TRANSGENIC MODELS OF RETINOBLASTOMA: WHAT THEY TELL US ABOUT ITS CAUSE AND TREATMENT*

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INTRODUCTION

IF WE ARE ULTIMATELY TO HAVE POWERFUL STRATEGIES FOR DEVELOPING anticancer therapies, we will need a full understanding of the molecular basis of different tumor types. The authors have utilized transgenic mice to understand the initiation and progression of retinoblastoma. These mice are also being used in the development of new therapies for this tumor.

The study of the function of the retinoblastoma susceptibility gene (*Rb gene*) has led to its recognition as a prototypic tumor suppressor gene, since absence of its function is tumorigenic. A major early insight into the role of the *Rb gene* was gained through Knudson's statistical analysis of the inheritance pattern of retinoblastoma.[1] Knudson proposed a "two-hit" model for the etiology of both the sporadic and hereditary forms of retinoblastoma, in which two mutations must occur in the same retinoblast. This model was expanded by Comings,[2] who in 1973 proposed that the two mutations were in the two alleles of the same gene, resulting in an absence of the gene product. This proposed model was supported by the isolation of the *Rb gene* and demonstration that both copies of the gene are mutated in retinoblastoma tumor cells.[3-5]

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Retinoblastoma is a tumor that occurs spontaneously only in humans.[6] The available data indicate a relatively simple and straightforward picture of the genetic initiation of retinoblastoma in humans. Here, retinoblastoma is a genetically homogeneous disease that segregates as a highly penetrant, autosomal dominant trait linked to mutations at the *Rb-1* locus in chromosome 13q14. When the mutations are inherited from an affected or carrier parent, or when the mutations occur early in embryogenesis, the disease is usually bilateral and multifocal, and affected individuals have a high probability of transmitting the trait to their offspring. When the mutation occurs late in embryogenesis, the disease is generally unilateral and unifocal, and affected individuals have a low probability of transmitting the tumor to their offspring.[7] This apparently simple molecular basis for retinoblastoma stands in contrast to many other tumor types, in which tumorigenesis appears to involve a complex, multistep process consisting of a combination of successive mutations, some of which activate cellular proto-oncogenes and others which inactive tumor suppressor genes. It remains to be seen in humans whether the cooperation of other mutations is involved in some later stage of retinoblastoma development. In the mouse, we shall present evidence suggesting that the production of a tumor resembling retinoblastoma cannot be achieved by inactivating the *Rb gene* alone.

In 1989, we described to the American Ophthalmological Society a transgenic mouse line that developed ocular neoplasms morphologically identical to human retinoblastoma.[8] The original male founder was identified in lines of transgenic mice derived from fertilized ova microinjected with a chimeric gene containing the protein coding region of the simian virus T-antigen (SV40-Tag) driven by the promoter of the luteinizing hormone β-subunit gene (LHβ). The phenotype of the founder was heritable with complete penetrance in transgenic offspring. We demonstrated a specific association between $p105^{Rb}$ and T-antigen protein in the mouse retinoblastoma tumor cells.[9] The transgene integration site is on chromosome 4, distinct from the Rb locus. We postulated that some random integration event placed the SV40-Tag under other unexpected retina-related regulatory control.[8]

We now provide additional data regarding this original (LHβ-Tag) mouse. In addition, we describe four other lines of transgenic mice that develop retinoblastoma-like tumors and/or give insights into the development of retinoblastoma in the genetically altered mouse. Finally, we give an example of the use of the original line (LHβ-Tag) in evaluation of new therapy for the treatment of human retinoblastoma.

MATERIALS AND METHODS

HARVESTING

Harvesting of tissues was performed as previously described.[8,10,11] Murine tissues were fixed in 10% buffered formalin and 3% phosphate buffered glutaraldehyde-sucrose or quick-frozen in liquid nitrogen. Processed tissue was sectioned for light microscopy and transmission electron microscopy or for immunohistochemical analysis.[12]

The transgene utilized in the LHβ-Tag transgenic line has been described in detail.[13] Methods of gene purification, injection into fertilized ovocytes, Northern analysis, and Southern analysis for this model have been described in detail.[9] The IRBP-Tag transgenic line, transgene construction, production and identification of transgenic mice, and Northern blot analysis follow the methods described by Howes and associates.[14] Transgene construction and generation and analysis of transgenic lineages for the αA-HPV-16 E6/E7 transgenic mice have been described by Griep and colleagues[15] and Lambert and co-workers.[16] IRBP-E7 transgene construction, production and identification of transgenic mice, and identification of p53 heterozygous and nullizygous mice are described by Howes and associates.[17]

Experimental treatment studies utilizing calcitriol (vitamin D_3), 1,25 dihydroxy-16-ene, 23-yne cholecalciferol (16,23 D_3), and 1,25 dihydroxy-16-ene cholecalciferol (16 D_3) and the combination of 16,23 D_3 and 16 D_3 follow the methods previously described.[18]

RESULTS

The results of the transgenic mouse experiments (1 through 5) are summarized in Table I.

