p53 and p21^waf-1^ Expression Correlates with Apoptosis or Cell Survival in Poorly Differentiated, but not Well-Differentiated, Retinoblastomas

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ABSTRACT

In human retinoblastomas, rare genetic mutations of the retinoblastoma gene cause massive cell proliferation, altered differentiation, and tumor formation; but paradoxically, this is accompanied by extensive apoptotic cell loss. We quantified the immunohistochemical distribution of p53, its downstream effector p21 (WAF-1), and apoptotic cells in 50 human retinoblastomas, within three concentric zones of sleeves of tumor cells surrounding blood vessels. In poorly differentiated retinoblastomas, both p53 expression and apoptosis increase toward the outer zone of tumor sleeves, whereas p21 expression occurs primarily within the inner zone. This staining pattern of p53 expression is reversed in well-differentiated tumors, whereas p21 staining and apoptotic cell distributions are unchanged. We detected no p53 mutations in four retinoblastomas and two retinoblastoma cell lines. We postulate that oxygen and cell “survival/growth factors” delivered via blood vessels protect retinoblastoma cells from apoptosis. In poorly differentiated tumors, apoptosis is spatially associated with increased p53 expression and may be p53 mediated, but in well-differentiated tumors, apoptosis does not colocalize with p53 and may be p53 independent. In retinoblastomas, p21 is involved not in cell death by apoptosis but in cell survival. Thus, p53 varies its expression (and by implication its function) with altered differentiation in retinoblastomas.

INTRODUCTION

Retinoblastomas, although rare, are the most frequent primary eye malignancy in children. Retinoblastomas arise within the retina to form soft white tumor masses that expand within the posterior chamber of the eye, and which (if untreated) commonly invade through the sclera or along the optic nerve. Distant metastasis is uncommon after 5 years. Without treatment, retinoblastomas are almost universally fatal. Histologically, the tumors often consist of primitive small retinoblasts (in poorly differentiated areas), although primitive photoreceptors may form as individual cells (fleurettes) or in organized groups of cells radiating around a central point (rosettes) in well-differentiated areas.

Retinoblastomas grow rapidly and consequently contain numerous mitoses histologically. The tumors are highly vascular, but there are often large areas of dead cells, which were classically described as areas of necrosis. However, there is increasing recognition that a large proportion of these dead cells result from apoptosis (individual programmed cell death; Ref. 1). The net growth of the retinoblastoma therefore depends upon the balance of cell division and cell death by apoptosis. Thus, if apoptosis were to become more frequent than mitosis, a given neoplasm would decrease in size; this is a possible mechanism to explain why retinoblastomas have a relatively higher incidence of spontaneous complete regression than do other malignant neoplasms (2).

Retinoblastomas are caused by inactivating mutations of both copies of the retinoblastoma (RB1) gene in individual retinal cells. This occurs more readily in “hereditary” cases, where an infant inherits a mutation of one RB1 gene copy/cell; rarer “sporadic” cases require two separate mutations in individual retinal cells (3). Disruption of pRb function contributes not only to the formation of hereditary retinoblastoma but also (in surviving patients) to the later and less frequent development of sarcomas, leukemia, and breast, bladder, and small cell lung carcinomas in retinoblastoma survivors (4–7).

Human retinoblasts (primitive cells that eventually differentiate into retinal cells during embryogenesis) are uniquely sensitive to the loss of the RB1 gene, causing subsequent retinoblastoma development, but retinoblastomas do not occur spontaneously in other species. Germ-line mutations in RB1 in knockout mice cause embryonic death, because of developmental abnormalities of the hematopoietic and central nervous system, rather than giving rise to retinoblastoma. However, mice that are heterozygous for RB1 develop pituitary adenomas as a result of somatic loss of the second allele (8–12). Because of the embryonic lethal phenotype of pRb$^{+/−}$ mice, the effects of pRb loss in the retina has been studied in chimeric mice; in these mice, pRb-deficient retinoblasts are lost by apoptosis (13). This observation was supported by the work of Howes et al. (14), who demonstrated that the loss of pRb (and/or p107 and p130) by the targeted expression of HPV-16 E7 oncprotein in photoreceptor cells resulted in cell death rather than enhanced proliferation. However, mice expressing E7 in a p53 null background developed retinoblastomas. Collectively, these results suggest that pRb loss does not lead to retinoblastomas in the mouse retina, because proliferation is counteracted by apoptotic cell death, which is attenuated in the absence of p53.

