Retinoblastoma : genetic considerations and report of a new animal model

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Retinoblastoma is the most common primary, intraocular neoplasm of childhood. Histologically, retinoblastoma resembles, in many respects, other pediatric malignancies such as medulloblastoma and neuroblastoma. These tumors are composed of small, basophilic cells with scanty cytoplasm and often form non-specific Homer Wright rosettes. Retinoblastomas frequently possess, in addition, the distinctive Flexner-Wintersteiner rosettes, a form of photoreceptor differentiation.

Retinoblastoma was uniformly fatal until the second half of the nineteenth century [1]. Improvement in prognosis occured as a result of earlier diagnoses made possible by the ophthalmoscope and the adoption of enucleation with an adequate segment of the optic nerve as the primary treatment for retinoblastoma [1]. As children with retinoblastoma survived to adulthood, the role of heredity in retinoblastoma was appreciated.

The Retinoblastoma gene

During the 20th century, it has been recognized that retinoblastoma occurs in heritable and non-heritable forms. The heritable form accounts for approximately 30 % of cases and appeared clinically to be transmitted as an autosomal dominant trait. The remaining 70 % of cases do not demonstrate transmission of disease. Patients with the heritable form develop bilateral, multifocal tumors, while patients with the non-heritable form develop unilateral and unifocal disease. In addition, heritable retinoblastoma occurs at an earlier age than non-heritable disease.

Knudson subsequently proposed that both non-hereditary and hereditary retinoblastoma arise as a result of two mutational events [11]. Knudson's « two hit » hypothesis suggested that in heritable cases, the first of these mutational events occurs through the inheritance of a mutant allele from a carrier parent or through a new germinal mutation. The second mutational event occurred in somatic, developing retinal cells. Knudson proposed that the somatic event occurred relatively frequently within developing retinal cells, but usually did not lead to tumorigenesis in normal individuals lacking the first inherited mutation. In heritable cases, every retinal cell contained the « first hit » ; the likelihood of the « second hit » therefore reflected the dominant transmission and multifocality of heritable retinoblastoma.

In contrast, Knudson proposed that the normal retinal cells of non-hereditary retinoblastoma patients do not possess either mutation. Thus, in sporadic retinoblastoma both mutational events (« hits ») occurred in postzygotic retinal cells. Both of these mutations were unlikely to occur within the *same* retinal cell ; non-heritable disease therefore develops unifocally and unilaterally [11].

The development of retinoblastoma in children with chromosomal deletions of the q14 band of chromosome 13 led to increased molecular genetic investigation of this region [17]. In 198b, the retinoblastoma (Rb) gene was isolated (cloned) from the 13q14 region [7]. The Rb gene was discovered to be present in normal retina and other tissues, but was grossly deleted in 30 % of retinoblastomas [7]. Fine mapping of the Rb gene reveals that both hereditary and non-hereditary retinoblastoma are caused by mutations of both alleles of the Rb locus. Mutations of both Rb alleles within developing retinal cells lead to a loss of gene function, resulting in tumorigenesis. These findings have confirmed that Knudson's « two hits » occur at the two homologous Rb alleles. The Rb gene has come to be regarded as the prototypical tumor suppressor gene or recessive oncogene in that its presence is sufficent to prevent the development of this ocular neoplasm.

The development of other non-ocular cancers, such as osteosarcoma and soft tissue tumors, in patients with the heritable form of retinoblastoma has been attributed to the constitutional inheritance of one abnormal Rb allele. Loss of both alleles have been recognized in secondary tumors of retinoblastoma patients and in various tumors among patients demonstrating no evidence of or predisposition to retinoblastoma. Rb gene loss has been observed in some osteosarcomas [7], soft tissue tumors [8], small cell lung cancer [9], breast carcinoma [12] and bladder carcinoma [10]. It is therefore apparent that presence of one or both normal Rb genes plays a broad and fundamental role in cellular growth regulation.