TABLE I: RESULTS OF TRANSGENIC MOUSE EXPERIMENTS

TYPE OF MOUSE	ONCOGENE	PROMOTER	STATUS OF Rb PRODUCT	STATUS OF p53 PRODUCT	RETINAL TUMORS	CNS TUMORS
LHβ-Tag	SV40-Tag	LHβ	–	–	+	+
IRBP-Tag	SV40-Tag	IRBP	–	–	+	+
αA-HPV-16 E6/E7	E6, E7	α-crystallin	–	–	+	?
IRBP-HPV 16-E7	E7	IRBP	–	+	–	+
IRBP-HPV 16 E7/null p53	E7	IRBP	–	–	+	+

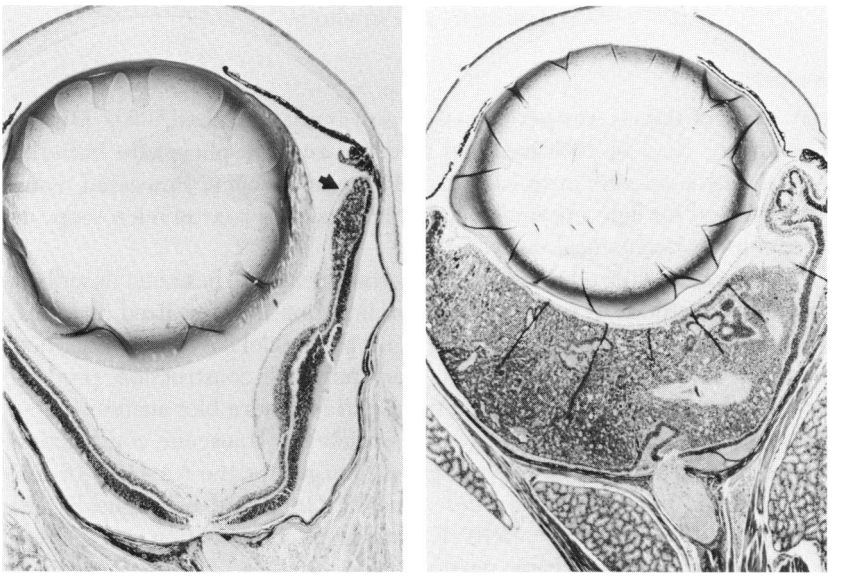

FIGURE 1
A: Early development of retinoblastoma in LHβ-Tag mouse. Tumor is seen arising from inner nuclear layer of peripheral retina (*arrow*) (hematoxylin-eosin, ×42.5). B: More advanced tumor in older mouse. Tumor has become confluent and involves almost entire retina (hematoxylin-eosin, ×42.5).

1. FURTHER FINDINGS ON THE LHβ-TAG MOUSE

The cell of origin for the ocular tumors appears to be the amacrine cell[19] (Fig 1A and B). Intraocular tumor reacted with antibodies to neuron-specific enolase and synaptophysin, while vimentin, glial fibrillary acidic protein, and S-100 were detected only in reactive glia derived from adjacent retina. The antigenic profile resembles human retinoblastoma, but differences in morphology and antigen distribution suggest a closer relationship to neurons of the inner nuclear layer than to photoreceptor cells. Bilateral multifocal ocular tumors have continued to be observed in 100% of transgene-bearing mice. Central nervous system neoplasms occur in 27% and closely resemble undifferentiated suprasellar or parasellar tumors occasionally observed in human trilateral retinoblastoma. Immunohistochemical and ultrastructural examination reveal that the transgenic brain tumors were undifferentiated and lacked all antigens associated with normal murine neuronal, glial, and ependymal cells.

The molecular basis for retina-specific gene expression in the LHβ-Tag transgenic mouse was studied by isolating several genomic clones of the

transgene integration site. These clones spanned the transgene integration site and more than 20 Kb of its flanking sequences. To identify the genes that were specifically expressed in the retina, we used a rapid screening procedure to search for exons of retina-specific genes in the genomic DNA flanking the transgene integration site. A systematic search of the 20 Kb of DNA flanking the transgene integration site revealed no retina-specific transcripts. These results indicate that one of two possibilities exists: (1) the retina-specific control element is in close proximity to the transgene, but the coding region of the gene that it controls is at a distance or (2) both the retina-specific control element and the coding region of the gene that it normally controls are located at a significant distance from the transgene integration site.