The cooperative role of pRb and p53 in tumor progression is not confined to the retina of the mouse but has been observed in a number of systems. In the developing ocular lens of embryonic mice, loss of pRb activity by the targeted expression of HPV-16 E7 oncprotein leads to unrestrained cellular proliferation and an inability of the lens fiber cells to differentiate in regions of the lens that should be postmitotic, resulting in high levels of apoptosis. Loss of p53 function, by expression of HPV-16 E6 oncprotein, fails to restore normal regulation of proliferation and differentiation in the lens of RB-deficient mice, but it does rescue cells from apoptotic cell death (11, 15). Further evidence that inactivation of pRb sensitizes cells to apoptosis has come from experiments using the E1A adenviral oncprotein, which binds and inactivates pRb. E1A has been shown to increase susceptibility to apoptotic cell death in many different cell lines, including primary rodent cells (16, 17) and mouse embryonic fibroblasts (18). Induction of apoptosis was shown to depend on functional p53 (18, 19). In some tumors caused by DNA viruses, viral oncoproteins bind to and inactivate both pRb and p53, causing cell proliferation and inhibition of apoptosis, respectively.

The mechanism by which pRb loss induces apoptosis is not fully understood. In normal cells (pRb$^{+/−}$/p53$^{+/−}$), cell proliferation can...
be inhibited through the action of cyclin-dependent kinase inhibitors, which inhibit cyclin-dependent kinase activity, thus maintaining the pRb/E2F complexes and consequently preventing S-phase entry. Cell cycle arrest is mediated in part by the p53-mediated transcriptional activation of the cyclin-dependent kinase inhibitor p21 (WAF-1). In pRb-deficient cells, E2F-1 is liberated from pRb control, causing unrestricted progression into late G₁ and S-phase of the cell cycle (20). It has been suggested that, in the presence of p53 (+/+), failure to establish growth arrest may lead to conflicting growth signals that result in the induction of apoptosis. However, in the absence of both pRb and p53, E2F-1 activation stimulates cell proliferation, which leads to tumor formation.

p21 expression is also induced in a p53-independent manner during cell differentiation (21) and on stimulation with serum and growth factors (22, 23). An increase in endogenous intracellular p21 concentrations prior to induction of the apoptotic pathway has also been reported in some systems (24) but not in others (21, 25). It has been shown that low concentrations of p21 promotes the assembly of stable cyclin D/cyclin-dependent kinase 4 complexes, whereas at high concentrations, it inhibits kinase activity (26). This suggests that the role of p21 is not limited to mediating a G₁-S arrest in the regulation of cell division. The p53-mediated apoptotic program functions by down-regulating “anti-apoptotic” genes such as the “survival” proto-oncoprotein Bcl-2 (27), insulin-like growth factor-I receptor gene (28), and the microtubule gene associated with protein 4 (29); and by up-regulating “pro-apoptotic” genes such as BAX (27), insulin-like growth factor-BP3 (30), and Fas/Apo-1 (31).

Robanus-Mandaag et al. (32) have reported that function of both pRb and p107 is required for the development of retinoblastoma in chimeric mice. In addition, escape from apoptosis (either through p53 mutation or by some other mechanism) is required for tumor progression in the mouse.

In the human, apart from pRb loss and a limited number of karyotypic rearrangements with unknown functional significance, such as in the i(6p) marker chromosome and the amplification of the 1q chromosome (33–35), no other specific mutations have been identified in the development of retinoblastomas. It therefore appears that the developing human retina is better protected from apoptotic cell death in response to pRb loss than its mouse counterpart.

