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In Vitro Models of Carcinogenesis: Expression of Recessive Genes by Chromosomal Mutations

By John B. Little*

There has been considerable recent interest in the mechanisms by which recessive mutations involving cancer genes may be expressed. We have developed an *in vitro* model to study this phenomenon in an endogenous autosomal gene in human cells. We have analyzed the molecular structural changes that lead to loss of heterozygosity at the thymidine kinase (tk) locus. The results indicate that expression of a recessive allele frequently occurs by loss of heterozygosity at that locus. Over 90% of spontaneous mutants at the tk locus arose by allele loss. The fraction of induced mutants that arose by this mechanism depended upon the inducing agent. Loss of the active tk allele was often accompanied by loss of linked genetic loci on the long arm of chromosome 17. These results suggest that large-scale chromosomal mutations resulting from events such as deletion or mitotic recombination may be an important mechanism for the expression of activated or mutated recessive genes in human cells. Such recessive mutations could involve oncogenes or other growth regulatory genes important in carcinogenesis.

It gives me great pleasure to participate in this Symposium that celebrates the 40th Anniversary of the Institute of Environmental Medicine and honors its leader for many of those years, Norton Nelson. I have been associated with the Environmental Health Center at Harvard for the past 25 years, and we have always felt a special relationship with our many friends at New York University. Jim Whittenberger was the founder and leader for many years of our Center. He and Norton Nelson are close personal friends as well as scientific colleagues, who together share a major responsibility for the development of the field of environmental health as we know it today.

Introduction

It has long been recognized that the development of a malignant tumor *in vivo* is a complex, multistage process that likely involves genetic and epigenetic events. With the development of cellular systems that allow us to study the neoplastic transformation of individual cells *in vitro*, it has become evident that this initial stage in the process of carcinogenesis is also a complex one (*1,2*). Studies in human and rodent cells indicate that at least two cellular processes are involved. The first is morphological transformation or the conversion of normal cells to those with the malignant phenotype in terms of their growth and cytologic characteristics. The second is immortalization or the development of a cell line with an unlimited potential for proliferation. Immortalization has proven difficult to induce in human cells, perhaps because of their karyotypic stability (*3*).

It appears likely that heritable mutational changes in target cell DNA are involved in neoplastic cell transformation, though the precise role of such mutations is not yet clear. In some cases, they appear to be involved in the initiation of carcinogenesis and in others at subsequent stages. Balmain (*4*), for example, presented evidence that activation of the *ras* oncogene by specific base mutations is an important mechanism for the initiation of mouse skin tumors by various chemical carcinogens. Cerutti (*5*) emphasized the potential importance of chromosomal mutations in the process of carcinogenesis. Chromosomal mutations result from large-scale changes including translocations, rearrangements or deletions, rather than point mutations such as those associated with *ras* gene activation (*4*). X-radiation, for example, is an efficient inducer of stable chromosomal rearrangements (*6*), and fully transformed, immortalized human cells *in vitro* are characteristically aneuploid with multiple chromosomal abnormalities (*7*).

There has been considerable recent interest in the loss of heterozygosity by such chromosomal mutations as a mechanism for the expression of recessive mutations in

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cancer genes. This hypothesis has been given impetus by the finding that certain hereditary tumors are characterized by loss of heterozygosity at specific chromosomal sites; retinoblastoma, for example, is associated with a locus on chromosome 13 (*8,9*). A model we have developed to study this phenomenon *in vitro* (*10*) will be described; in particular, to analyze the molecular structure of chromosomal changes associated with the loss of heterozygosity at an autosomal locus in human cells.

Methodology

The detailed methodology and results of these experiments are described elsewhere (*10,11*). The characteristics of the human lymphoblastoid cell line designated TK6 are shown in Table 1 (*12*). Of particular note is the fact that these cells are heterozygotic at the thymidine kinase (tk) locus. They contain two copies of the tk gene in their native locations on chromosome 17, but one of these has been inactivated by a frameshift mutagen. We thus study the induction of changes in the chromosome containing the active gene that lead to expression of the inactive gene, yielding the tk−/− phenotype.

