Developmental Basis of Retinal-specific Induction of Cancer by RB Mutation

Brenda L. Gallie, Christine Campbell, Hollie Devlin, Allison Duckett, and Jeremy A. Squire

Abstract

Understanding why children with RB mutations specifically develop retinoblastoma will contribute to the understanding of the fundamental principles of cancer. Only a subset of developing retinal cells are at risk for developing cancer when RB is mutant because rod photoreceptor and bipolar cells never normally express RB. Retinoblastomas are observed to arise commonly in the inner nuclear layer, where they can show features attributed to outer nuclear layer cells (photoreceptors). The best-studied function of RB is control of the cell cycle, and the usual tissue consequence of loss of RB is apoptosis. Perhaps the specificity of RB mutation for retinal cancer resides in the dependency of this tissue on programmed cell death to achieve a precise architecture of individual types of interconnecting neurons. The additional mutations that are present in all retinoblastoma, such as the i(6p) marker chromosome, may interrupt signals that normally would induce apoptosis when RB is absent. A combination of loss of cell cycle control and loss of signals that delete extra cells would result in retinoblastoma.

Introduction

The prototype embryonal tumor, retinoblastoma, has revealed much about fundamental processes of cancer. What is not yet clear is how children with mutations in the RB gene are specifically susceptible to this particular rare tumor. In fact, a human that is heterozygous for a RB germ-line mutation has a 40,000-fold relative risk of developing retinoblastoma (1). The same individual has a 500-fold relative risk to get other tumors, particularly sarcomas. If such children are treated with radiation as infants, the risk for second tumors is greatly increased (2). However, the germ-line RB mutation does not increase the risk for leukemia or many other tumors. We know that the RB gene is important in the cell cycle in most cells and is inactivated or mutated in many human cancers. But what is the basis for the unique, strong specific induction of cancer in human developing retina? RB<sup>+/−</sup> mice, with exactly the same genotype as the human RB<sup>−/−</sup> patients, do not develop retinoblastoma but, instead, develop tumors of a part of the pituitary gland that is vestigial in humans (3). This suggests that subtle tissue specific developmental factors are critical in the balance between nonmalignant outcomes and cancer in cells that lose the RB gene product, pRB.

The basic function of pRB is to hold cells in G<sub>1</sub> or G<sub>0</sub> phase of the cell cycle and prevent entry into S phase until pRB is appropriately inactivated by phosphorylation, at a time when the cell cycle is supported by various factors that allow normal cell cycle progression. If pRB is absent because of mutation or because of constitutive phosphorylation due to dysregulation of the pathway inactivating pRB, the common cellular outcome is apoptosis, when a cell progresses into S phase unsupported by appropriate factors (4–6). In certain tissues, for example, muscle, in addition to apoptosis, endoreduplication occurs, suggesting that S phase is completed in myotubes, but a deficiency of G<sub>1</sub> or M phase factors prevents completion of the cell cycle (7). This was demonstrated by the partial rescue of RB<sup>+/−</sup> mice, which allowed them to live past E13.5, when they usually die. These mice were not completely rescued but died at birth with a severe muscle defect, including extremely large nuclei indicating endoreduplication.

A pathognomonic feature of retinoblastoma is calcification, which can be detected in even isolated cells in areas of viable tumor. Perhaps these represent fossilized cells that have undergone endoreduplication, packing the nucleus full of DNA, leading to calcification.

Expression of RB in Developing Retina

To learn what might be the basis of the specificity of induction of cancer in the retina in the absence of functional pRB, we explored the pattern of RB expression in developing murine and adult human retina. The RB gene is expressed in all adult tissues, but specific cell types initiate expression of RB at specific developmental times (8). Surprisingly, we discovered that RB is expressed in all retinal cells, from the time of initiation of differentiation, except in bipolar cells and rod photoreceptors, which never express RB (4). As the retina develops, terminal differentiation occurs at the outer margin of the retina, and the postmitotic neurons migrate from this surface through the neuroblastic layer to their final destination as functional neurons. Using both in situ hybridization and immunohistochemistry, we showed that the first expression of RB in murine retina was at E15, in the first cell type of retina to differentiate, the ganglion cells. Expression was evident within a small subset of cells in the neuroblastic layer, presumably the differentiating, migrating cells, and was expressed strongly in every cell in the GCL. RB expression became obvious in the emerging INL around day E17. The INL also contained numerous cells that did not express RB, even in adult murine retina. The ONL, consisting of photoreceptor cells, showed only sparse cells expressing RB, even in adult mice.