In this study, we have investigated cellular proliferation, cell death, and differentiation in human retinoblastomas in an attempt to determine the consequences of aberrant cell cycle control that result from the loss of functional pRb. We have studied the spatial distribution of these events in relation to the expression of pRb, the “proliferation” marker Ki–67, E2F-1, Bcl-2, p53, and its downstream effector p21wat-1. Many retinoblastomas have areas where there are “sleeves” of viable tumor surrounding blood vessels; these are surrounded in turn by nonviable areas of confluent apoptotic cells. We believe that within these structurally simple sleeves of tumor tissue there may be a spectrum of microenvironmental conditions ranging from relatively high concentrations of oxygen, growth factors, and cytokines (near the central blood vessel) to low concentrations at the periphery of the sleeves (distant from the blood vessel), where there is a confluent apoptosis. We aimed to determine whether there are differences between the distributions of apoptotic cells and cells expressing p53 and p21 within tumors and to establish how such changes might relate to blood vessels within a tumor. Because p53 is the most commonly mutated gene found in human cancer (36), we have also sequenced exons 2–11 of the p53 gene to determine the mutational status of p53 in retinoblastomas.

**Materials and Methods**

**Microscopy and Quantitation.** We examined 50 formalin-fixed, paraffin-embedded retinoblastomas from our archives, stained with H&E and by standard immunohistochemistry (Avidin-Biotin peroxidase method; Vectastain Elite ABC kit; Vector Laboratories, Peterborough, United Kingdom; after microwave-mediated antigen retrieval) with a brown diaminobenzidine reaction product (Peroxidase Substrate kit; Vector Laboratories) and light hematoxylin counterstain. Primary antibodies included pRb (G3–245; BD PharMin gen, San Diego, CA), MB1 antibody for the Ki-67 antigen (Dianova, Hamburg, Germany), E2F-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), p53-D07 (Novacastra Laboratories, Ltd., Newcastle, United Kingdom), WAF1 (Ab1) p21 (Onogene Science, Inc., Nottingham, United Kingdom), and Bcl-2 (Dako Corp., Copenhagen, Denmark) at dilutions of 1:250, 1:200, 1:250, 1:100, 1:100, and 1:250, respectively.

We identified apoptosis at light microscopy using the criteria described by Wyllie and Kerr (37). Initially, we examined the stained sections conventionally and recorded our qualitative impressions of the distributions of cells that have already undergone apoptosis (“apoptotic cells”) and cells staining positively for p53 and p21, within the sleeves of tumor around small blood vessels. Because this method is subjective, we devised a more objective method to quantify “events” in the sections. We took random (unmatched) photomicrographs of tumor sleeves stained for p53 of p21 and drew 30 degree segments radiating from the central blood vessel out to the periphery of the viable tumor (at the line of confluent apoptotic cells; Fig. 1). We divided each segment into three concentric zones of equal thickness, the “proximal” (inner, peripheral), “middle,” and “distal” (outer) zones, and then counted and analyzed (in the same segments) the proportion of apoptotic cells and the cells expressing p53 or p21, within each of these tumor zones. We determined the proportion of apoptotic cells (to viable cells) and the cells expressing p53 and p21 (to viable cells) in the three zones, between 3 and 25 segments of each of the first eight retinoblastomas with well-defined circular cross-sections of tumor sleeves.

At the next stage of analysis, we returned to the segments of tumor sleeves we had analyzed and divided tumors into three groups according to their level of differentiation (“well,” “moderately,” and “poorly” differentiated, depending on the relative proportions of photoreceptor-like Homer-Wight or Flexner-Wintersteiner rosette formation). We used two methods to assess the effect of differentiation on the level and distribution of apoptosis and expression of p53 and p21 in tumors: by analyzing differentiation in the individual segments of tumors (quantified in the initial phase of the study); and by grouping segments from several tumors according to their level of differentiation (to relate differentiation in segmental microenvironments from several tumors to microscopic events). Segments were analyzed “blind” by M. Andrew Parsons using coded photographs, with no tumor identification codes.

