; nuclear) for p130 of differentiated (**a**, **b**) and undifferentiated (**c**, **d**) human retinoblastomas. (**d**, *arrow*) Inner positive control of endothelial cells and fibroblasts. Original magnification, ×970.

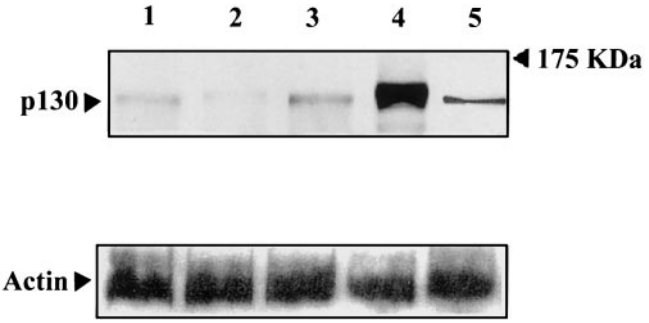

FIGURE 2. *Top*: Western blot analysis for p130 in primary retinoblastomas and normal retina. *Lanes 1* and *3*: $p130^+$ tumors by immunohistochemistry in 20% and 70%, respectively, of neoplastic cells; *lane 2*: $p130^-$ tumor by immunohistochemistry; *lane 4*: Jurkat cells, used as the control; *lane 5*: normal retina. Expression of p130 was strongly decreased in $p130^-$ tumors, compared with that in $p130^+$ tumors and normal retina. *Bottom*: actin control.

Degree of Differentiation and AIs of Retinoblastomas

The degree of differentiation per case, evaluated by test point analysis, is shown in Figure 3 as a function of the respective AI. In this plot, two distinct but overlapping groups can be seen: one with low AIs and a very limited grade of differentiation, comprising all $p130^-$ tumors, and one with significantly higher AIs ($P < 0.001$) and generally more pronounced differentiation ($P < 0.005$).

Comparison of the Expression of p130 with the AIs

In the 14 cases in which the retinoblastoma cells did not express p130 ($p130^-$ group) the AI was significantly lower than in the $p130^+$ group ($P < 0.001$; Fig. 4). In the latter, there was a positive linear correlation between the AIs and the percentages of $p130^+$ tumor cells ($r^2 = 0.5358$).

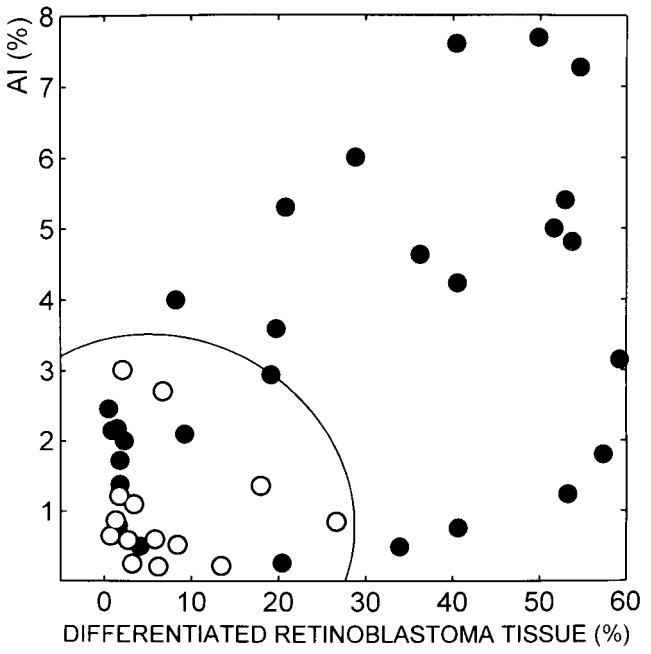

FIGURE 3. Apoptosis and differentiation in human retinoblastomas. Plot of AIs per case against percentage differentiation of the respective tumors. (●) $p130^+$ retinoblastomas; (○) $p130^-$ retinoblastomas. The cluster of $p130^-$ retinoblastomas is indicated by a 95% tolerance ellipse.

