The Rb Gene and the Negative Regulation of Cell Growth

By Robert A. Weinberg

In recent years, several lines of evidence have converged to support the idea that loss of genetic information is a critical step in the pathogenesis of many types of tumors. According to this model, cells shed certain critical growth regulatory genes during their evolution from normalcy to malignancy. Such growth regulators may normally act to constrain cellular proliferation; once lost, normally existing barriers to cell growth are removed, leading in turn to the unregulated proliferation of neoplasia.

Such carcinogenic mechanisms involving loss of genetic information stand in stark contrast to the lessons taught over the past decade by the field of oncogene research. More than a dozen oncogenes have been associated with human tumors. Without exception, these oncogenes derive from pre-existing normal cellular genes, the proto-oncogenes, which have been implicated in regulation of normal cell growth and differentiation. The genetic changes that convert normal protooncogenes into oncogenes invariably involve alterations that potentiate the activity of the normal genes, creating deregulated, hyperactive alleles. It is these hyperactive genes that then drive malignant proliferation.

Somatic cell genetics has provided the first body of evidence indicating the importance of loss of genetic information from tumor cell genomes. The results all stem from a simple experimental plan involving the fusion of tumor cells with more normal, nontumorigenic cells. The resulting cell hybrids, which carry chromosomes from both parents, are generally nontumorigenic. This has led to the conclusion that the normal partners in the fusion are supplying genes that reimpose normal growth control on the malignant cell.

Various control experiments have been performed to demonstrate that this loss of tumorigenicity is not attributable to trivial mechanisms deriving from tissue or species incompatibility between the two parent cells. Ultimately the most persuasive experiments are those showing that reversion from tumorigenicity correlates with the presence in the hybrid cell of a specific chromosome originating from the normal parent cell. It would seem that this normal chromosome carries a specific gene (or genes) that imposes normal growth control on the malignant cell. Such genes are presumed to replace similarly acting genes that were lost previously during the genetic events that led initially to the creation of the malignant partner in the cell fusion.

This point is made most directly by experiments in which single chromosomes are transferred individually from a normal cell to a malignant recipient. Such an experiment represents a more focused experimental strategy than earlier manipulations in which an entire complement of normal chromosomes are comingled with those of the tumor cell. For example, such experiments have shown that a normal chromosome 11 causes loss of tumorigenicity when introduced into cells of a Wilms' tumor line. Experiments using other single-chromosome transfers are in progress.

A second line of evidence pointing toward genetic loss during tumorigenesis derives from karyotypic studies of specific human tumors. A decade ago, study of chromosomes prepared from retinoblastoma tumor cells revealed interstitial deletions affecting the q14 band of chromosome 13. Analogous experiments on Wilms' tumor demonstrated a specific loss of genetic material associated with the p13 band of chromosome 11.

In the case of retinoblastoma, this karyotypic abnormality suggested the loss of a hypothetical gene, termed Rb, associated with the 13q14 chromosomal band. Insight into the origins of retinoblastomas was extended by others who addressed the fate of the surviving intact Rb allele on the paired chromosome 13. A series of indirect but elegant genetic analyses made it clear that this allele is also lost during the formation of a retinoblastoma. Taken together, this body of work strongly suggested that both copies of the 13q14-associated Rb gene need be lost or inactivated in order to trigger tumorigenesis.

The observed loss/inactivation of both copies of the Rb gene during tumorigenesis carried a number of implications about the function of the normal Rb alleles. Most important, this result indicated that either copy of the Rb gene can serve effectively to control normal cell proliferation. However,
when both copies are lost, then an essential repressor of proliferation is deleted from the cell, leading in turn to the unconstrained growth of the cell and its descendants.

The loss of functional Rb copies can occur at two distinct points in the human life cycle. At conception, a gamete may contribute a defective Rb gene to the zygote; the resulting conceptus is constitutionally hemizygous for Rb function. Subsequent development is essentially normal since the intact Rb allele present in all somatic cells suffices to orchestrate normal development. However, in some cells of the fetus, a rare somatic mutational accident may occur that deprives a cell of its remaining intact Rb allele. Such an Rb nullizygous cell, should it reside in the developing retina, will spawn a retinoblastoma. Children who are constitutionally hemizygous for Rb are strongly predisposed to retinoblastomas since one of the two normally present Rb gene copies is missing from all their retinal cells.

An alternative mechanism for generating Rb nullizygosity depends exclusively on somatic mutational events. Children afflicted with “sporadic” retinoblastoma are genetically intact at conception. In these individuals, two rare somatic genetic alterations occurring within the same retinal precursor cell serve to knock out the two copies of the Rb gene. The unlikelihood of two rare mutations occurring within the same retinal cell clone explains the fact that children suffering from sporadic retinoblastoma exhibit only a single focus of tumor formation. In contrast, those afflicted with the familial disease usually exhibit multifocal disease in both eyes since one of the essential mutations is implanted in every cell of their retina.