We used the nonparametric Kruskal-Wallis test to determine the significance of differing proportions of apoptotic cells and the cells expressing p53 or p21 within the proximal, middle, and distal zones of tumor sleeves, both in individual tumors and groups of tumors. We also used the nonparametric Spearman rank test to determine possible correlations between apoptotic cells and the cells expressing p53 or p21 in the three tumor zones. We also measured the distance (in μm) between the central blood vessel and the apoptotic edge (line of apoptotic cells) in 143 segments, grouped as a whole, and also in groups according to differentiation.

**p53 Gene Sequencing.** We extracted DNA from two frozen and two formalin-fixed, wax-embedded retinoblastomas and from the established retinoblastoma cell lines Y79 (The Center For Applied Microbiology and Research, Porton Down, United Kingdom) and WERI Rb-1 (American Type Culture Collection, Rockville, MD).

We amplified the DNA samples from exons 2–11 of the p53 gene using intron-specific primers (MWG Ltd., Milton Keynes, United Kingdom; the sequences of the primers used are shown in Table 1). We amplified exons 5 and 6 together (but singly in the paraffin wax-embedded tumors) and exons 7 and 8 individually. Each amplification was performed at a 100-μl reaction medium containing both sense and antisense primers at a final concentration of 0.1 pmol, 0.2 mM of each deoxynucleotide triphosphate (MBI Fermentas, Helena Biosciences, Sunderland, United Kingdom), reaction buffer (20 mM (NH₄)₂SO₄ and 75 mM Tris-HCl (pH 9.0); Advanced Biotecnologies, Paisley, United Kingdom), 0.5 unit Thermoprime Plus DNA polymerase (Advanced Biotecnologies, United Kingdom), Hot Wax 1.5 mM MgCl₂ beads (Invitrogen,
the Netherlands) and −1 μg of target DNA. We processed the reaction mixtures for 35 cycles, each at 94°C, 60°C, and 72°C for 1, 1, and 2 min, respectively, with a final extension of 72°C for 3.5 min in a DNA Thermal Cycler 480 (Perkin-Elmer, Warrington, England). We purified the amplification products using Microcon-100 micro-concentrators (Amicon, Stonehouse, United Kingdom) and sequenced using the ABI Prism Dye Terminator Cycle Sequencing Reaction kit (Perkin-Elmer), using the PCR primers.

RESULTS

Apoptosis, p53, and p21 Expression. The 50 retinoblastomas had varying proportions of confluent solid tumor tissue and areas of cylindrical tumor sleeves around blood vessels; these were separated from adjacent viable tumor by areas of nonviable tissue (Fig. 1). Most tumors were poorly differentiated, with only very occasional Homer-Wright or Flexner-Wintersteiner rosettes, but a few tumors with larger proportions of these rosettes were classified as moderately or well differentiated. In tumors with sleeves of tumor tissue around central blood vessels, the cells immediately adjacent to the vessel were viable, with very few (or no) apoptotic cells (Fig. 1). With increasing distance from the blood vessel, however, the proportion of apoptotic cells increased, until they became confluent at the periphery of the distal zone. The radius of the sleeves of viable cells surrounding the central blood vessel ranged from 75 to 250 μm, with the majority of the segments falling in the range of 100–200 μm. We noted that a tumor sleeve radius of 200 μm was generally associated with very vascular tumors. There was no demonstrable effect of differentiation on the radius of tumor sleeves.
around the central blood vessels in the proximal zone (Fig. 1 e), but by contrast cells expressing p21 tended to be located nantly affected cells in the outer (distal) zone of the tumor sleeves (Fig. 1). The cells that have already undergone apoptosis (apoptotic cells) do not stain for p53.