Adult human retina clearly delineated which cell types express RB and which do not. It was obvious that the cone photoreceptors strongly expressed RB, whereas the rod photoreceptors never expressed RB. In the peripheral retina, the INL consists of mixed horizontal, amacrine, and bipolar cells and showed a mixture of RB-expressing and non-RB-expressing cells. Examination of the INL in the macular region revealed that the amacrine cells at the inner edge and the horizontal cells at the outer edge strongly expressed RB, but the very numerous bipolar cells occupying the bulk of the middle part of the macular INL did not express RB.

Retinoblastoma tumors show many morphological features and characteristics of photoreceptors. The Flexner-Wintersteiner rosette is a spherical monolayer of columnar epithelial cells, with the apical surface central and with features of nonmotile cilia that are considered evidence that retinoblastoma arises from photoreceptor cells (9). Our new data on retinal expression of RB suggest that retinoblastoma

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3 The abbreviations used are: E, embryonic day; GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PCD, programmed cell death.


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could not arise from rod photoreceptor cells or bipolar cells because RB expression is not important in those cells. Retinoblastoma could arise from cone photoreceptor cells, consistent with the expression of cone photoreceptor-specific transducins (10) but not rod photoreceptor-specific transducins in retinoblastoma tumors. In the few human retinoblastoma specimens that are small when the eye is surgically removed, the layer of the retina in which the tumor appears to arise is evident. Such tumors demonstrate nuclear morphology of the INL rather than the ONL, even if the tumor appears to be arising within the ONL (Fig. 1).

Chimeric mice with RB-/- cells did not develop retinal abnormalities, although the proportion of RB-/- cells in retina was lower than in other tissues, suggesting that apoptosis may be an outcome of the absence of pRB in developing retina (11). However, p107-/- mice that were chimeric for RB-/- cells developed tumors of the INL of the retina but not of the ONL (12). This suggests that p107 partially compensates for RB function in murine retina to suppress tumorigenesis.

**Additional Genetic Events in Retinoblastoma**

All retinoblastoma have mutations of both RB alleles, but they always display additional non-RB mutations (13). The most common cytogenetic marker (70% of retinoblastoma) is an isochromosome, i(6p), which results in two extra copies of chromosome 6p in each tumor cell (14). When a retinoblastoma that does not have an i(6p)
was examined with a probe for chromosome 6p, a small fragment of 6p was noted to be translocated to a different chromosome (Fig. 2). More studies like this might identify a small fragment of 6p that would contain a gene for which low-level amplification was a selective advantage in RB<sup>−/−</sup>-developing retinal cells. Also frequent in retinoblastoma are extra copies of chromosome 1q. All retinoblastoma examined showed either i(6p) or +1q (Fig. 3).

Other changes occur later in a smaller subset of retinoblastoma. Expression of telomerase is a late event in retinoblastoma and is more common in retinoblastoma cell lines than primary cultures (15). The genomic amplification of MycN occurs in a few retinoblastomas (16).

Many types of human tumors have p53 mutations, probably the most common tumor suppressor gene mutations, which contributes to tumor growth by eliminating a major mechanism of apoptosis. Retinoblastoma are highly necrotic tumors, perhaps because they outgrow their blood supply, but apoptosis is well documented (17). Consistent with the intact apoptotic process, we found no p53 mutations in retinoblastoma (Fig. 3) by single-strand conformational polymorphism screening for mutation, protein half-life, quantitative mRNA assessment, or the ability of radiation to induce p53 and apoptosis.

Developmental Signals in Retinal Development

Notch is a membrane receptor that, when activated in developing neural systems, blocks neurogenesis and maintains neuroblasts in a proliferative state. The activation of Notch is regulated by membrane-bound ligands, such as Serrate and Delta. Fringe regulates the interaction between Notch and these ligands. In Drosophila wing and eye development, Notch is activated at the borders between Fringe-expressing and non-Fringe-expressing cells.

In mammalian developing retina, the undifferentiated progenitor cells express Notch 1 and Delta-like-1 (18). The expression of activated Notch appears to block neuronal cell differentiation in a reversible manner, perhaps by regulating the ability of cells to respond to differentiation signals. In addition, newly differentiated retinal neurons, by expressing Delta-like-1, appear to inhibit neighboring progenitors from entering the neuronal differentiation pathway (19). In Drosophila retina, Notch also directs PCD to remove excessive, unneeded precursor cells (20). Such local signaling is critical to accomplish the precision of retinal architecture, with a precise number and interrelationship of the neuronal subtypes, on which full retinal function is dependent. Perhaps this requirement sets the retina apart from even brain.