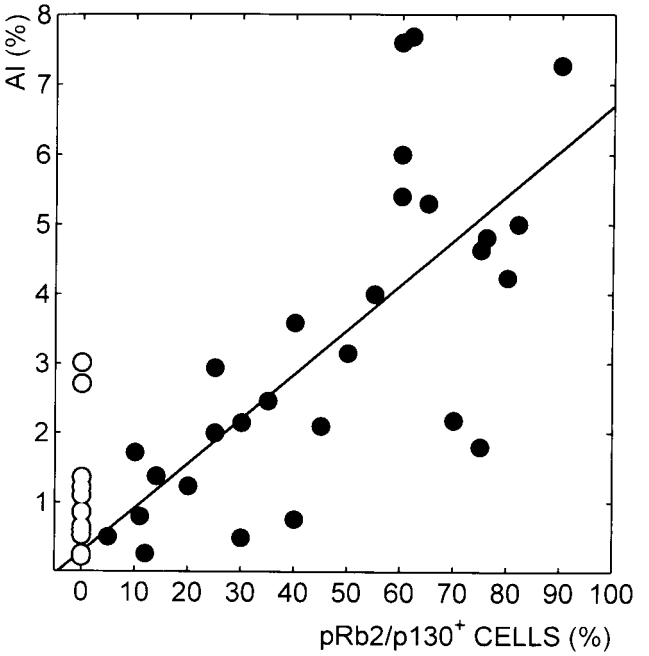

FIGURE 4. Apoptosis and expression of p130 in human retinoblastomas: plot of AI per case against the respective percentages of $p130^+$ tumor cells. The regression line for $p130^+$ retinoblastomas is also shown. (●) $p130^+$ retinoblastomas; (○) $p130^-$ retinoblastomas. See also legend to Figure 3.

AIs Plotted against MIs

The cluster of $p130^-$ retinoblastomas in the lower left of Figure 5 differed significantly (Hotelling test; $P = 0.004$) from

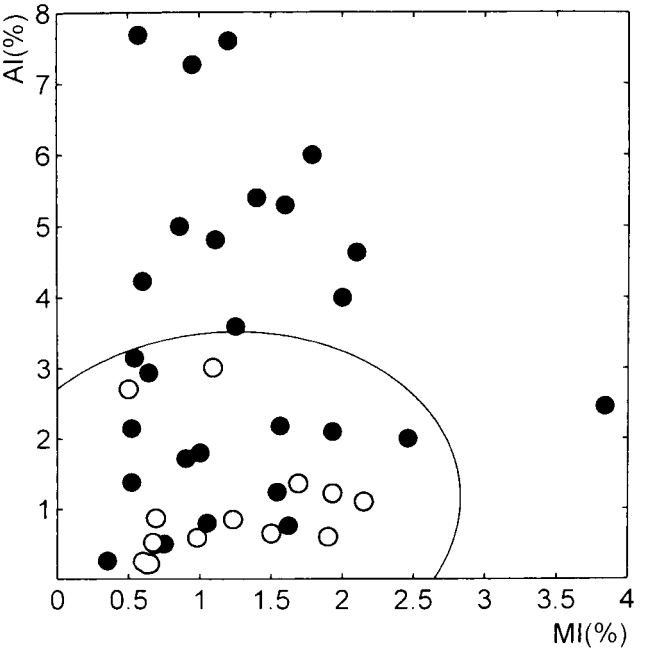

FIGURE 5. Apoptosis and mitotic activity in human retinoblastomas: AI per case plotted against the respective MIs. (●) $p130^+$ retinoblastomas; (○) $p130^-$ retinoblastomas. See also legend to Figure 3.

the cohort of tumors that contained $p130^+$ cells, in that the cells showed distinctly lower AIs, although some overlap was observed. The MIs were almost the same in both subgroups. The correlations between MI and AI in both the $p130^-$ and $p130^+$ neoplasms were not significant.

Effect of Overexpression of p130 on Apoptosis in Saos-2 Cells

Forty-eight hours after transfection, Saos-2 cells that overexpressed p130 appeared to be irregularly shaped, and the medium contained membranous debris, by light microscopy. The ISEL method used on the pellet of cells overexpressing p130 detected many apoptotic cells and bodies compared with the number of apoptotic wild-type cells (Fig. 6). Supravital PI staining, which recognizes early apoptotic cells even before the appearance of a hypodiploid peak,[21] showed the presence of such cells in the sample overexpressing p130, whereas no apoptosis was observed in wild-type cells and in those containing the empty vector (Fig. 7). These results suggest that overexpression of p130 dramatically enhances the percentage of apoptosis in Saos-2 cells.