On quantitative analysis, taking all of the tumors as a group, the vast majority of apoptotic bodies and the cells expressing p53 were confined to the distal zone of the tumor sleeves (Table 2), whereas cells expressing p21 were most numerous in the proximal zone, with some in the middle zone, but very few in the distal zone (Table 2). There was a strong positive correlation between apoptosis and p53 expression (r = 0.43, P < 0.01, Spearman rank test) and a strong negative correlation between apoptosis and p21 expression (r = −0.45, P < 0.01, Spearman rank test).

The “combined” group of tumors had cells expressing p53 mainly in the distal zone, but in some individual tumors (Table 3) this pattern was either reversed or modified so that the proximal zone contained many positive cells. Examination of the histology of these tumors revealed that they were well- or moderately differentiated, whereas the majority of the tumors were poorly differentiated. We found that well-differentiated tumors expressed p53 in a pattern that was the inverse of the majority of tumors that were poorly differentiated (with p53 expressed in perivascular proximal zone rather than the distal zone). This was statistically significant when p53 expression was related directly to the differentiation of the local tumor segments, rather than the tumor as a whole. Interestingly, there is no effect of altered differentiation on either the proportion or apoptotic cells or cells expressing p21 in any of the tumor zones.

**pRb Localization.** Of the 32 tumors studied for pRb, two contained slight nuclear staining of pRb in viable tumor cells. Eight tumors expressed pRb in the endothelial cells of the tumor blood vessels, although adjacent tumor tissue contained no demonstrable pRb. We noted that the inner nuclear layer cells of the retina stained for pRb in some eyes.

**Tumor Proliferation.** We used the MIB1 antibody to demonstrate Ki-67 antigen (which is expressed in the nuclei of cells at all stages of the cell proliferation cycle except G0) in 81% of the tumors (Fig. 1). In poorly differentiated tumors, this proliferative activity was uniformly distributed throughout the tumor and across the proximal, middle, and distal zones in tumor sleeves. Similarly, proliferation was detected in the majority of cells in the rosettes of well-differentiated tumors.

**E2F-1 Localization.** E2F-1 was demonstrable in 37 of the 39 stained tumors. E2F-1 expression was uniformly distributed throughout the tumor across the three zones in most tumors (Fig. 1), but occasional “sleeves” contained stronger staining in the distal regions. Similarly, the majority of the cells of rosettes were positive for E2F-1 in well-differentiated areas. Generally, strong E2F-1 expression was associated with very vascular tumors.

**Bcl-2 Localization.** We detected Bcl-2 expression in only occasional tumor cells close to the central blood vessels in 16 of the 50 retinoblastomas (not illustrated).

**p53 Gene Sequencing.** All four tumors and cultured cells (Y79 and WERI-Rb1) sequenced for exons 2–11 contained wild-type p53 (results not shown). We did not detect any p53 mutations in any of the samples tested.

### DISCUSSION

There is a considerable body of evidence that suggests that the loss of the functional retinoblastoma gene product pRb results in the activation of the p53-dependent apoptotic pathway. This has been demonstrated in mutant mouse retinoblastoma models where, under conditions of pRb loss, proliferation is restrained by apoptosis in the presence of functional p53. It is clearly advantageous for p53-mediated apoptosis to be inactivated for a neoplasm to develop. Such inactivation has been demonstrated in mouse models of retinoblastoma and during embryological lens development in mice, and it also occurs in naturally occurring tumors caused by DNA viral proteins, which target both pRb and p53 for inactivation.

In our study, however, we have shown that persistent proliferation is possible in human retinal cells in the presence of wild-type p53, and that loss of pRb does not spontaneously induce tumor retinoblasts to undergo apoptosis. This is not a consequence of a defect in the apoptotic pathway, because we have identified all stages of apoptosis.