We examined the expression patterns of the Notch ligands, Serrate-1 and -2 and Delta like-1, and the three mammalian Fringe genes in murine retinal development. The earliest ligand to be expressed was Serrate-1. Strong expression was detected from E11 onward, particularly in the rapidly proliferating anterior borders of the developing retina. Although high Serrate-1 expression was maintained in the lens, as retinal differentiation occurred, Serrate-1 expression decreased and was replaced by that of Delta-like-1 in the still proliferating neuroblastic layer and by Serrate-2 in differentiated cells.

In E15 murine retina, both Delta like-1 and Lunatic Fringe were

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Fig. 3. Frequency of genetic events in retinoblastoma.
prominently expressed in the proliferating neuroblastic outer layer of the retina, with less expression in the differentiating GCL. The opposite was true for Serrate-2, Manic and Radical Fringe, which were expressed predominantly in the differentiating GCL with minimal expression in the neuroblastic layer. Lunatic Fringe was strongly expressed in the neuroblastic layer. Postnatally, Radical and Manic Fringe were expressed in the differentiated ganglion cells and INL, where they remained expressed into adulthood. Lunatic Fringe expression in adult eyes was eventually limited to a subset of cells at the innermost INL, which have yet to be identified. Whereas Radical and Manic Fringe were both expressed in most differentiated cells of the GCL and INL layer, of particular note was their absence from the photoreceptor layer, the same as the pattern of expression of RB.

Table 1 summarizes this data. Serrate-1, Notch 1, Delta-like-1, and Lunatic Fringe were initially expressed in the proliferating cells and then turned off, except for the Lunatic Fringe expression in a narrow layer of the INL throughout adult life. In contrast, Manic and Radial Fringe and Serrate-2 were prominently expressed in differentiated cells. None of these signaling molecules appear to be important for rod photoreceptor cells because they were not expressed in the ONL. In addition, expression of an activated form of Notch-1 from a retrovirus in developing rat retina caused abnormal growth of the INL and GCL differentiated progeny of the infected clone but did not appear to alter the differentiation of the rod photoreceptors (18).

Model for Retinoblastoma

We can start to assemble a model for the induction of cancer in developing retina. Proliferating neuroblasts do not express RB until terminal mitosis, at the ventricular margin of the retina (4 Fig. 4). The RB-positive developing cells then migrate through the RB-negative cells that are still proliferating and expressing Notch, Delta-like-1, and Lunatic Fringe. Many extra differentiated cells arrive in the GCL and INL, but the final precise architecture of these layers is achieved by extensive PCD (21), induced by intercellular signaling, perhaps including regulators of Notch family genes, such as Serrate-2, Manic Fringe, and Radical Fringe, which are expressed in the surviving GCL and INL neurons. Determination of which cells survive and which undergo PCD is complex and may depend on trophic factors achieved by the cell acquiring axonal connections, which may trigger survival but may also involve Notch-like short range intercellular signaling. A neuroblast with both RB alleles mutated may still undergo a ventricular zone “last” mitosis and migrate to the GCL or INL. Because there is no pRB to prevent reentry into S phase, further cell division may occur within the normally postmitotic layers, inducing extra cells within the differentated layer. Intercellular signaling from the neighboring terminally differentiated neurons would result in PCD of many of these cells, either eliminating the RB-deficient mutant cells or allowing only linear expansion of the clone. The clinical entity of “retinoma” may be an expression of such a clone of RB-/- cells, which form a benign retinal mass that does not change in adulthood (22). However, if any RB-deficient cell also acquired a mutation of its receptors that receive the PCD signal, it could escape cell death and proliferate exponentially as a malignant clone that is unresponsive to terminal differentiation signals, because it has no pRB, and unresponsive to death signals because of a defect in the PCD signaling pathway. In such a retinoblastoma tumor, apoptosis in response to radiation or other stimuli of p53 or non-p53 pathways leading to apoptosis would remain intact, despite defective upstream signals for PCD. Malignant retinoblastoma has been observed to emerge from a retinoma, and indeed, many retinoblastomas have underlying retinoma, which are overgrown long before diagnosis and, therefore, undetectable clinically or pathologically. Tissues in which PCD is not a critical part of extensive remodeling of postmitotic cells would not be susceptible to induction of malignancy in the absence of pRB.

