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Towards an animal model for retinoblastoma

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Within the past decade a large repertoire of cellular oncogenes has been implicated in the genesis of many types of cancers. These oncogenes all function to promote the neoplastic growth of cells in which they act. Indeed, they all appear to derive from normal cell genes, proto-oncogenes, which act to stimulate normal cell proliferation.

During this period, evidence has also been presented to suggest that elements that normally function to inhibit cell proliferation are also playing a role in the process of carcinogenesis. For instance, in a number of distinct types of tumors it was found that genetic material from specific chromosomal loci was absent in tumor DNAs, but present in the adjacent normal cell DNA. This indicates that loss of genetic material represents at least one step in the pathogenesis of these tumors (Klein, 1987). It seems likely that the normal function of the genes that are lost in these tumors is to restrain the growth of cells from which the tumors originate. For this reason such genes have been named recessive oncogenes or anti-oncogenes.

Retinoblastoma is an early childhood tumor occurring in about 1 out of every 20,000 live births and is probably the best-studied tumor in which loss of genetic material constitutes a critical step in tumorigenesis (reviewed by Klein et al., 1987; Friend et al., 1988; Hansen and Cavenee, 1988). Retinoblastoma occurs in two forms: An hereditary form in which an infant inherits one defective allele of the retinoblastoma (Rb) locus. The remaining wild-type allele is then lost relatively frequently through somatic mutation, resulting in a multifocal, bilateral form of retinoblastoma. The hereditary form of the disease constitutes about 30% to 40% of all cases. In the sporadic form, a new somatic mutation occurs in a retinal cell followed by the loss of the remaining normal allele through a second somatic event. The chance of spontaneous loss of both Rb alleles in a single cell is relatively low; as a consequence, non-hereditary retinoblastoma is unilateral and only a single focus of tumor growth is observed.

Another major difference between the familial and non-hereditary forms of retinoblastoma is that infants with familial retinoblastoma have only one functional copy of the Rb gene in all their somatic cells. The presence of only one functional allele of the Rb gene predisposes the patients with inherited

retinoblastoma to a variety of tumors later in life, with osteosarcoma being the most frequently observed second tumor. In these second tumors, it was again found that the remaining functional Rb allele was lost in the tumor DNA, strongly implicating the loss of the Rb gene in the genesis of also these second tumors. These data furthermore suggest that the retinoblastoma gene plays a role in growth regulation of a much larger spectrum of tissues than just the retina.

Cytogenetic studies have shown that deletions in chromosome 13 region q14 are frequently found in the tumor genomes of retinoblastoma patients (Potluri et al., 1986). Recently we and others have isolated a cDNA clone from the q14 region of chromosome 13 that has many of the characteristics of the retinoblastoma gene (Friend et al. ,1986; Fung et al., 1987; Lee et al., 1987). Most importantly, Southern blot analysis of retinoblastoma tumor DNAs using the isolated cDNA as a probe revealed that as many as 30% of retinoblastomas have gross structural alterations in the locus that encodes this gene (Friend et al., 1986, 1987; Fung et al., 1987; Lee et al., 1987; Dunn et al., 1988; Goddard et al., 1988). Fine mapping of the deletions within this locus strongly suggested that the cDNA isolated indeed encodes the Rb gene.

Recently it has been shown that the Rb gene specifies a nuclear phosphoprotein of 105 kilodaltons (pp105 Rb) that binds to DNA in vitro (Lee et al., 1987; Whyte et al., 1988). Evidence that this gene product indeed mediates the growth suppressing effects of the intact Rb gene was recently provided by Huang et al. (1988), who showed that introduction of a retrovirus encoding pp105 Rb into a tumor cell line that lacks a functional Rb gene caused these tumor cells to become growth-arrested. Conversely it seems likely that the homozygous inactivation of this gene underlies the deregulated growth that initiates retinoblastoma.

