GENOMIC INSTABILITY AND CANCER: CAUSE AND EFFECT

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The remarkable insights gained in cancer research in the last decade demonstrate that cancer is a genetic disease; that is, alterations in the structure and/or function of genes are a fundamental feature of the malignant cell. The genomic alterations associated with cancer include gene amplification, point mutations, chromosomal translocations, deletions, insertions and loss, as well as epigenetic changes such as DNA methylation. These genomic alterations are linked to a variety of DNA metabolic processes including packaging, replication, recombination, repair, and modification. A recent Keystone Symposium entitled "Genomic Instability and Cancer,"* was held to foster interaction between investigators who share a common interest in these topics but who are using very different experimental systems.

Errors in DNA Metabolism

Errors in DNA metabolism represent potential sources of genomic instability (Table 1). For each cellular process affecting DNA, there is a base-line frequency of errors. Errors may give rise to inappropriate gene expression, manifested as under- or over-expression of the normal gene product or synthesis of a mutant product. The targets of these errors that may be important to cancer development include senescence genes, oncogenes, tumor suppressor genes, and metastasis genes. A knowledge of the *types* of changes observed may point one in the direction of the *process(es)* responsible for those changes.

**Organized by C. Harris, P. Hanawalt, and J. Rowley and held in Tamarron, Colorado, February 4–10, 1991.*

Sources of Spontaneous Mutations

An illustration of this concept was provided by L. Loeb (Univ. Washington, Seattle) in the context of DNA replication and damage. He pointed out that a substantial fraction of human cancers may arise from spontaneous errors in normal cellular processes and from the intrinsic chemical instability of DNA. The likely sources of spontaneous mutations can be deduced by comparing the spectrum of spontaneous mutations observed in cancer with that caused by individual potential sources of those mutations. From such a comparison, Loeb concluded that spontaneous mutations in cancer are most likely to arise from unrepaired DNA polymerase errors and oxygen damage. Because polymerases are known to misincorporate bases during DNA replication, errors persisting after exonucleolytic proofreading and postreplicative repair can contribute to the generation of point mutations in cancer genes. Oxygen damage can result from the reaction of DNA with stray free radicals generated during normal oxidative metabolism.

An example of a link between a potential mechanism of mutagenesis and changes in oncogenes and tumor suppressor genes is the finding that a large number of mutations resulting in *ras* activation in squamous cell carcinomas and in p53 inactivation in lung and liver cancers are G→T transversions (C. Harris, NIH). Work in the laboratories of J. Essigman (MIT), A. Grollman (Univ. New York, Stony Brook) and L. Loeb has shown that a very common guanine adduct, 8-hydroxyguanine, gives rise to precisely that mutation. 8-hydroxyguanine can be generated spontaneously, indirectly by chemical agents (e.g., in tobacco smoke), or physiologically via inflammatory processes. Thus, oxidative damage of this form may contribute to carcinogenesis (as may other sources of G→T transversions, such as aflatoxin B_1 and benzo[*a*]pyrene).

Similarly, the other processes listed in Table 1 have a basal error rate and can generate mutations or loss of heterozygosity. Molecular epidemiological studies of the genomic alterations associated with cancer may suggest which processes are involved.

Topoisomerases and Cancer

Work in diverse areas points to the importance of DNA topoisomerases in the organization and recombination of DNA, as well as in cancer. U. Laemmli (Univ. Geneva) reminded participants of the role of topoisomerase II (topo II) in maintaining chromosome structure. As judged by immunofluorescence, about 90% of this abundant protein is localized on the chromosomal axis. Remarkably, exogenous yeast topoisomerase can

TABLE 1
Errors in DNA Metabolism as Sources of Genomic Instability

Processes
genome organization
replication/replicative proofreading
DNA damage
DNA repair
chromosome transmission
recombination
epigenetic modification
Effects of errors
DNA amplification
point mutation
insertion
deletion
chromosomal translocation
chromosomal loss
inappropriate gene activity
fragile sites
Critical target genes in cancer
oncogenes
tumor suppressor genes
metastasis genes
senescence genes

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promote chromosomal condensation in chicken erythrocyte nuclei. In fact, AT-rich DNA sequences that comprise scaffold attachment regions (SARs), also designated matrix attachment sites (MARs), aggregate in the presence of topo II; this aggregation is abolished by topo II inhibitors. R. Rothstein (Columbia Univ.) reported on mutations in a new gene that stimulate recombination in a variety of repeat sequences in the yeast *Saccharomyces cerevisiae*. This gene, which he calls *top3*, is homologous with the genes encoding both bacterial type I topoisomerases, *topA* and *topB*, but not with either of the two known yeast topoisomerase genes, *top1* or *top2* (mutants of which are also recombinogenic for repeat sequences). Many anticancer drugs target topo II, as reviewed by L. Zwelling (M.D. Anderson Cancer Ctr., Houston).