### Table 1 Sequences for primers used for exons 5, 6, 7, and 8 of the p53 gene

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer</th>
<th>Sense/Antisense</th>
<th>Sequence</th>
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<td>Sense</td>
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</tr>
<tr>
<td>5`-</td>
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<td>Antisense</td>
<td>-CAATTGTAACTTGAACCATC-3</td>
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<tr>
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<td>5`-</td>
<td>Sense</td>
<td>-CTTTCCAACCTAGGAAGGCA-3</td>
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<tr>
<td>5`-</td>
<td>5`-</td>
<td>Antisense</td>
<td>-GACTGGAAACTTTCCACTTGAT-3</td>
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*Statistical significance with reference to the three zones.*

### Table 2 Apoptotic cells (as a proportion of total live + apoptotic cells) and cells expressing p53 or p21 (as proportion of live cells; median values) in proximal (inner), middle and distal (outer) zones of the sleeves of retinoblastoma tumor cells surrounding blood vessels

<table>
<thead>
<tr>
<th>Segments</th>
<th>Proximal zone (P)</th>
<th>Middle zone (M)</th>
<th>Distal zone (D)</th>
<th>Kruskal-Wallis</th>
<th>Interpretation</th>
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<td>Apoptosis</td>
<td>118</td>
<td>0</td>
<td>29.5</td>
<td>P &lt; 0.001</td>
<td>M = &lt;D</td>
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<tr>
<td>p53</td>
<td>59</td>
<td>0</td>
<td>15.8</td>
<td>P &lt; 0.001</td>
<td>M = &lt;D</td>
</tr>
<tr>
<td>p21</td>
<td>59</td>
<td>33.3</td>
<td>11.8</td>
<td>P &gt; 0.001</td>
<td>M &gt; D</td>
</tr>
</tbody>
</table>

*Statistical significance with reference to the three zones.*
in retinoblastoma tumor cells, which confirms that the apoptotic pathway is fully functional. This suggests that in humans pRb and p53 do not “co-operate” in the development of retinoblastomas, but instead that retinal cells must be protected from p53-mediated apoptotic loss, in the absence of functional pRb.

We have demonstrated that retinoblastomas frequently have sleeves or cuffs of viable tumor cells arranged around a central blood vessel. It has not been recognized that, because there is only one blood vessel in these tumor sleeves, this vessel must be part of a vascular loop for blood to flow (as seen in angiogenesis and vasculogenesis in other tumor and embryological systems). Within these structurally very simple tumor sleeves, we have demonstrated that tumor cells undergo apoptosis 100–200 μm away from the blood vessel; at this distance from the vessel, tissues are likely to have low oxygen concentrations [reviewed by Sutherland (38) and Brown and Giaccia (39)]. It is not surprising that cells that have already undergone apoptosis, and hence extensive proteolysis, do not contain demonstrable p53. We therefore used methodology that localized p53 expression in viable cells within the tumor zones associated with the highest proportion of apoptotic cells.

We developed a new quantitative and statistical analysis of tumor zones to demonstrate for the first time that in retinoblastomas as a whole, there is a strong positive correlation between apoptosis and p53 expression and a strong negative correlation between p21 and apoptosis. This strongly suggests that the “wild type” p53 in these tumors actively mediates apoptosis by a mechanism that does not involve p21. The presence of mitotic figures and demonstrable Ki-67 antigen indicates that cell proliferation occurs in all three zones of the tumor sleeves, causing thickening of the sleeves. However, this causes the cells in the distal (outer) zone to move further away from the central blood vessel of the tumor sleeve, with a presumed consequent reduction in the levels of oxygen, nutrients, and growth factors, which induces p53-associated/mediated apoptosis in the distal zone.

**Poorly Differentiated Retinoblastomas.** We have shown that apoptosis occurring in the distal zone of poorly differentiated areas of retinoblastomas correlates with p53 and is probably p53 mediated, but that this does not involve p21 (although we accept that it is theoretically possible that p21 may be functioning at some level in the outer zone, at a concentration below the level of detection by immunohistochemistry; conversely, p53 may also theoretically be functioning in the inner zone, again below the level of detection by immunohistochemistry). This finding is in agreement with the recent observation that mouse embryo fibroblasts that lack p21 are still susceptible to apoptotic cell death (40).