An important lesson derives from the mechanisms that lead to the elimination of the second Rb allele. In principle, this loss may occur through some random somatic mutation event such as a point mutation or deletion occurring at low frequency (approximately $10^{-6}$ per cell generation). However, another mechanism can yield the same end result with far higher probability. Through mitotic recombination or gene conversion, the entire region of the chromosome carrying the initially defective allele may become doubled; the duplicated chromosomal segment may then replace homologous sequences on the hitherto intact chromosome. To the extent that genes in this chromosomal region were initially present heterozygously, they have now become homozygous, since one of the two allelic copies is now present in two identical copies.

By this logic, reduction to homozygosity may be a frequent genetic mechanism operating during the formation of retinoblastomas that serves to remove a surviving wild type Rb allele and thereby unmask a recessive, defective Rb allele. More generally, such reductions-to-homozygosity occurring in other chromosomal regions may signal the presence of other genes that behave like the Rb gene in tumorigenesis. Accordingly, a number of groups have searched for reductions-to-homozygosity of various chromosomal regions as a means of discovering hitherto unrecognized genes whose homozygous inactivation triggers tumor formation in one or another tissue. By now at least a dozen such chromosomal regions and associated hypothetical loci have come to light using this type of genetic analysis. Reductions-to-homozygosity of each of these loci is associated specifically with one or several types of malignancy. In each of these cases, these chromosomal changes are presumed to result in homozygosity of nonfunctional (ie, null) alleles. By logical extension, the normal versions of these genes, when present in at least single copy, are presumed to act as guardians of normal cellular growth regulation. Some have called these “tumor suppressor” genes or “anti-oncogenes.” Neither term appropriately describes the normal role of such genes, but these terms are already well-embedded in a rapidly growing literature and will likely stick.

Only one of these genes, that associated with the Rb locus, has been isolated by molecular cloning. The Rb gene encompasses about 190 kilobases of DNA on chromosome 13. While it is widely assumed that other tumor-suppressor genes function in a manner analogous to that of Rb, these other genes do not appear to share close sequence homology with Rb. As such, the isolation of these other genes will not be facilitated by the availability of the cloned Rb gene and will thus depend on difficult and labor-intensive structural analysis of large numbers of tumor cell DNAs.

Use of the Rb clones, represented by both genomic (ie, chromosomal) and cDNA sequences, as probes in Southern blot analyses has already revealed interesting lessons about the mechanisms that cause Rb gene inactivation. Large scale rearrangements or deletions are responsible for less than one third of the gene inactivation events. Much more frequent are subtle changes in gene structure including point mutations. These findings are not unexpected in that they reflect genetic mechanisms that operate widely to knock out the functioning of a variety of well-studied genes. The apparent frequent involvement of subtle changes in Rb gene structure is in consonance with karyotypic analyses of the chromosomes of retinoblastomas that only rarely reveal microscopically visible anomalies in the long arm of chromosome 13; only changes involving millions of DNA base pairs are large enough to affect the banding structure of chromosomes as seen through the light microscope.

Use of cloned Rb gene probes has revealed that Rb gene inactivation occurs in far more tumors than simply retinoblastomas. Such an outcome was predictable in the case of osteosarcomas and soft-tissue sarcomas since children suffering multifocal retinoblastoma and cured of this tumor early in life have greatly increased risk for these sarcomas in later years. In light of this, the term Rb would seem to be a misnomer in that null alleles of this gene predispose to more than just retinal tumors. Recent work has shown frequent Rb inactivation in sarcomas occurring even in patients having no history of retinoblastoma. Indeed, it becomes possible that somatic inactivations of Rb alleles may be the triggering events in most human sarcomas.

More recent analysis of the genomes of small cell carcinomas of the lung and bladder carcinomas also reveal frequent inactivation of Rb alleles, although these events are not as common in these tumors as in retinoblastomas. A small percentage of mammary carcinomas may have similar lesions. Nonetheless, Rb inactivation is not ubiquitous in all
types of cancer since study of most types of tumor DNAs has failed to reveal any alterations of chromosomal Rb sequences.

Like virtually all other genes, Rb imposes its effects on the cell through the actions of an encoded protein. The nucleotide sequence of the Rb gene allowed prediction of the amino acid sequence of its protein. This sequence was used in turn to design several synthetic oligopeptides that served as immunogens that were useful for inducing antisera reactive with the Rb gene product. It is a protein of a 105 kD that is found in the nucleus and is readily labeled with radioactive phosphate. Accordingly, the Rb gene constrains cell growth through the actions of a nuclear phosphoprotein, termed here p105-Rb. Since this protein has some binding affinity for DNA, one might speculate that it serves as a regulator of gene expression; further evidence on this point is lacking at present.

The antisera against this protein serve as useful reagents for determining the frequency of Rb inactivation in retinoblastomas. While the protein is readily detectable in normal retinoblasts, work in my own laboratory has failed to reveal it in 18 of 18 retinoblastoma lines tested to date (Horowitz JM and Weinberg RA: in preparation). This provides strong support for the notion that Rb inactivation is central to the genesis of all retinoblastomas. This point could not emerge from structural analyses of retinoblastoma DNAs using Southern blotting in which only a minority of tumor samples gave evidence of change in gene structure.