Over the past two years, the function of the protein product of the Rb gene (p105-Rb) has been investigated. p105-Rb is a nuclear phosphoprotein hypothesized to act as an inhibitor of cellular proliferation. p105-Rb is underphosphorylated in non-proliferating cells and is phosphorylated in proliferating cells. This information suggests that the retinoblastoma protein has properties of a cell cycle regulatory element [4, 6].

Oncogenes

The function of tumor suppressing genes, such as Rb, appears to be distinct from that of the oncogenes. Oncogenes are present in the normal human genome in the form of proto-oncogenes. Proto-oncogenes appear to play a fundamental role in promoting normal cellular proliferation. Mutation within proto-oncogenes converts them into oncogenes which specify many of the malignant characteristics of tumor cells. Whereas tumor suppressing genes are inactivated in certain malignancies, somatic mutation of proto-oncogenes result in oncogene activation [14]. Unlike Rb inactivation, oncogene activation appears to occur only in somatic cells and has not served as a model for cancer predisposition.

Viral Oncogenes

A subset of oncogenes are contained within various viruses. After viral infection, viral oncogenes incorporate into the genomic material of target cells and produce oncoproteins which elicit indefinate cellular growth (transformation) or malignant behavior. The oncogenic viruses are well characterized and include the papillomavi-

ruses (HPV), polyomaviruses (JCV, BKV, simian virus 40) and the adenoviruses. The transforming genes which encode these oncoproteins have also been studied and include E7 (HPV 16), T-antigens (JCV, BKV, SV-40), and EIA (adenovirus) [2].

Rb as an Antioncogene

Viral oncoproteins function by complexing with host cellular proteins. Over the past two years, various investigators have demonstrated that HPV 16-E7, adenovirus EIA, and the T-antigens associate with and presumably inactivate the protein product of the retinoblastoma susceptibility gene [5, 6]. This suggests that tumor viruses acquire their malignant potential, in part, by inactivating the Rb gene at the protein level. This interaction has contributed to the Rb gene being referred to as an antioncogene.

An animal model of retinoblastoma

The creation of transgenic mice has allowed scientists to study the biological effects of various genes incorporated within the mouse genome. Introduction of genes into the mouse germline is performed by the microinjection of DNA into the pronuclei of fertilized ovum. Transgenic eggs are transferred to pseudo-pregnant females and allowed to develop and give rise to transgenic mice.

We have reported the development of heritable retinoblastoma in transgenic mice created by the retinal specific expression of simian virus 40 (SV40) large T-antigen (T-ag). These mice represent the first heritable model of retinoblastoma and demonstrate an *in vivo* inactivation of the retinoblastoma protein product by a viral oncoprotein [16].

Transgenic mice with retinoblastoma were created by microinjection of the coding region of SV40 T-ag linked to the luteinizing hormone beta (LHB) subunit promoter gene. This construct was designed with the intention of directing expression of T-ag and the resultant T-ag oncoprotein, to the anterior pituitary. Pituitary tumors developed in the majority of mice created. A single male founder, however, developed bilateral ocular tumors. This founder gave rise to a colony of transgenic mice demonstrating inheritance of ocular tumors with SV40 T-ag [16].

We have characterized these ocular malignancies and find that they represent the mouse counterpart to human retinoblastoma. Histologically, these tumors contain Homer Wright and Flexner-Wintersteiner rosettes together with less differentiated tumor cells (fig. 1). Ultrastructural features such as neurosecretory granules, triple membrane structures, cilia with a 9+0 pattern, and microtubules, which all occur in human retinoblastoma, are also demonstrated in murine tumors. Mouse retinoblastoma also resembles the human case immunohistochemically. Like the human tumor it expresses neuron specific enolase and does not express glial fibrillary acidic protin [16].