In the past year, other studies have shown that the human Rb gene product is bound by the transforming proteins of adenovirus SV40 and human papilloma virus (DeCaprio et al., 1988; Whyte et al., 1988; Dyson et al., 1989). These data suggest that these tumor viruses acquire their oncogenic potential at least in part by complexing and inactivating the Rb gene product. Since these DNA tumor viruses have the ability to transform cells from a variety of tissues, it appears likely that the Rb gene product constitutes a critical regulator of growth in more cells than just the retina. Support for this view was also provided by recent observations made by several groups which investigated the status of the Rb gene in a number of tumors from patients that did not have a family history of retinoblastoma. They found homozygous inactivations of the Rb gene in such diverse tumors as breast cancer, small cell lung cancer, bladder cancer and a variety of mesenchymal tumors, again indicating the relevance of the Rb gene product in the normal growth control of breast, lung, bladder and mesenchymal tissues (Friend et al., 1987; Harbour et al., 1988; T'Ang et al., 1988; Horowitz et al., 1989).

The Rb gene is the first example of a gene whose inactivation leads to deregulated cell growth. The availability of a cloned copy of this gene should allow us to study the growth-inhibitory effect of its gene product in detail. One obstacle to the study of the function of the Rb gene is that to date no animal model for retinoblastoma exists. The reason for the apparent absence of retinoblastoma in domestic, agricultural and laboratory animals is at present unclear. For this reason, we have undertaken to develop an animal model for Rb-gene associated carcinogenesis, the first results of which are described here.

Structure and expression of the mouse Rb gene.

We recently isolated a nearly full length cDNA of the murine Rb gene. DNA sequence analysis of this clone revealed that the predicted structure of the murine Rb gene product has a 95% similarity to its human homologue (Bernards et al., 1989). Both the Rb protein of mouse and man were found to contain a leucine repeat motif (commonly referred to as a "leucine zipper") which is also found in the fos, myc and jun nuclear oncogenes, and appears to play a role in protein-protein interaction (Landschultz et al., 1988). The significance of the presence of this motif in the Rb protein is at present not clear.

To determine the pattern of expression of the mouse Rb gene, we have used Northern blot analysis of RNAs derived from mouse embryos at different periods of gestation. These data demonstrate that the Rb gene is expressed as early as 8 days of gestation, with a maximum level being observed at 14 days. When RNA derived from individual tissues of adult mice was tested for Rb expression we found that all tissues tested expressed the Rb gene, with brain, kidney, spleen, thymus and lung having relatively high levels and liver having relatively low levels of Rb mRNA (Bernards et al., 1989). These data again suggest that the Rb gene is involved in controlling cellular proliferation in a wide spectrum of cell types. On the other hand, these data raise the question why inactivation of the Rb gene leads to cancer in only a limited subset of tissues.

A mouse model for retinoblastoma.

We have recently been involved in the characterization of transgenic mice which harbor a transgene carrying the coding region of SV40 T-antigen oncogene coupled to the luteinizing hormone beta subunit gene promoter. Although one would expect that such transgenic mice would express the T-antigen gene in the cells of the anterior pituitary, a single founder mouse was found to develop bilateral ocular tumors. The retinal specificity of tumor formation was apparently caused by the fact that the SV40 T-antigen gene in this particular founder mouse was expressed only in the retina. Subsequent breeding of this founder mouse demonstrated that the ocular tumors were heritable and occurred with very high penetrance in offspring (Windle et.al., 1989).

One of the most striking histological characteristics of human retinoblastoma cells is that they spontaneously undergo photoreceptor cell differentiation in vivo to form Flexner-Wintersteiner rosettes. These rosettes are compsed of cuboidal cells which surround a central lumen. As can be seen in the photomicrograph in Fig. 1, rosettes that are very similar to those observed in human retinoblastoma were also found in every transgenic mouse ocular tumor examined. Furthermore, it was found that the transgenic mouse ocular tumors contained characteristic Homer Wright rosettes, retinoblastoma-characteristic ultrastructural features like lamelliform nuclear membranes, neurosecretory granules, cytoplasmic microtubules and cilia with a typical 9+0 pattern, thus lacking central tubules (Windle et al., 1989). All of these features are characteristic of human retinoblastoma, indicating that the ocular tumors observed in the transgenic mice closely resemble human retinoblastoma.