Discussion

In the present study, we showed for the first time that, taken together, human retinoblastomas that do not express (i.e., do not show immunoreactivity to) p130 have, as a group, significantly lower AIs than do the $p130^+$ tumors. This suggests that p130 may exert a proapoptotic effect in vivo on the tumors that gave the Rb family of cell cycle regulators its name. In particular, among retinoblastomas exhibiting $p130^+$ cells, the positive linear correlation between the percentages of these cells and the AI per case is in line with this conclusion. The observation that the same protein promotes apoptosis when transiently overexpressed in the human Saos-2 cell line also supports the idea that p130 may have some role in the control of apoptosis. It thus seems that the enhancing effect of p130 on apoptosis is a more general phenomenon, which contradicts the notion that all members of the Rb family tend to suppress this type of cell death.

The second pertinent result was that all $p130^-$ retinoblastomas were not, or were only poorly, differentiated, which suggests that p130 promotes cell differentiation. Additional observations made in the present study are of lesser importance. The MI of $p130^-$ and $p130^+$ tumors did not differ noticeably, suggesting that the cell production rate was approximately the same in the two groups. This suggests that the presence or absence of p130 had a greater influence on the

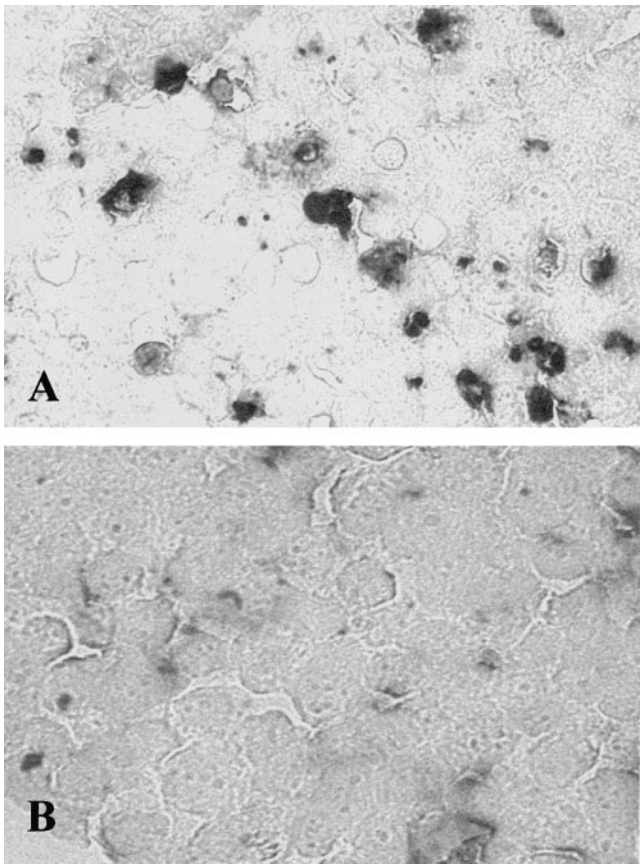

Figure 6. ISEL of (**A**) the pellet of Saos-2 cells overexpressing pRb2/p130 and (**B**) the wild-type cells. Original magnification, ×970.