Perhaps the most intriguing evidence of the importance of topo II in the etiology of malignant transformation is the observation that many cancer patients treated with topo II inhibitors such as the epipodophyllotoxins develop secondary acute leukemia; interestingly, many of these leukemic cells contain chromosomal translocations with breakpoints at 11q23 (J. Rowley, Univ. Chicago).

Gene Amplification

Amplification of proto-oncogenes and drug-resistance genes can be used as a prognostic indicator in a subset of human cancers (reviewed by J. Biedler, Memorial Sloan-Kettering Cancer Ctr.). T. Tlsty (Univ. North Carolina) asked whether the ability to amplify specific segments of the genome correlates with the malignant phenotype. Using Luria-Delbrück fluctuation analysis of specific drug-selected gene amplification events, she showed that amplification is a spontaneous, rather than an induced, event; she also showed that established tumor cell lines exhibit a more than 100-fold greater frequency of gene amplification as compared to primary diploid cell populations. Whether this phenomenon occurs in other transformed cells or human cancers, and to what extent this phenomenon reflects other forms of genomic instability, remain to be established.

DNA Repair

The ground-breaking work of P. Hanawalt and colleagues (Stanford Univ.) demonstrating preferential repair of the transcribed strand of active genes has now been extended to other systems by many laboratories. Persistent damage in non-transcribed strands may also contribute to genomic instability. Experiments by de Cock and colleagues (Univ. Leiden, The Netherlands), showed that, at least in one system, there is no correlation of repair with transcription. These workers studied the repair of three genes, *gart*, *white*, and *notch*, in an embryonic *Drosophila* cell line. They observed that only *gart*, which is associated with house-keeping functions, is actively transcribed, although repair of UV-induced pyrimidine dimers in all three genes occurred at the same rate. It will be interesting to see whether this exception to strand-specific repair is specific to the cell type or to the organism.

Patients with the human autosomal recessive skin cancer disorder, xeroderma pigmentosum (XP), are known to be defective in their ability to repair UV-damage (e.g., pyrimidine dimers) to DNA. A. Sarasin (Inst. de Rech. Scient. sur le Cancer, Villejuif, France) reported that skin cancers in XP patients not only appear to have a higher incidence of *ras* gene mutations (55% cf. 22% for controls), but also that the *ras* mutations (T → A at codon 61 and C → T or C → A at codons 12 and 13) occur at the 5′ base of pyrimidine dinucleotides; these results agree with published UV mutagenesis data.

Mismatch Correction

A.-L. Lu-Chang (Univ. Maryland), using a classical biochemical approach, detected three different mismatch-specific nicking activities in HeLa cells. The T/G mismatch-specific nicking activity has already been described by J. Jiricny. The other two activities, which co-purify in early steps of purification, have been separated into A/G-specific and "all-type" nicking activities. Such enzymes may be involved in both post-replicative mismatch repair and recombination. Mutations in these genes are expected to increase genomic instability, as is the case with the *mutHLS* mismatch repair mutants of *E. coli*.

Checkpoints for DNA Damage

When damage occurs to DNA, it behooves the cell to recognize the damage and stop DNA replication until repair is completed. Elegant studies of such "checkpoint" regulation in the cell cycle in the yeast *Saccharomyces cerevisiae* were reported by T. Weinert (Univ. Arizona). When the DNA of normal cells is damaged, cell cycle arrest occurs in G_2 prior to cytokinesis. However, when *rad9* (radiation-sensitive) mutants are irradiated, they continue to progress through the cell cycle, and die, unless artificially delayed by chemical or genetic means. Using cell cycle (*cdc*) mutants with known points of action in the cell cycle, Weinert has pinpointed the time of checkpoint activity in the cell cycle. In addition to finding checkpoint activity in *rad9*, *rad17*, and *rad24* genes, he has identified three new checkpoint genes by screening for lack of G_2 arrest upon irradiation; he has called these genes *mec1*, *mec2*, and *mec3*, for mitosis entry checkpoint mutants. The *mec1* and *mec2* mutants exhibit hydroxyurea sensitivity, which suggests defective S checkpoint activity. Checkpoint mutants also exhibit an increased frequency of chromosomal loss.