Molecular structural changes at the active tk allele were examined by restriction enzyme mapping on Southern blots hybridized to a full-length cDNA tk probe. The active allele was identified by means of a SacI restriction fragment length polymorphism (RFLP) located within the tk gene. By analyzing changes in the polymorphic SacI band, as well as the restriction patterns for four other enzymes, the structural changes in mutant clones could be separated into three categories (*10*). The first is characterized by no change in the restriction patterns, indicating that the mutant phenotype arose from a point mutation (or more specifically, a change involving less than 200 base pairs). Loss of the polymorphic band, associated with no other change in restriction patterns, indicates that loss of heterozygosity resulted from loss of the entire active tk allele. The appearance of new bands indicates that an intragenic rearrangement had occurred.

Molecular Structural Changes Leading to Loss of Heterozygosity

The distribution of the structural changes amongst over 200 mutant clones arising either spontaneously or following treatment with four different classes of mutagens are shown in Table 2. As can be seen, loss of heterozygosity in approximately two-thirds of spontaneously arising and x-ray induced mutant clones resulted from loss of the entire active gene. This fraction was lower for UV light or mitomycin-C induced mutants. None of the ethylmethane sulfonate (EMS) mutants were apparently associated with loss of the entire allele. The low fraction of mutant clones in this category (8%) were probably spontaneously arising; the induced frequency of mutations in the EMS treated cultures was only 6-fold above background (as compared with 10-fold for the other mutagens) such that 3 of the 24 mutants scored should be spontaneous ones. Most of the remaining mutant clones were associated with no change in the restriction patterns, indicating that a small scale change or point mutation had occurred.

In the course of these experiments, it became evident that a second class of slow growing mutants occurred at the tk locus (*11*). These mutant colonies arose after 20 days of incubation in selective medium, whereas normal growth mutants were scored after 12 days. When these slow growth mutants were isolated and serially subcultivated *in vitro*, slow growth rate remained a stable phenotypic characteristic over many cell doublings. The mean cell doubling time was approximately 34 hr (range 21–44) among slow growth mutants as compared with 16 hr (range 14–18) in normal growth mutants. When 100 different clones were analyzed, the range of doubling times was highly distinct for each of the two types of mutants with no overlap (Liber, Yandell, and Little, unpublished data).

The distribution of the molecular structural changes associated with over 200 slow growth mutant clones are shown in Table 3. In contradistinction to the results for normal growth clones (Table 2), loss of heterozygosity in slow growth mutant clones was associated almost entirely with loss of the entire active gene. This was true for EMS-induced mutants as well as those that arose spontaneously or were induced by X-rays or mitomycin-C. These results suggest that slow growth mutants arise as the result of a separate and distinct class of mutational events. The relative size of the slow growth mutant fraction

Table 1. Characteristics of TK6 human lymphoblastoid cell line.

Near diploid, stable karyotype with serial sub-cultivation
Immortal
Rapid growth in suspension
High cloning efficiency, measured in 96-well microtiter dishes
Single X chromosome (XY)
Thymidine kinase heterozygote ($TK^{+/-}$), one active and one nonactive but unrearranged tk gene both in native locations on chromosome 17

Table 2. Molecular structural changes leading to loss of heterozygosity at the tk locus in 218 mutant clones.

Inducing agent	Number analyzed	Structural changes at active tk locus		
		No change	Rearranged	Loss (%)
Spontaneous	51	10	5	36 (71)
X-irradiation	56	18	3	35 (63)
UV light	20	10	1	9 (43)
Mitomycin C	67	45	4	18 (27)
EMS	24	20	2	2 (8)

Table 3. Molecular structural changes leading to loss of heterozygosity at the tk locus in 203 slow growth mutant clones.

Inducing agent	Number analyzed	Structural changes at active tk locus		
		No change	Rearranged	Loss (%)
Spontaneous	120	4	1	115 (96)
X-irradiation	22	1	1	20 (91)
Mitomycin C	37	1	0	36 (97)
EMS	24	2	0	22 (92)

varied with each mutagen, ranging from approximately 30% of all mutations for EMS to 80% for X-rays. About one-half of spontaneously arising mutant clones were of the slow-growth type.