Murine retinoblastoma develops within the retinal inner nuclear layer in a multifocal, bilateral pattern. Foci of tumors coalesce to fill the globe ; there is subsequent optic nerve invasion and metastatic spread. This progression pattern displays a marked similarity to human retinoblastoma. These mice therefore represent an appropriate system for investigation of therapeutic interventions in retinoblastoma.
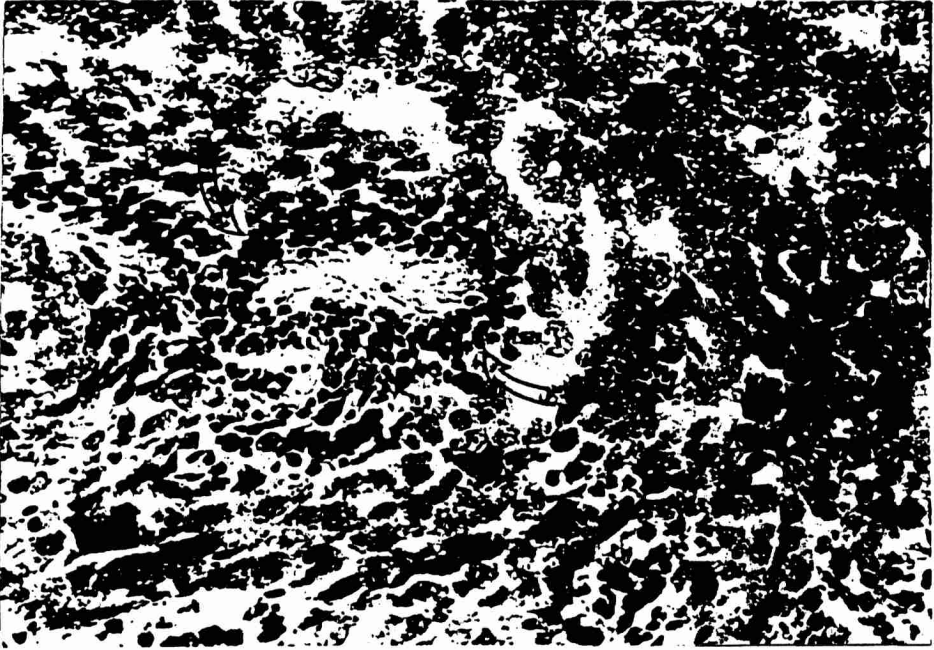
Fig 1. Photomicrograph of retinoblastoma from a transgenic mouse demonstrating Flexner-Wintersteiner (open arrows) and Homer Wright (closed arrows) rosettes amongst less differentiated tumor cells

How and why did retinoblastoma develop in these animals ? The site of transgene integration was mapped on metaphase chromosomes by in situ hybridization to a single integration site on chromosome 4. The murine Rb gene is located on chromosome 14 and was not altered by transgene integration. Specific expression of SV40 T-ag within the retina was demonstrated by northern blot analysis. Immunoprecipitation and western blot analysis revealed that p105-Rb and T-ag were intact and bound together within ocular and metastatic murine retinoblastoma. This information provides an alternative mechanism for retinoblastoma tumorigenesis through the retinal specific expression of a viral oncoprotein leading to inactivation of the retinoblastoma protein [16]. We are, therefore, investigating the role of viruses in the pathogenesis of human retinoblastoma.

In search of a retinal specific enhancer

The majority of transgenic mice created with the above construct did not demonstrate retinal expression of SV40 T-ag and did not develop retinoblastoma. The distribution of T-ag expression to the pituitary was determined solely by the promoter of the LHB gene. Transgene expression may also be determined by the site of chromosomal insertion. We believe that in our line of mice with retinoblastoma, the retinal specific expression of T-ag is most likely caused by transgene integration adjacent to an unidentified regulatory sequence of retinal expression [16].

We have isolated the transgene integration site possessing flanking genomic DNA, which may include a retinal specific enhancer gene and/or a novel retinal gene. The cloning of a retinal specific enhancer may prove useful in directing various genes to the retina.

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