Rod photoreceptor cells and bipolar cells do not express RB, do not express the Notch pathway ligands and modifiers of ligand function and do not show much PCD. Unlike the non-bipolar INL and CGL cells, which require exquisite intercellular signaling, photoreceptor cells do not interact with each other, and the more there are, the better vision might be. Interestingly, the cone photoreceptors express RB and have a precise architecture in their eventual distribution in retina, similar to the neurons of the INL and GCL. The rod photoreceptor and the bipolar cells are very different from the other retinal neurons and extremely unlikely to be the origin of retinoblastoma.

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References

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Discussion

Dr. Sharon Murphy: Could you expand a little more on this concept of how the normal retina during development and differentiation selects some cells to stay in place and differentiate and others to undergo PCD, so that you have just the right number, and what regulates those signals again?

Dr. Gallie: We don’t know precisely the answer to your question, but there are many models to approach this problem. For example, Drosophila retina has far too many cells on the proliferative side of the morphogenetic furrow (20). By the time you get to the other side, fully differentiated, there is a very precise anatomical arrangement and the extra unneeded cells have been deleted. The ligands signaling PCD act at a very short range to fine-tune the critical number of cells necessary for the challenges of precise visual function. We must develop precise experimental systems to manipulate these genes before we can answer those questions more precisely.

Dr. Sharp: Dr. Sharp.

Dr. Phillip Sharp: You project in the model that absence of RB allows a sort of expansion of a pool but homeostasis through some intercellular signaling pathway remains and that disruption of that pathway is the second critical step in the development of a full-blown malignancy.

Dr. Gallie: Yes, malignancy would result from disruption of homeostasis for cell number by PCD.

Dr. Sharp: Is that distinct from another mutation that, in essence, just allows more proliferation? Is there something unique about breaking down the homeostasis patterns of this tissue in progression to a tumor? Is it one of many possible other mutations that have to happen?

Dr. Gallie: Not necessarily. The reason I have come to this model is the very unique profile of the third mutation in retinoblastoma tumors, where there aren’t a whole array of different mutations and the very high tissue specificity for this general cell cycle gene (the RB gene) to cause this particular tumor. That is why I postulate that the third event is very precise and not sort of a general tumor mutation. Otherwise, we would see all sorts of mutations, which we don’t in these tumors. Retinoblastoma is the equivalent of a very, very early tumor, which the uncommon expression of telomerase may tell us. So in retinoblastoma, not only do we absolutely know the first and second mutation (both RB alleles), but we have a chance to look at M3, separate from M0 and M10 and whatever, which are to me less interesting. I am interested for progression, maybe targets for therapy, but the PCD signaling pathway would be an exciting target for prevention.

Dr. Carol Thiele: Carol Thiele, National Cancer Institute. There’s some recent evidence emerging from the lab of Barbara Osborne that Notch may be a survival signal in T cells in cell-to-cell communication (23). Have you looked to see whether or not Notch signaling is operant in retinoma or retinoblastomas?

Dr. Gallie: No, we haven’t looked at Notch signaling in retinoblastoma yet.

Speaker: Do you see any evidence of excessive genome instability in these cells at the level of the retinoma or later on? I mean, there’s certainly some chromosomal abnormalities that tend to crop up like i(6p) from time to time.

Dr. Gallie: We see no evidence of instability in retinoblastoma, which are remarkably stable. In fact, we can recognize the tumors that Jeremy Squire grew in the 1980s by their karyotype today, so they are very stable. Retinoblastoma can have as few as a single marker chromosome, for example, only i(6p). Retinoblastoma is really never aneuploid unless you irradiate the cells or pull them out of a metastatic site. Now retinoma has never been studied for mutations because only very rare pathology samples have been obtained. Most patients with retinoma are very happy to keep their eyes with the benign tumors.

Unidentified Speaker: Have you done an LOH analysis or comparative genomic hybridization systematically, looking for mutant genes?

Dr. Gallie: A full screen for regions of LOH has not been done properly in retinoblastoma. Interestingly, when LOH was first identified, we got away without a full screen because LOH fitted so well with the Knudsen two-hit hypothesis (24, 25). Subsequent tumor suppressor genes identified by LOH have been put to a more rigorous test!

Speaker: This may be a simple question, but you said early in development there’s no expression of RB in the photoreceptor cells. Is there any expression of PI07 or P130?

Dr. Gallie: In very early retina, there’s no expression at all of RB. The photoreceptor cells never express RB. The other RB family members, p107 and p130, are expressed differently than RB, but we have not done a detailed developmental expression study. The work of Anton Berns and group indicates that p107 can partially compensate for RB (12).

The abbreviation used is: LOH, loss of heterozygosity.
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