A mammalian protein possibly associated with checkpoint activity is, of all things, p53. Evidence for this possibility, presented in a poster by M. Kastan

(Johns Hopkins Med. Sch.), stemmed from experiments in which he simultaneously measured, by flow cytometry, p53 levels and bromodeoxyuridine incorporation. In response to X-irradiation, cells with wild-type p53 showed a drop in the proportion of cells in S phase, with accumulation of G_1 cells. In contrast, cells containing mutant or no p53 showed no change in the proportion of S phase cells, and a drop in the number of G_1 cells, suggesting defective G_1 checkpoint activity (in contrast to the yeast G_2 checkpoint mutants). It will be interesting to determine the mechanisms of checkpoint activity and their role in tumorigenesis.

Li-Fraumeni syndrome is transmitted as an autosomal dominant defect. Affected individuals are characterized by a high incidence of cancer (50% by age 30–35), multiple tumors in single individuals, p53 germline mutations, and frequent loss of heterozygosity of the p53 locus in tumors. M. Tainsky (Univ. Utah) showed that primary Li-Fraumeni fibroblast cultures display early chromosome loss and aneuploidy, with concomitant loss of cellular senescence. This is in contrast to normal primary fibroblast cultures, which stop growing after a limited time in culture and maintain an essentially normal karyotype in the process. The Li-Fraumeni cultures also exhibit enhanced susceptibility to *ras* transformation. Late-passage cells develop a staggering number of chromosomal aberrations including double minutes, translocations, deletions, and ring chromosomes. Although these characteristics are consistent with the predicted phenotype of a checkpoint mutation, they may also represent indirect phenotypes. The observation that germline mutations in p53 are generally different from those found in tumors (C. Harris, NIH) suggests the possibility that there are limitations on the p53 mutations tolerated in the germline; the dominant/recessive character of these mutations is currently under study in in vitro systems. Mechanisms responsible for the generation of p53-associated aneuploidy also remain to be identified.

Recombination and Chromosomal Translocation

The discovery of immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangement, which normally involves genetic recombination, with concomitant deletions and insertions, has led to the realization that aberrant recombination may be responsible for some of the gross chromosomal abnormalities associated with lymphoid malignancies. F. Alt (Columbia Univ.) has shown that transcriptional activity influences the rate of recombination in TCR and Ig gene segments. He now plans to test whether inappropriate expression of immune recombinases, encoded by the *rag1* and *rag2* genes, in transgenic animals can result in an increased frequency of chromosomal translocations or other alterations, and thus an increased frequency of cancer.

C. Croce (Temple Univ., Philadelphia) reminded participants that gene fusion is the most common consequence of translocations in B and T cell tumors, and often leads to oncogene activation. Such translocations are frequently associated with the aggressive lymphomas that occur in 25% of all AIDS patients and nearly half of AIDS patients treated with azacytidine for more than 3 years. The remarkable correlation of chromosomal translocations with immune recombination now appears to apply to a majority of lymphoid malignancies.

J. Sklar (Harvard Med. Sch.) asked what determines the participation of non-antigen receptor sites in lymphoid translocations. In some cases, the answer appears to be that consensus heptamer/nonamer sequences present in Ig and TCR genes can be found adjacent to other genes involved in translocations. To test whether such aberrant recombination occurs with higher frequency in ataxia telangiectasia (AT), a human multisystem disorder characterized by an increased incidence of chromosomal aberrations and cancer, he used the polymerase chain reaction (PCR) to look for predicted interchromosomal recombinant products of antigen receptor genes. Sklar found that the frequency of such recombinant products was 100 times greater in lymphocytes from AT patients versus normal individuals. This finding supports the idea that translocations in lymphoid malignancy are variants of normal recombination. However, the mechanisms involved in chromosomal aberrations associated with non-lymphoid malignancies remain to be determined.

R. Gatti (Univ. Calif., Los Angeles) discussed the results of a large collaborative effort to clone the gene responsible for AT. Using statistical analysis of the association of AT with restriction-fragment length polymorphism (RFLP) markers, investigators have localized the gene to a 2 megabase region in 11q22-23. J. Murnane (Univ. Calif., San Francisco) has narrowed it down to a single cosmid clone. Thus the elusive AT gene belonging to one of the AT complementation groups may soon be cloned.