2. IRBP-TAG TRANSGENIC MICE

We produced transgenic mice that express the SV40 Tag oncogene specifically in photoreceptor cells, giving rise to retinoblastoma tumors of photoreceptor cell origin. One line of mice was generated in which all the animals develop both retinal photoreceptor cell and pineal tumors by as early as 7 days of age. Initially, Homer Wright rosettes are seen in the outer nuclear layer. By 4 weeks of age, the photoreceptor cells are largely replaced by hyperchromatic cells of small size and scanty cytoplasm containing numerous mitoses (Fig 2A). The majority of cells resemble undifferentiated retinoblastoma cells. The neoplastic cells invade the inner layers of the retina, and soon invade the optic nerve, ciliary body, iris, and vitreous as well (Fig 2B). Areas of necrosis, some focal calcification, and extension through the sclera, as well as invasion of the optic nerve, are present. This transgenic mouse model is similar to that described by Al-Ubaidi and associates.[20]

Examination of the brain of an embryonic 18-day-old IRBP-Tag mouse shows complete replacement of the pineal by tumor cells arranged almost entirely in Homer Wright rosettes (Fig 2C). By 2 weeks of age, the tumor fills the transverse fissure of the cerebrum. By 9 to 12 weeks, the tumor has extended diffusely throughout large portions of the brain.

Cell lines derived from retinal tumors and pineal cell tumors give positive immunostaining for the neuronal markers synaptophysin and neuron-specific enolase. Cultures were negative for glial fibrillary acidic protein, vimentin, and S-100 protein.