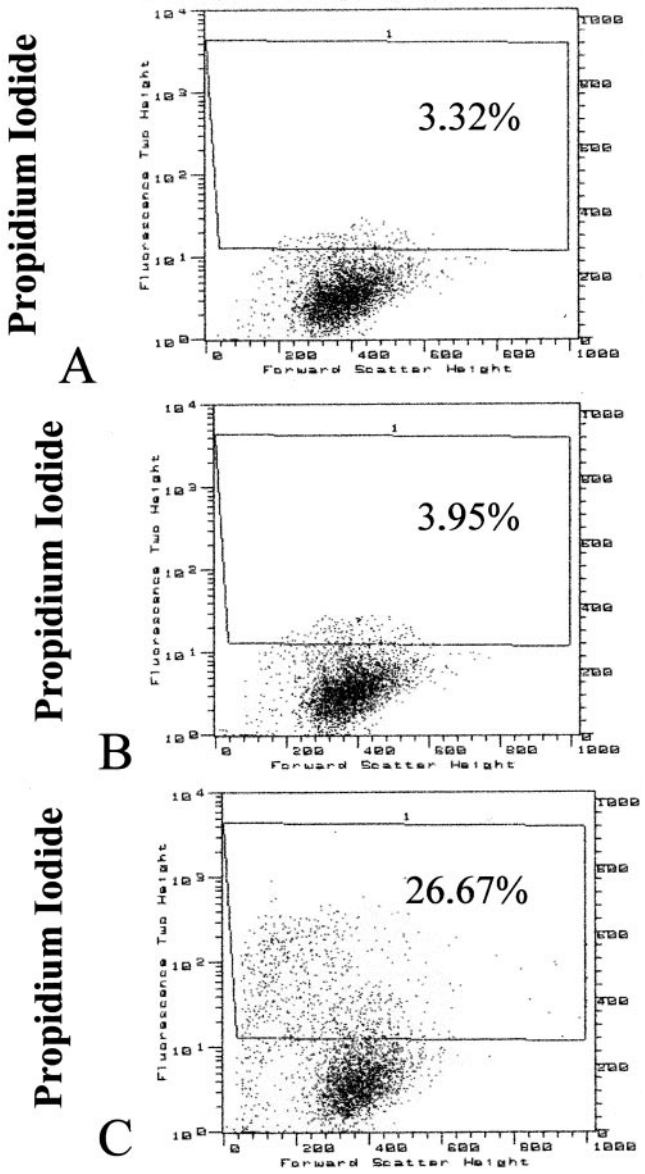

Figure 7. Flow cytometry analysis of Saos-2 cells after supravital PI staining. (**A**) Wild-type Saos-2 cells. (**B**) Saos-2 cells containing the empty vector. (**C**) Saos-2 cells overexpressing pRb2/130. Percentages of apoptosis for each sample are also shown.

earlier phase of the cell cycle than on phases close to cell division.

To better understand the context of our present results, it is necessary to recall certain essentials of the cell cycle.[23] The cell cycle is the result of a series of complex events that regulate cellular life, to permit the accurate transmission of genetic information in a precise and correct way. The retinoblastoma proteins and their cyclin partners control cell cycle progression in the G_1 phase, and hyperphosphorylation of Rb proteins allows cells to enter the S phase and proceed with cell division or undergo cell death. The principal targets of the hypophosphorylated RB proteins are the transcription factors of the E2F family and their co-actors, which consist of at least eight genes, *E2F1* to *E2F6*, *DP1*, and *DP2*. Individual E2F-DP heterodimers associate specifically with each member of the retinoblastoma family of proteins in the pocket region, and repression of their activity during the G_0-G_1 phase is exerted by masking of the E2F activation domain, as shown by in vitro binding experiments.[24] As the cycle moves from the G_1 to the S phase, the RB proteins become progressively phosphorylated and release E2F transcription factors. Free E2Fs bind to E2F sites on the promoters of many cell cycle genes, such as cyclin A, cyclin E, and cdc2 and S-phase–promoting genes such as DNA polymerase-α, *TK*, and *DHFR*.[25]

This summary of the roles of the Rb family of genes and their products and of cyclins in the cell cycle reflects, in a simplified manner, only the essential functions of these regulators, the best known of which is pRb/p105. Although the latter and the two other members of the Rb protein family share considerable sequence homology, in that they can interact with and regulate E2F transcription activity[26] and may complement each other, they are not completely functionally redundant.[27] In fact, these three proteins differ in various respects—for example, in the mechanisms that control their accumulation and phosphorylation,[28,29] in their interaction with distinct members of the E2F transcription factor family,[24,30] in the degradation of the latter,[31] in the mechanisms of cell growth control in malignant lymphomas,[32] and in other features.

The results of the present study, demonstrating that expression of p130 correlates with higher apoptosis in human retinoblastomas, are the first to show such an effect in vivo. At present, however, we do not know through which pathway overexpression of p130 leads to increased programmed cell death. It should be mentioned in this context that pRb/105, the principle member of the Rb family, not only plays the role of an antiapoptosis factor, but may in certain systems and under distinct circumstances favor this type of cell death.[33,34] Thus, the impression is that the members of the Rb family do not each have fixed functions but may, depending on a given situation of the cell cycle machinery, either prevent or induce an apoptotic response.[5,35] This notion conforms to the existence in nuclear Rb family products of multifunctional domains that permit complex interactions with the cellular transcription apparatus.[36]

Our finding that the absence of expression of p130 correlated inversely with the degree of differentiation came as no surprise. It corresponds to observations made in other disease entities—lung[37] and endometrial cancers,[38] choroidal melanoma,[39] and certain non-Hodgkin's lymphomas,[32]—in which an inverse association between the expression of p130 and intensely proliferating neoplasms has been noted. That our study included a considerable number of tumors with a low degree of differentiation and a very restricted expression of p130 arouses the suspicion that, in these cases, the p130 gene may be mutated.

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