We have demonstrated that p53 expression increases from the inner to the outer zones of the tumor sleeves in poorly differentiated areas of the tumor, but that p21 expression is confined primarily to the inner zones of the tumor sleeve, adjacent to the blood vessel. The spatial distribution of p53 and p21 expression suggests that p21 is induced in a p53-independent manner, possibly in response to the high concentration of oxygen, nutrients, and growth factors derived from the adjacent blood vessel.

p21 expression may also be induced through the deregulated expression of E2F-1. Hiyama et al. (41, 42) have shown that high levels of E2F1 can transactivate the p21 gene through binding sites located in its promoter. In this way, E2F1 and p21 could oppose each other to control entry into S-phase of the cell cycle. However, in retinoblastomas the primary target for p21 G1 arrest function (pRb) is absent. LaBaer et al. (26) have reported that low concentrations of p21 promote the assembly of stable cyclin D/cyclin-dependent kinase 4 complexes, whereas high concentrations inhibit kinase activity. Although the role of p21 in retinoblastomas is unclear, the localization of p21 in proliferating cells around blood vessels suggests that it has a role in cell survival. However, if p21 expression is induced by the deregulated expression of E2F-1 (which we localized in all three zones of the retinoblastoma sleeves), we postulate that p21 is stabilized by relatively high concentrations of factors diffusing from the central blood vessel. In areas away from the blood vessel, such factors are at relatively low concentration, which leads to p53 induction and stabilization in distal zones. High concentrations of p53, possibly in combination with high E2F-1 expression, results in apoptosis induction (43–45). E2F-1 would therefore serve as a signal to initiate the apoptotic pathway, unless this pathway is blocked by cell survival factors. We have demonstrated increased E2F-1 expression in the distal zone of some tumor sleeves, which would support such a link between p53-mediated apoptosis and E2F-1 expression.

We believe that the fate of a pRb-deficient tumor cell in poorly differentiated retinoblastomas depends upon a balance between two opposing signals that promote either cell survival or apoptotic cell death. Under conditions of high nutrient and oxygen concentrations, the apoptosis-promoting activity of E2F-1 is suppressed by survival factors delivered by blood vessels to the tumor. A number of reports indicate that, under conditions of high cell proliferation, E2F-1 can transactivate p19ARF (46), which associates with p53 and/or Mdm-2 to stabilize p53 (47–49), and which in turn promotes apoptosis. It is possible that E2F-1 is unable to activate p19ARF expression in the presence of survival factors, and that consequently p53 is maintained at low levels by the targeted degradation of p53 by Mdm2 (50, 51). Subsequently, in response to the exhaustion of oxygen/growth factors, this repression is withdrawn and p53 is stabilized (Fig. 1).

Only one-third of our tumors expressed demonstrable Bcl-2, and for this reason it seems unlikely that retinoblastoma cells are protected by this “survival factor” proto-oncogene. Bcl-2 acts to inhibit the cell cycle by reducing E2F concentrations. Loss of pRb function may abrogate the effect of Bcl-2 (52) and may also relate to the relatively low levels of Bcl-2 in retinoblastomas.
Well-differentiated Retinoblastomas. The pattern of p53 expression in retinoblastomas appears to depend on the state of differentiation of the tumor, which reflects the developmental stage of the retinoblasts. The majority of the tumors are poorly differentiated (representing primitive retinoblasts), with p53 expressed in the outer zone of the tumor sleeves, as discussed above. However, in well-differentiated areas of the tumor (representing a relatively later developmental stage of retinoblasts) this pattern is reversed, so that the p53-positive cells lie in the proximal perivascular zone, and there is almost no p53 expression distally. Despite this reversal of the zonal position of p53-expressing tumor cells, there was no associated alteration of the zonal position of either p21 expression or apoptosis.