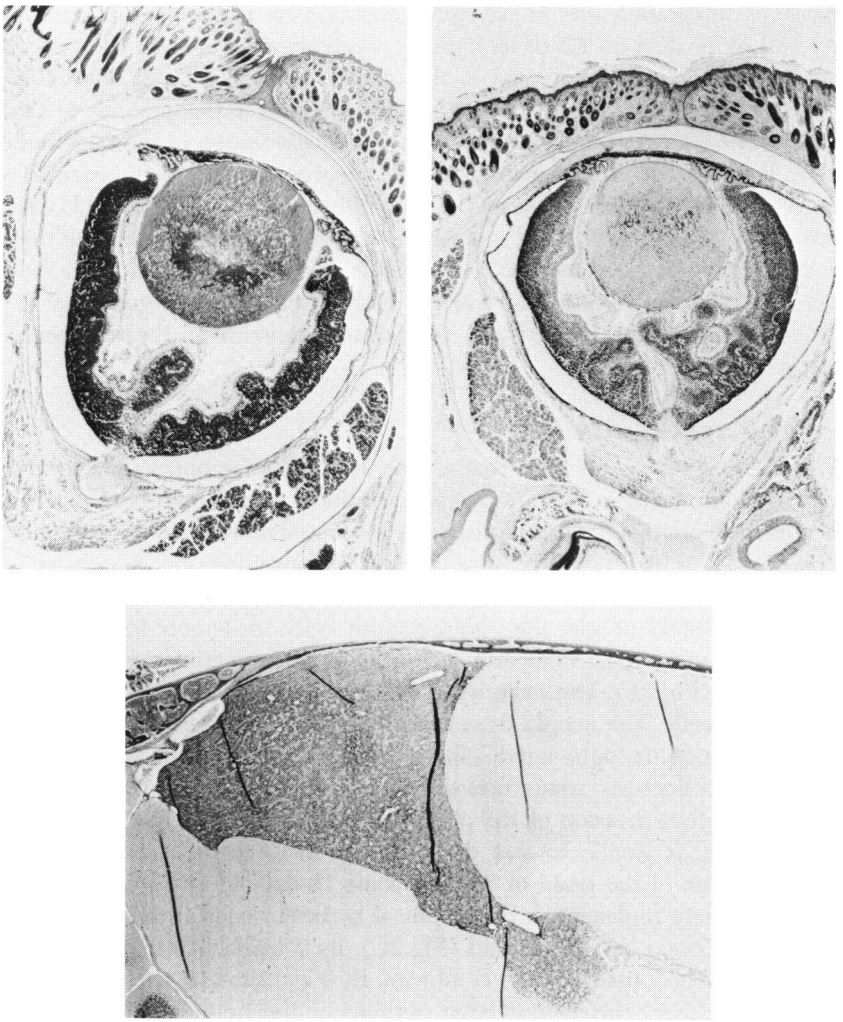

FIGURE 2

A: Diffuse development of retinoblastoma in IRBP-Tag mouse. Ganglion cell layer is spared at this stage of tumor development. Note presence of cataractous lens change (hematoxylin-eosin, ×37.5). B: More advanced tumor in older animal. Tumor involves full thickness of retina in some areas (hematoxylin-eosin, ×37.5). C: Pineal gland is replaced by tumor containing multiple Homer Wright rosettes (periodic acid-Schiff, ×28.3).

3. αA-HPV-16 E6/E7 TRANSGENIC MICE

Human papillomaviruses are associated with cervical and anogenital other carcinomas that commonly express two papillomaviral genes, E6 and E7. Transgenic mice were generated in which expression of E6 and E7 was directed to the developing ocular lens by virtue of the transcriptional regulatory activities of the murine αA-crystallin gene promotor. Three αA-HPV-16 E6/E7 mouse lineages were derived. All three lineages had overt phenotypes of microphthalmos and cataract.[15] Lens tumors developed late in the lives of these mice in one transgenic line, line 19. Line 19 also expressed E6 and E7 in the retina and other tissues outside the lens. The line 19 αA-HPV-16 E6/E7 transgenic mice were generated on the inbred FVB mouse genetic background. On this genetic background, we observed the infrequent occurrence of retinoblastomas in old adult animals in line 19 (ie, > 12 months of age) (Fig 3A and B). However, when the transgenic mice were mated to mice of other genetic strains, retinoblastoma developed at high frequency within 3 to 6 months of age. Thus, incidence of the retinoblastomas was dependent on mouse genetic background. The tumors appeared to originate from the bipolar cell layer of the retina. They closely resembled human retinoblastomas with numerous Homer Wright rosettes present under light microscopy. Electron microscopy revealed triple membrane structures and basal bodies. In situ hybridization experiments verified the high levels of expression of E6 and E7 transgenes in these tumors and that these tumors were not of lens origin. Histologic analysis of early foci of tumor cells in the retina of these animals indicates that the retinoblastomas originated from the bipolar layer of the retina. In numerous mice, the tumor was found to extend through the optic nerve into the brain (Fig 3C), and metastases to the cervical lymph nodes were observed. Studies are in progress to determine if primary central nervous system tumors occur as well.

4. HPV16-E7 WITH INTACT p53

The transgenic mice described above (Results 1 through 3) would be predicted to have the function of both the *Rb gene* and the p53 gene at least in part inactivated. To investigate the role of p53 in the development of retinoblastoma in mice, transgenic mice expressing HPV16-E7 in the retina were generated to potentially inactivate pRb while leaving p53 intact. The HPV16-E7 expression was directed to retinal photoreceptor cells using the promoter of the interstitial retinol binding protein (IRBP) gene. These mice failed to develop evidence of retinal tumors but showed the presence of pineal tumors (Fig 4A). The retinas, however, showed degeneration due to photoreceptor cell death and exhibited the histologic and ultrastructural features of apoptosis (Fig 4C and D).