There are several different mechanisms by which loss of heterozygosity at the tk locus on chromosome 17 could occur. These include loss of the active allele through nondisjunction (with or without reduplication of the chromosome containing the inactive gene), mitotic recombination, or gene conversion. These are mechanisms that are presumably not available at X-linked or hemizygous loci. Loss of the active gene could also occur as a result of a deletion or a point mutation.

Extent of Large-Scale Changes Leading to Loss of Heterozygosity

To approach the question of which of these mechanisms may be operating in our *in vitro* system, we have estimated the extent of the structural changes associated with the loss of the active gene in mutant clones by examining polymorphic markers located elsewhere on chromosome 17. These include a XhoI polymorphism within the erb A1 gene located proximal to tk on the long arm of chromosome 17, and a PstI polymorphism within the sequence recognized by the D17S2 probe presumably located on the short arm of chromosome 17. Analysis of changes at these loci allow us to determine whether loss of the active tk allele in mutant clones extends to involve a proximal locus on the long arm of chromosome 17, or may in addition involve loss of a short arm marker suggesting that nondisjunction had occurred.

The results of experiments in which changes at the erb A1 locus were examined in tk mutant clones arising from loss of the active gene are shown in Table 4. About one-third of normal growth mutants and 50% of slow-growth mutants showing loss of the tk gene also showed loss of the erb A1 gene on the same chromosome. The fraction showing loss of erb A1 did not differ significantly among spontaneously arising mutants and those induced by three different mutagens. These results suggest that loss of heterozygosity at the tk locus is associated in many cases with a large scale change on the long arm of chromosome 17 involving another genetic locus some distance away.

On the other hand, in only 5 of more than 300 tk mutant clones analyzed was loss of the short arm marker revealed by the D17S2 probe observed. This finding suggests that nondisjunction is a relatively rare cause of loss of heterozygosity at the tk locus. In a preliminary analysis of cytogenetic changes associated with these tk mutants, karyotyping has been carried out in 18 spontaneously arising mutant clones. Both copies of chromosome 17 were present in all clones; in 16 of the 18, chromosome 17 was karyotypically normal. Presumptive evidence for very small deletions involving the long arm of chromosome 17 were found in the other two. These preliminary results suggest that multi-locus changes as identified by molecular structural analyses are not usually associated with cytogenetically detectible deletions or rearrangements.

Table 4. Structural changes at the erb AI locus in 190 tk mutant clones showing loss of active tk gene.[a]

Mutant phenotype	Inducing agent	Number analyzed	Structural changes erb AI	
			No change	Loss
Normal growth	Spontaneous	28	17	11
	X-irradiation	18	11	7
	UV light	6	4	2
	Mitomycin C	12	9	3
Slow growth	Spontaneous	95	45	50
	Mitomycin C	31	17	14

[a]Data from Yandell et al. (*11*).

Conclusions

These results indicate that large-scale chromosomal mutations resulting from events such as deletion or mitotic recombination may indeed be an important mechanism for the expression of recessive mutations in cells. Such recessive mutations could involve oncogenes or other growth regulatory genes important in neoplastic transformation. This phenomenon could, for example, represent a mechanism by which a mutant gene is expressed in the later stages of carcinogenesis; it is well known that the appearance of multiple chromosomal abnormalities is associated with the later stages of transformation and tumorigenesis. The occurrence of such chromosomal mutations would thus facilitate the expression of recessive cancer genes that have been mutated either at the time of the initial exposure to the carcinogen or have been carried as a germ-line mutation as in hereditary retinoblastoma.

Finally, the system described offers a useful model for studying this phenomenon *in vitro*. We are currently identifying other markers in and near the tk gene in order to better map the extent and type of molecular changes involved. Of particular interest is the question of whether mitotic recombination is an important mutational mechanism at an endogenous gene in its normal position in the human genome.

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