The mechanism by which ocular tumors arise in the transgenic mice is at present not clear. It seems likely that through a fortuitous integration the SV40 T-antigen gene was juxtaposed to a retinal-specific gene, bringing the transgene under the transcriptional control of this retinal-specific gene. Since SV40 T-antigen has been shown to bind to pp105 Rb (DeCaprio et al., 1988), it is likely that the expression of T-antigen in the retinal cells of the transgenic mice depletes these cells of functional Rb protein, thereby mimicking the loss of Rb by homozygous deletion.

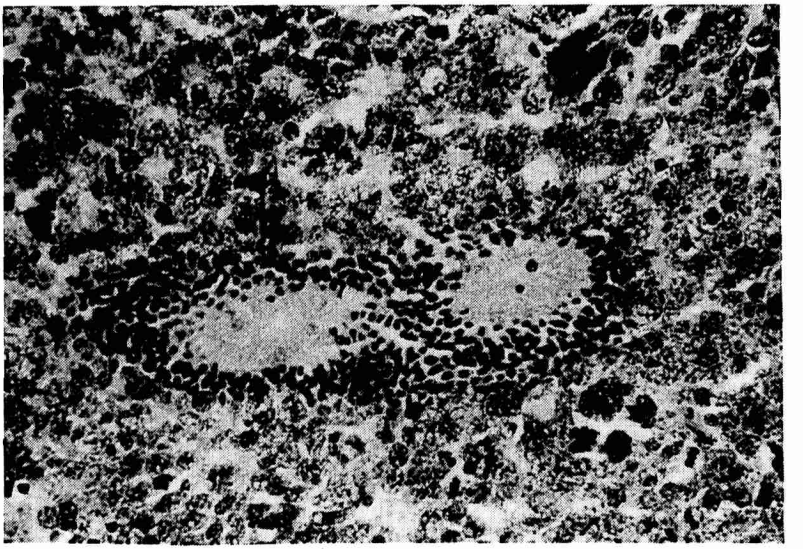

Figure 1. Photomicrograph of transgenic mouse ocular tumor section. Shown are two differentiated Flexner-Wintersteiner rosettes composed of photoreceptor-like cells, surrounded by more undifferentiated tumor cells.

Targeted mutagenesis.

Although the precise mechanism by which SV40 T-antigen induces ocular tumors in this line of transgenic mice is not known, it is obvious that a tumor that is by many criteria very similar to human retinoblastoma can be induced in mice. Because of this, it is surprising that mice never seem to develop spontaneous retinoblastoma. One possible explanation could be that not sufficient target cells for oncogenic transformation are present in the mouse retina, making retinoblastoma a very rare disease in mice. Alternatively it could be that loss of Rb is associated with a different spectrum of tumors in mice than in humans, or that Rb loss does not result in an increased risk for cancer in mice at all.

To answer these questions, we have begun experiments aimed at inactivating the Rb gene in transgenic mice. One technique to achieve this goal, targeted mutagenesis, was recently developed (See Capecchi, 1989 for a review). In short, this technique involves the inactivation of one allele of Rb in mouse embryo-derived stem cells (ES cells) in culture by homologous recombination, followed by the introduction of these Rb+/Rb- cells in mouse blastocysts. The resulting chimeric animals are then bred to derive mouse strains that are hemizygous for Rb.

As a first step towards this goal, we have cloned a part of the genomic copy of the mouse Rb gene that encodes exons 13 through 16. Using recombinant DNA techniques, we have introduced several mutations in exon 16 of this clone of the mouse Rb gene. The mutations consisted of either the insertion of a stopcodon in the reading frame of the Rb coding sequence or the insertion of a gene coding for neomycin resistance. These mutant Rb clones have been used for either transfection or microinjection into mouse ES stem cells.

At present, we have identified by use of the PCR technique several pools of ES cells that contain cells that appear to have undergone at least one homologous recombination event. It should now be possible to isolate ES cells with only one functional copy of the Rb gene. Such cells, once isolated, should allow the generation of chimeric mice with only one functional copy of the Rb gene. The availability of these mice should allow us to ask whether loss of Rb leads to oncogenic transformation in mice and should also allow us to evaluate which additional factors (if any) are required to induce malignant transformation of murine cells in the absence of functional Rb protein.

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