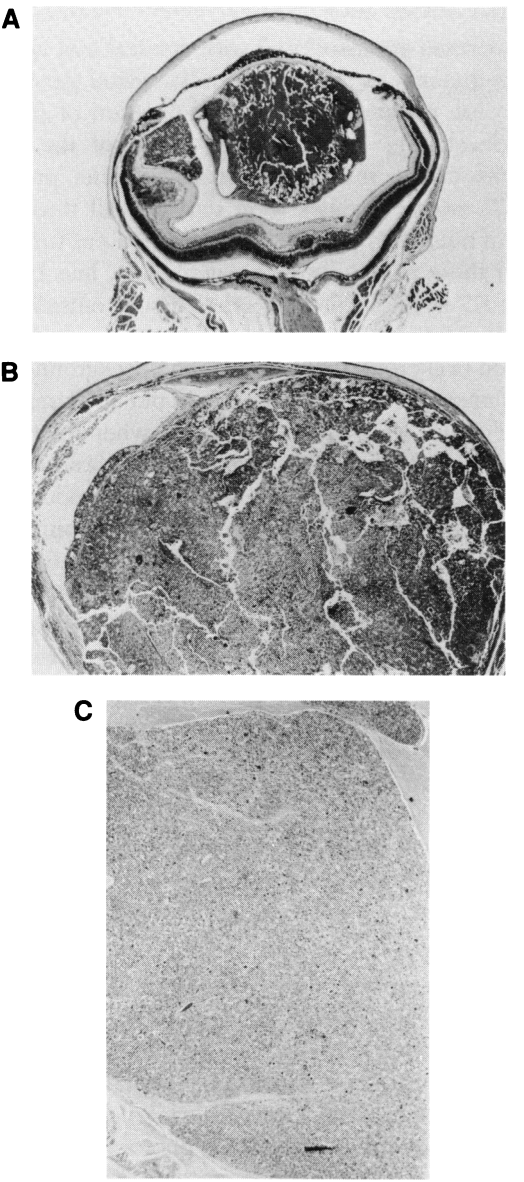

FIGURE 3
A: Retinoblastoma occurring in αA-HPV-16 E6/E7 transgenic mouse (hematoxylin-eosin, ×37.5). B: More advanced tumor in older mouse (hematoxylin-eosin, ×37.5). C: Retinoblastoma-like tumor in central nervous system (hematoxylin-eosin, ×37.5).

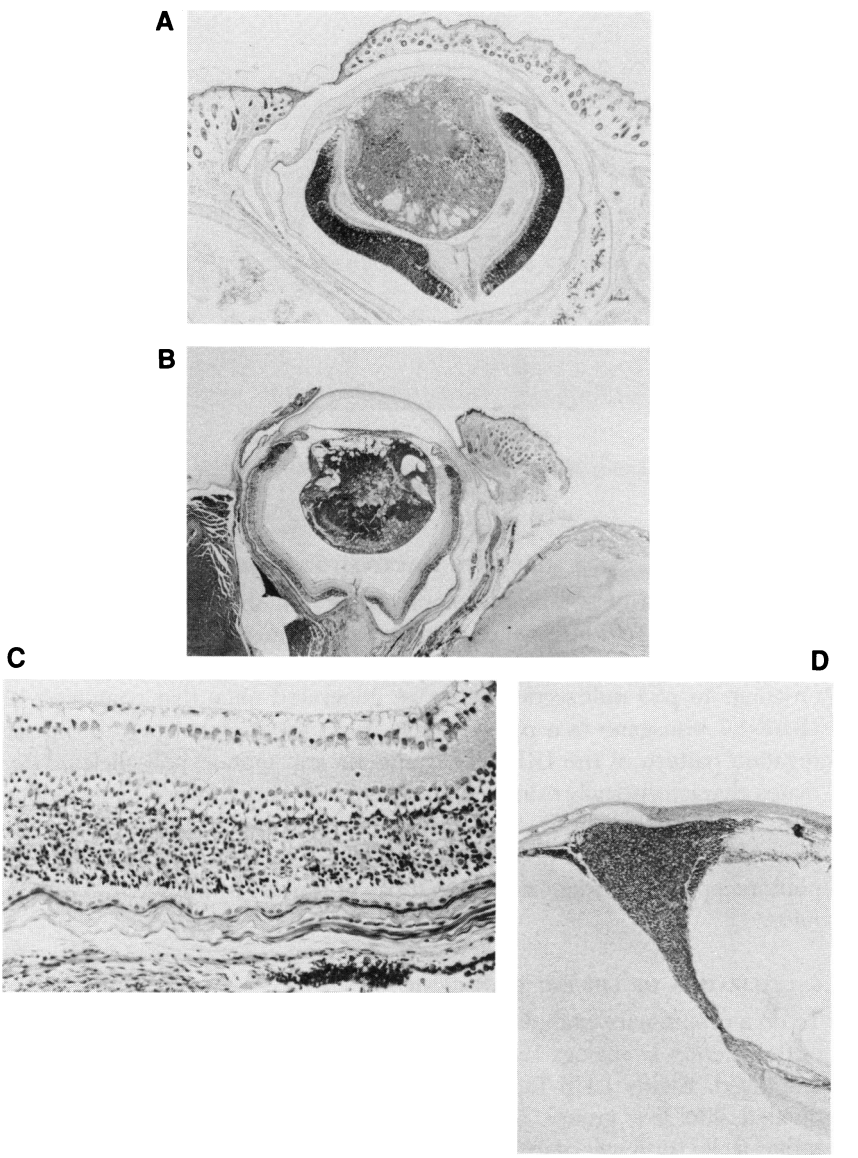

FIGURE 4

A: Developing retina in HPV16-E7 mouse with intact p53. No evidence of retinal tumor is seen (hematoxylin-eosin, ×37.5). B: Older animal showing evidence of degeneration of photoreceptor cell layer (hematoxylin-eosin, ×37.5). C: Higher-power view of photoreceptor cell death; cells have histologic features of apoptosis (hematoxylin-eosin, ×187.5). D: Tumor developing in pineal of IRBP-HPV-16 E7 mouse with intact p53 genes (hematoxylin-eosin, ×37.5).