This alteration in the spatial distribution of the p53-positive cells in retinoblastomas, without any change in the position of apoptotic cells, suggests that the control of apoptosis in retinoblasts may change during retinal development. At the periphery of poorly differentiated retinoblastoma tumor sleeves, expression of p53 is associated with “survival factor deprivation” apoptosis. In well-differentiated retinoblastomas, this “survival factor deprivation” apoptosis still occurs peripherally; but the almost total absence of p53 expression at this site suggests that the “survival factor deprivation” apoptosis is p53 independent. This may be via the activation of the E2F-1 apoptotic mechanism, which does not involve p53, similar to that described by Pan and Grien (53) in the developing ocular lens of embryonic mice.

The presence of colocalizing p53 and p21 in proximal zones of well-differentiated retinoblastomas suggests that p53 may mediate cell cycle arrest in differentiating retinoblasts, in which a differentiation program can be initiated by induction of p21. However, we have demonstrated that Ki-67 is expressed in the majority of the most differentiated cells (photoreceptor rosettes), which indicates that p21 is unable to mediate a complete cell cycle arrest, presumably overridden by loss of pRb. It is unclear, then, how retinoblastoma cells undergo differentiation in the absence of terminal mitosis and in the absence of functional pRb. It is possible that other members of the pRb family (such as p107 and p130) partially compensate for pRb, allowing some cells to differentiate. Alternatively, differentiation may be a result of the action of differentiating agents in the tumor environment acting via a pRb-independent pathway. It is also possible that a degree of differentiation may be initiated from a p53-mediated G2-M cell cycle arrest.

Humans are unique in being the only animal species in which retinoblastomas occur naturally. Perhaps human retinoblasts are uniquely susceptible to the loss of functional pRb because they depend solely on this protein during a short window of opportunity during embryological or early postnatal development (although some retinoblastomas are present at full-term birth). In theory, the loss of pRb function in a retinoblast should cause the cell to undergo apoptosis, thus preventing tumorigenesis. However, our findings suggest that human retinoblasts can be rescued sufficiently by survival factors to allow malignant growth. The mechanism for this aberrant survival is unknown but may involve additional mutations in retinoblastomas such as (6p) or +1q (35).

In this study, we have used spatial analysis to show that the function of p53 changes with the state of differentiation in retinoblastomas. In poorly differentiated areas, p53 mediates apoptosis but shows little or no p21 transactivation activity, whereas in well-differentiated areas, p53 possibly initiates cell cycle arrest via p21 transactivation. It is interesting to speculate that p53 cycles between two different conformations with mutually exclusive functions: one that promotes apoptosis and the other that promotes a cell cycle arrest and differentiation. Such different conformations could be determined by growth factors that are differentially expressed at specific stages of differentiation or embryological development. In this model, specific growth factors might act either directly or indirectly to activate the “growth arrest” p53 conformation and suppress the “apoptotic” p53 conformation in the well-differentiated areas. By contrast, the “apoptotic” conformation would be activated in the poorly differentiated areas, and the growth arrest conformation suppressed.

In conclusion, we suggest that apoptosis is induced in retinoblastomas in response to the exhaustion of growth/survival factors, in combination with a lack of supply of oxygen, within a neoplasm that is growing rapidly and has high metabolic requirements for these factors. We believe that the poor and well-differentiated areas of these tumors contain retinoblasts that are analogous to retinoblasts occurring at relatively earlier and later stages of their embryological development, respectively, and that these cells respond differently in relation to p53 function. In the poorly differentiated areas, high p53 expression is associated with apoptosis but, in well-differentiated areas of the tumor, p53 induces p21 in an attempt to mediate a cell cycle arrest from which a differentiation program can be initiated. We postulate that tumor progression in retinoblastomas is strongly dependent on availability of “survival factors” such as oxygen, nutrients, and growth factors, without which the tumor is likely to undergo involution by apoptosis. Because these factors are delivered by blood vessels, neovascular activity of the tumor is vital for tumor progression. Thus, therapeutic strategies targeting “survival factors” and tumor angiogenesis are likely to enhance preexisting apoptotic mechanisms and promote involution of this highly malignant eye cancer in children.

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p53 AND APOPTOSIS IN RETINOBLASTOMA


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