Cytogenetic Aberrations as Tools

The correlation of common cytogenetic aberrations with particular human tumors has repeatedly aided our understanding of cancer, either by pointing us in the direction of tumor suppressor genes such as *Rb* in retinoblastoma, or oncogenes such as *myc* in Burkitt's lymphoma. M. Diaz (Univ. Chicago) described unbalanced translocations and deletions on chromosome 9p that are commonly associated with acute lymphoblastic leukemia, gliomas, melanoma, and lung carcinoma. Diaz and co-workers are mapping these chromosomal aberrations more precisely by using molecular probes derived from the nearby interferon -α and -β_1 gene cluster. Submicroscopic deletions of the region, some homozygous, have been

found, and mapping of the putative tumor suppressor gene is in progress.

Cellular Senescence

In contrast to the limited life span exhibited by normal diploid cells in vitro (cellular senescence), neoplastic cells are frequently immortal. In cross-species somatic cell hybridization experiments between neoplastic and normal cells, the senescence phenotype proved dominant, and mapped to four complementation groups (O. Pereira-Smith, Baylor Coll. Med., Houston). In a follow-up of earlier work localizing one of these senescence genes to human chromosome 1, J.C. Barrett's group (NIEHS) has now further defined its location to the region 1q23-1q31. In another fascinating link between cellular senescence and tumor suppressor gene biology, he found that senescent hamster cells are defective in RB protein phosphorylation in response to serum; only unphosphorylated RB protein was found in senescent cells.

Metastasis: Enhancers and Suppressors

The most common cause of cancer death is tumor metastasis, or spread of tumor cells from primary to distant sites in the body. Since metastasis is a complex multistep phenomenon, we might expect to see the emergence of many genes that affect metastasis in a positive or negative fashion.

P. Herrlich (German Cancer Res. Ctr., Heidelberg) and co-workers have generated a monoclonal antibody that binds to the surface of a spontaneously-arising metastatic variant of a rat pancreatic tumor cell line, but not to non-metastatic parental cells. The antibody specifically recognizes the core region of a large surface glycoprotein (four bands on a Western blot), whose carboxy- and amino-terminal domains (expressed on both metastatic and non-metastatic cells) are homologous with the CD44 lymphocyte homing receptor. The metastatic cell-specific epitope recognized by the monoclonal antibody appears to be a core region encoded by an extra exon whose sequence origin is currently unknown. Co-injection of the antibody with metastatic cells inhibits metastasis, whereas nonspecific antibody does not have this activity. Overexpression of the CD44 variant cDNA in non-metastatic *ras*-transformed (but not non-transformed) fibroblasts results in overwhelming lung metastasis. It will be interesting to determine to what extent expression of this gene segment correlates with metastasis in human tumors (the current antibody unfortunately does not cross-react with mouse or human proteins).

P. Steeg (NIH) appears to have found the first metastasis suppressor gene. The *nm23* (non-metastatic, clone 23) gene (now known to be two closely-related genes, *H1* and *H2*) was discovered as a cDNA that was expressed at high levels in mouse melanoma cells of low metastatic potential, and at low levels in highly metastatic derivatives. Transfection of this gene into metastatic cells lowered, but did not abolish, their metastatic potential. Furthermore, she has observed concomitant loss of heterozygosity and onset of metastasis in some, though not all, human tumors. These results are highly suggestive of metastasis suppressor function in melanoma cells. The deduced protein sequence of nm23 reveals homology to the *Drosophila* developmental mutation *awd* (A. Shearn, Johns Hopkins Univ.) and to nucleoside diphosphate (NDP) kinase genes from the bacterium *Myxococcus xanthus* (J. Munoz-Dorado, Robert Wood Johnson Med. Sch.) and slime mold (M. Veron, Pasteur Inst., Paris). NDP kinases play a role in maintenance of nucleoside triphosphate pools, synthesis of nucleic acids, microtubule polymerization, and signal transduction via G proteins. Which of these pathways is important to the antimetastatic action of *nm23* remains to be determined.

Epigenetic Phenomena and Cancer

Loss of heterozygosity of tumor suppressor genes has emerged as an important mechanism in cancer development. Although loss of allelic heterozygosity in tumors can sometimes be related to loss of chromosomal heterozygosity, epigenetic changes, such as those associated with differential gene expression in tissue differentiation, X-chromosome inactivation, and genomic imprinting, provide an equally plausible way in which functional heterozygosity can be lost despite the persistence of heterozygosity at the DNA level.