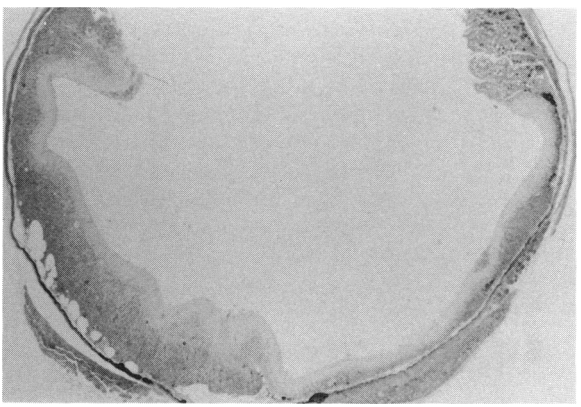

FIGURE 5
Retinal tumor developing in eye of trangenic mouse with E7 gene and absence of p53 (toluidine blue, ×37.5).

5. HPV16-E7 TRANSGENIC MICE WITH INACTIVATION OF p53

To further determine if the development of retinoblastoma required inactivation of IRBP-E7 positive p53, we generated mice expressing the IRBP-E7 transgene in a p53 nullizygous background. Through two generations of crossings to p53 nullizygous mice, we generated mice that contained the IRBP-E7 transgene in a p53-deficient background. According to the segregation pattern of the IRBP-E7 transgene and mutant p53 alleles, littermates characteristically exhibited either normal, degenerative, or retinoblastoma phenotypes (Fig 5). Southern and representative histologic analyses show that retinas from p53 nullizygous mice exhibited bilateral tumors of photoreceptor cell origin similar to those described in the IRBP-SV40 Tag mice.

6. UTILIZATION OF LHβ-TAG TRANSGENIC MICE IN EXPERIMENTAL DRUG TESTING

To do a preliminary analysis of the antineoplastic effect and level of toxicity of the vitamin D analogs 16,23 D_3 and 16 D_3, the LHβ-Tag mouse line was employed. Eighty LHβ-Tag transgenic mice, 8 to 10 weeks of age, were divided into five groups of 16 animals. Each group was treated intraperitoneally for 5 weeks with daily intraperitoneal injections of a vitamin D analog or control vehicle. Group 1 received the vehicle alone (negative control). Group 2 received vitamin D_3 (calcitriol), 0.05 μg/injection. Group 3 received the analog 16,23 D_3 0.05 μg. Group 4 received 0.05 μg of the related analog 16 D_3. Group 5 received a combination of 0.05 μg 16,23 D_3

and 0.05 μg 16 D_3. Eyes were enucleated at 1 week following termination of injection and were examined histologically in a masked fashion. All control animals demonstrated involvement of retinoblastoma. 16,23 D_3 analog showed essentially no toxicity at the doses used and inhibited the growth of retinoblastoma to a greater extent than did vitamin D_3, which was included as a positive control (Fig 6).