While these analyses have strongly supported the role of Rb inactivation in the pathogenesis of a variety of tumors, they shed little light on the most interesting question in this field: how does the Rb gene function in the physiology of the normal cell, and by extension, how does its inactivation trigger neoplasia? While the answers are hardly in hand, a striking and powerful insight came last summer from an unexpected quarter. Those studying the workings of a human adenovirus oncogene uncovered a molecular interaction that tied their own studies closely to the mechanism of action of p105-Rb.

The adenovirus oncogene in question is termed EIA. Many studies have demonstrated its ability to specify several distinct functions. In human cells, in which adenovirus infection is generally lytic, the oncogene plays an essential role in programming the expression of other viral genes; in this way, the E1A oncoprotein can serve as a transcriptional regulator. By contrast, in rodent cells the E1A gene acts as an oncogene: it can immortalize embryo cells, thereby enabling them to grow indefinitely in culture. Nonetheless, these oncogenic powers do not suffice to transform a fully normal cell. The viral oncoprotein may alter their functioning, and by so doing, trip a series of critical switches in the cell's regulatory circuitry.

Monoclonal antibodies against the E1A-encoded oncprotein have been developed as a tool for understanding the multiple roles of E1A. When used on lysates of adenovirus-transformed cells, these antibodies bind the E1A proteins, as expected, but the immunoprecipitates also contain as many as six other proteins whose nature was initially unclear. Subsequent analysis explained these results by showing that the E1A oncoproteins exist within the virus-transformed cell in complexes with these other proteins, which are in fact of host cell origin.

These complexes between viral and cell proteins would appear to provide clues into how E1A is able to induce a large variety of changes in cell behavior. Thus, each of the host cell target proteins with which the E1A oncoprotein complexes may represent a normal regulator of one or another cellular response pathway. By complexing with such cellular proteins, the viral oncprotein may alter their functioning, and in so doing, trip a series of critical switches in the cell's regulatory circuitry.

The importance of Rb is highlighted by these observations involving two other DNA tumor viruses, SV40 and human papillomavirus type 16. SV40 is a monkey virus that is unrelated to adenoviruses, but like the latter it is tumorigenic in rodents. Human papillomavirus type 16 represents a third, essentially unrelated class of viruses. It and its close relatives are implicated in the etiology of most carcinomas of the uterine cervix. The large T (LT) oncoprotein of SV40 and the E7 oncoprotein of HPV16 also bind to Rb-p105 in virus-transformed cells. This is most striking since the three viral oncoproteins (E1A, SV40 LT, HPV E7) are structurally unrelated to one another and thus have apparently acquired this common tropism for p105-Rb through a process of convergent evolution.

One critical ancillary observation deserves mention here, as it illustrates the importance of p105-Rb complex formation to the transforming powers of a viral oncoprotein, specifically the protein of SV40. A single amino acid substitution is known to knock out its oncogenic powers; this subtle change in oncoprotein structure also prevents this protein from complexing with p105-Rb. This shows that the SV40 LT:p105-Rb complex is not reflective of some adventitious aggregation process; instead it represents a functional interaction that is essential to the transforming powers of SV40 LT.

The importance of Rb is highlighted by these observations showing that this gene and its encoded protein are involved in a number of distinct mechanisms leading to cancer. It appears that strong selective pressures have worked to encour-
age the evolution by a number of tumor viruses of oncoproteins able to complex with p105-Rb. When viewed from this perspective, it would seem that the p105-Rb protein sits at a central and critical point in the cell’s growth regulatory pathways.

The inactivation of genes like Rb must underly pathogenetic mechanisms in a wide variety of tumors, including perhaps hematopoietic malignancies. How can one conceptualize the workings of these genes and their encoded proteins? At the simplest level, one can say that the Rb-p105 protein functions as a signal transducer. For example, in many cells it may serve to inhibit growth through its ability to repress the expression of a bank of genes whose expression normally favors cell growth. Certain growth-stimulatory signals may antagonize Rb function and in this way allow the turn-on of these genes. Indeed, such inhibition of Rb function may be achieved by endogenous cellular analogs of the DNA tumor virus oncoproteins. Cells that have lost p105-Rb may lose the ability to shut down these critical growth-related genes and, as a consequence, the ability to enter into a growth-arrested state. In the absence of shutdown, cell proliferation may continue indefinitely, resulting in the large cell clones that are seen as tumors.

These speculations have little substance behind them at present, but the models they suggest make it possible to design specific experiments. With the molecular reagents currently available, these models can be subjected to critical testing. Because of this, we may be only several years away from a precise understanding of how the Rb protein functions as a critical governor of normal cell growth. This is only a beginning, for in the coming years a host of similar growth-constraining genes will be isolated. Their characterization will provide a whole new panorama on the molecular mechanisms governing cell growth.

REFERENCES

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