C. Laird (Univ. Washington) presented his paradigm for understanding a human model of genomic imprinting, the fragile X syndrome. Although the syndrome itself is not linked with cancer, it provides an important model for epigenetic suppression of gene activity. The fragile X defect, named for a cytologic gap or break that is induced when DNA synthesis is inhibited during cell division in vitro, is associated with a large proportion of cases of mental retardation; it occurs at a high frequency in the human population (approximately 1 in 1000 births) and exhibits a peculiar pattern of inheritance. In Laird's model, the mutation causes incomplete reactivation of a particular region of the X chromosome during female gametogenesis. Although the molecular basis of the block to reactivation is unclear, the model predicts that hypermethylation of CpG islands renders genes at the fragile X site inactive in affected individuals. Recently published data from international collaborative efforts have shown that methylation of CpG islands in the region of the fragile X lesion correlates with the mental retardation phenotype. Inability of individuals to express genes in this segment in enough cells during development results in mental retardation. Similar methylation patterns may well apply to cancers where there is phenotypic loss of heterzygosity despite the persistence of genetic heterozygosity of tumor suppressor genes.

A cytologic phenomenon similar to, but even more pronounced than that seen in the fragile X syndrome, can be induced in normal tissue culture cells by more stringent inhibition of DNA synthesis (such as by treatment with the DNA polymerase inhibitor, aphidicolin). As described by T. Glover (Univ. Michigan, Ann Arbor), the resultant fragile sites are associated with an increase in the number of sister-chromatid exchanges and deletions or translocations in somatic cell hybrid test systems. Although the direct relevance of this phenomenon to cancer is controversial, the types of changes associated with the induction of these fragile sites are similar to those important in human neoplasia. Glover is attempting to clone the most fragile of these sites in the human genome, 3p14.2 (*FRA3B*), with the help of several tools including: (1) *neo* gene integrants at the site; (2) clones containing deletions or interspecies chromosomal translocations at or near the site; and (3) a microdissection library of the 3p14 region.

Experiments leading to localization of *FRA3B* were presented in a poster by F. Rassool (Univ. Chicago). After transfection of a pSV*neo* plasmid into aphidicolin-treated somatic cell hybrids containing human chromosome 3, Rassool observed preferential integration at human chromosome 3p14, as well as another specific site on hamster chromosome 1. Detection of consistent features (such as consensus sequences) at these inducible fragile sites may help us to understand the basis of chromosomal breakage.

Pharmacologic Damage Control

While it is hard to imagine that the changes in nucleotide sequence involved in oncogene activation can be reversed, it may be possible to reverse the action of oncogenes in a manner similar to that imagined for tumor suppressor genes. Such was the assumption of S. Nishimura (Natl. Cancer Ctr. Res. Inst., Tokyo) in screening some 200 compounds from *Streptomyces* for activities that specifically inhibit the growth of *ras*-transformed NIH-3T3 cells. The first surprise is that the compound found to do just that is a derivative of the amino acid tyrosine, azatyrosine, which differs from tyrosine by a nitrogen substitution for a ring carbon. The second surprise is that *ras*-transformed cells able to grow after azatyrosine treatment revert to a normal morphology even in the absence of this agent and despite continued expression of *ras*; this suggests an epigenetic mechanism. The effect appears to be oncogene specific, since the reversion occurs with *ras*-, *erb*B2/*neu*-, and *raf*-transformed cells, but not with *src*-, *hst*-, or *ret*-transformed cells. Azatyrosine was found to suppress the ability of the carcinogen/mutagen methylnitrosourea (MNU) to cause tumors in rodents. How azatyrosine inhibits the growth of cancer cells and whether it will be suitable in the treatment of human cancer remain open questions.

Epilogue

A better understanding of how the processes involved in DNA metabolism relate to one another and to malignancy will enhance our understanding of carcinogenesis and may ultimately provide us with more reasonable and precise weapons with which to do battle against cancer. Because so many different cellular mechanisms can cause genomic alterations in a large number of cancer genes, the task will not be easy. However, meetings such as this one hold the promise of stimulating novel approaches to the study of genomic instability. A telling comment of several participants was "I had no idea that the research in other areas was so relevant to my own."