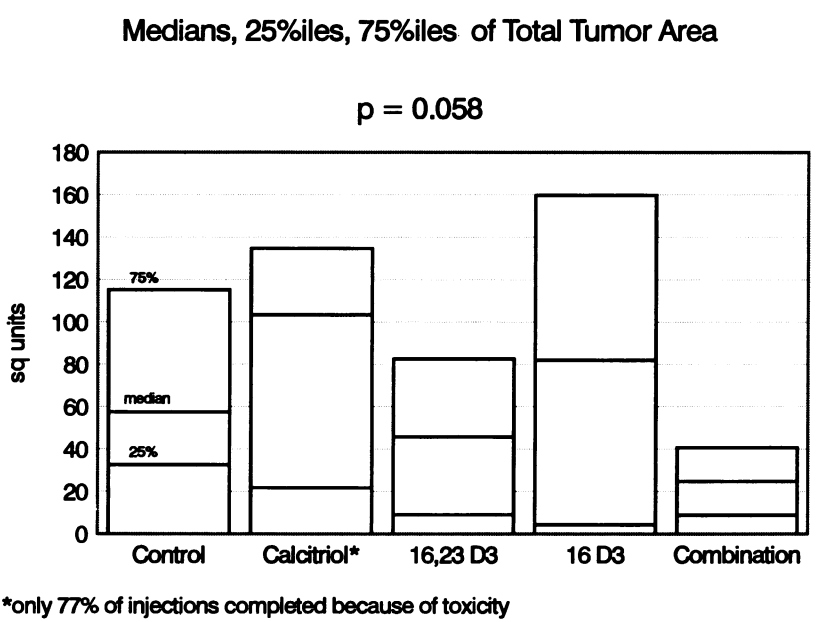

FIGURE 6
Summary of activity of calcitriol (0.05 μg/injection), 16,23 D_3 (0.05 μg/injection), 16 D_3 (0.05 μg/injection), and combination of 0.05 μg/injection 16,23 D_3 and 0.05 μg/injection 16 D_3 utilizing LHβ-Tag transgenic mice. Tumor area is expressed in square units measured through reticule.

DISCUSSION

Transgenic mice are created by the introduction of new genes into the pronuclei of recently fertilized eggs. The embryos are transferred to pseudopregnant mothers and allowed to develop to term. In a fraction of births, the injected gene or genes are incorporated into the genome of all cells, including the germ cells, thereby creating a transgenic animal.[21] The LHβ-Tag mice represent the first animal model of human retinoblastoma.[8,9] We previously provided evidence of a specific association between SV40-Tag

and p105-Rb within tumor cells by co-immunoprecipitation.[9] It has been demonstrated that the oncoproteins of a number of DNA tumor viruses, including SV40-Tag,[22] human papillomavirus E7,[23] and adenovirus E1A,[24] form specific complexes with the *Rb gene* product pRb. In addition, SV40-Tag binds the product of the p53 gene.[25,26] The p53 protein is now understood to be a tumor suppressor gene product.[27] Thus, the Tag oncoprotein is thought to mediate its tumorigenic properties, at least in part, by binding to and functionally inactivating the pRb and p53 tumor suppressor proteins. This would appear to be the basis for the development of retinoblastoma in the LHβ-Tag and IRBP-Tag transgenic mice.

Analysis of human cervical carcinoma has lead to the identification of specific papillomaviral genes E6 and E7, which appear to play a role in carcinogenesis. Tissue culture studies have demonstrated that E6 and E7 genes are oncogenic.[28-30] Like the T antigens (Tag) of simian virus 40 (SV40), the E6 and E7 gene products belong to a family of oncoproteins that affect cell growth and differentiation at least in part through their interaction with cellular tumor suppressor genes. The E7 protein associates with the retinoblastoma susceptibility gene product, Rb,[23] inactivating Rb function. The E6 protein is capable of binding the p53 protein[31] in the presence of a cellular protein E6 AP[32] and targeting p53 for degradation via a ubiquitin-dependent pathway.[33]

In the HPV-16 E6 and E7 transgenic mouse, the papillomaviral genes E6 and E7 expressed in the retina. Retinoblastomas developed in this line. In the HPV-16 E7 line with intact p53, retinoblastoma did not develop. Mice expressing E7 in a p53 nullizygous background did, however, develop retinal tumors.

These findings are consistent with studies described elsewhere.[34] Here, mice carrying mutations in both the Rb and p53 genes had reduced viability and exhibited novel pathology, including retinal dysplasia, pinealoblastoma, islet cell tumors, and bronchial epithelial hyperplasia.

These findings also are consistent with various proposed functions for p53. These include negative growth regulation,[35,36] response to DNA damage,[37,38] and induction of cell death.[39,40] In the development of neoplasia, the importance of coupled inactivation of Rb and p53 is evident.[41] It has been suggested that of the occurrence of sequential mutation of Rb and p53 may be a critical component in human multistep carcinogenesis.[34] Various human tumor types, including some sarcomas and carcinomas of the lung, breast, cervix, and pancreas,[42] show a high frequency of mutation of both genes. Interestingly, p53 mutations have rarely been identified in retinoblastomas[17,43] (D. Yandell, personal communication, 1994).

The infrequent observation of amplification of the N-myc gene in human retinoblastoma[44-46] is intriguing, since N-myc amplification in neuroblastoma has been correlated with poor clinical prognosis.[47] While the N-myc gene appears to be amplified in only approximately 10% of retinoblastoma,[47,48] it has been shown to be highly expressed in most retinoblastomas.[47] This possibly reflects the normal level of N-myc expression in the embryonic retinoblasts from which the tumors are derived.[48] The maintenance of high levels of N-myc expression may be important in keeping the cells in a mitotically active state, as has been shown to be the case of c-myc.

The possible involvement of N-myc in retinoblastoma is further suggested by the molecular biologic indications that pRb and N-myc act antagonistically to regulate cell growth. The N-myc protein has been shown to directly bind pRb in vitro, suggesting that direct interaction of these two proteins may be involved in cell cycle control.[49] In addition, pRb downregulates the expression of a number of S-phase genes, including N-myc, through its inhibitory interaction with the cellular transcription factor E2F.[50,51] Finally, injection of pRb into cells in the early G1 phase of the cell cycle results in a cell cycle arrest, but coinjection with c-myc inhibits the ability of pRb to arrest the cells.[52]

The utility of the retinoblastoma transgenic model in testing new chemotherapeutic agents is seen in the experiments regarding an analog of vitamin D_3, 16,23 D_3. The role of vitamin D as an antineoplastic agent is well described.[53,54] We have previously demonstrated in vivo and in vitro inhibition of human retinoblastoma by vitamins D_2 and D_3 and have discussed the mechanisms for the antineoplastic properties of these compounds.[18,55-57] In a previous study of vitamin D_3 in the LHβ-Tag mice,[18] vitamin D_3 was found to inhibit the local extension of the tumors in a dose-dependent fashion. The toxicity of vitamin D_3, however, was marked. The 16,23 D_3 analog showed essentially no toxicity at the doses used and inhibited the growth of retinoblastoma to a greater extent than did vitamin D_3, which was included as a positive control.

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DISCUSSION

DR DEVRON H. CHAR. As usual, Dr Albert and colleagues have produced an elegant, scholarly study, and he has presented it in his usual lucid manner.

A number of points are raised by this study, and I will try to comment on those that I think are the most important. As Dr Albert has stated, in most human tumors the development and progression of neoplasia are driven by multiple genetic abnormalities. While this is best described in the malignant transformation and progression of colonic carcinoma, in several other human tumors a similar pattern is observed.

In the first of the animal models he describes, retrospective analysis shows that loss of both p53 and the retinoblastoma gene is required to produce ocular tumors. Several other laboratories have shown with a variety of different techniques, including the production of chimeric animals, that loss of the Rb1 gene alone is not sufficient to produce eye neoplasms.

In collaboration with Drs J. Gray, J. O'Brien, K. Gordon, and N. Aldrich-Wolf, my co-workers and I have noted similar findings in human retinoblastoma, namely, that multiple genome changes occur in this tumor.

This slide is a graphic representation of a technique that Dr Gray termed comparative genomic hybridization (CGH) (*Eye*, In press). Basically, the entire tumor genome is hybridized onto a normal metaphase spread. Using appropriate labels, areas of genomic addition or amplification are shown in green, and areas of genomic loss or deletion are highlighted by red staining. In retinoblastoma tumors we have observed a myriad of different reproducible genetic alterations in addition to loss of the Rb1 gene. The importance of these various genetic changes in tumor progression and prognosis remains to be elucidated.

Drs O'Brien and Aldrich-Wolfe have performed DNA sequencing on a group of retinoblastomas that have been characterized regarding their DNA cell cycle status as well as immunohistochemical staining for presumably abnormal p53. The tumor suppressor gene p53 appears to have an abnormal gene product in over 80% of the human retinoblastoma cells we have studied. The nature of that abnormality is not yet clear. Unlike the Rb1 gene, p53 has areas that are "hot spots" for alterations, and sequencing of those regions does not show a detectable amino acid alteration. Most likely, there is either abnormal p53 nucleoprotein production or aberrant binding of the p53 protein. In either case, a posttranscriptional stabilization of the protein is likely. It appears that like the animal models, there are abnormalities in both RB1 and p53 in this human disease (*Nature Genetics*, In press).

Given these findings, along with the reproducible diversity of other genomic changes in retinoblastoma, it is likely that while RB1 alteration is necessary for tumor development, it is only one of several alterations that are important in the pathogenesis and progression of this tumor.

DR W. RICHARD GREEN. I too would like to congratulate Dr Albert and co-authors on this superb study. The animals with the simian virus-IRPB developed photoreceptor cell degeneration. I wonder if there is any possible clinical counterpoint to this and if that is being investigated.

DR DANIEL ALBERT. I would like to thank Dr Char for his useful discussion and amplification of our findings. It replied to Dr Richard Green's question. I think the role of the p53 gene in certain types of retinal degeneration may be an important one. Further studies of this are in progress.