Environmental and Molecular Mutagenesis 45:206–213 (2005)

Perspective

Mutator Phenotype in Cancer: Timing and Perspectives

Jason H. Bielas and Lawrence A. Loeb*

Joseph Gottstein Memorial Cancer Research Laboratory, Department of Pathology, University of Washington, Seattle, Washington

Normal human cells replicate their DNA with exceptional accuracy. During every division cycle, each daughter cell receives a full and accurate complement of genetic information. It has been estimated that approximately one error occurs during DNA replication for each 10^9 to 10^{10} nucleotides polymerized. Stem cells, the cells that are progenitors of cancer, may replicate their genes even more accurately. In contrast, the malignant cells that constitute a tumor are markedly heterogeneous and exhibit multiple chromosomal abnormalities and alterations in the nucleotide sequence of DNA. To account for the disparity between the rarity of mutations in normal cells and the large numbers of mutations present in cancer, we initially hypothesized that during tumor progression, cancer cells must exhibit a mutator phenotype. In this perspective, we summarize the evidence supporting a mutator phenotype in human cancer, analyze recent measurements of mutations in human cancer, consider the timing for the expression of a mutator phenotype, and focus on the important consequences of large numbers of random mutations in human tumors. Environ. Mol. Mutagen. 45:206–213, 2005.

Key words: cancer treatment; clonal expansion; DNA repair; metastasis

HISTORICAL PERSPECTIVE

The current concept of a mutator phenotype in cancer is founded on observations in cell biology and genetics. More than 100 years ago, Bovieri [1902] surmised that the disrupted growth patterns characteristic of human cancer could result from chromosomal aneuploidy. Foulds [1954] observed that cancers progress in a stepwise fashion. Muller [1951] proposed that cancers arise when a single cell receives multiple mutations. These observations, coupled with the observations on mutator DNA polymerases [Kunkel, 1992] and the identification of mutations in DNA repair genes associated with human cancers [Cleaver and Kraemer, 1989], have reinforced the hypothesis that cancer cells exhibit a mutator phenotype. The underlying premise is that normal mutation rates are insufficient to account for the multiple mutations found in human cancers, and that cancers must exhibit a mutator phenotype early in their evolution [Loeb et al., 1974; Loeb, 1991]. We proposed that errors in DNA replication and deficits in DNA repair accounted for multiple mutations in cancer. Nowell [1976] proposed that tumor progression is driven by sequential rounds of clonal evolution, in which single cells expand by stepwise selections to populate a tumor. Successive waves of clonal expansion of mutant cells could drive tumor progression. Recent evidence suggests that both enhanced mutagenesis and clonal selection occur during tumor progression, and that both contribute to the frequency of mutations observed in tumors.

MUTATION FREQUENCY IN NORMAL HUMAN CELLS

Prior to considering the number of mutations in tumors, it is instructive to substantiate the exceptional accuracy of DNA replication in normal mammalian cells. A tabulation of studies using *Hprt*, *Aprt*, and the *cII*, *lacI*, and *lacZ* transgenes indicates that the overall mutation frequency in mammalian cells varies between 10^{-4} to 10^{-7} nucleotides/gene (Table I). It should be noted that most of the studies have been carried out in cultured cells or in transgenic cells and that there is considerable variation in the data. Furthermore, the frequency of mutation may be underrepresented when using targets that confer a selectable phenotype (e.g.,

Grant sponsor: the National Institutes of Health; Grant Number: CA78885.

*Correspondence to: Lawrence A. Loeb, Joseph Gottstein Memorial Cancer Research Laboratory, Department of Pathology, University of Washington, Seattle, WA 98195. E-mail: laloeb@u.washington.edu

Invited article: 25th anniversary of *Environmental and Molecular Mutagenesis*

DOI 10.1002/em.20111

Published online 25 January 2005 in Wiley InterScience (www.interscience.wiley.com).

TABLE I. Spontaneous Mutant Frequencies in Mammalian Cells

Organism	Cell type	Mutational target	Frequency (mutations/gene)	Study
Mouse	Somantic tissues	*lacZ* transgene	8×10^{-5}	[Swiger et al., 1999]
	Somantic tissues	*cII* transgene	6×10^{-5}	[Swiger et al., 1999]
	Embryo fibroblasts	*lacI* transgene	6×10^{-5}	[Bielas and Heddle, 2003]
	T-lymphocytes	*Aprt*	1×10^{-6}	[Wijnhoven et al., 1998]
Human	Cultured fibroblasts	*HPRT*	5×10^{-7}	[DeMars and Held, 1972]
	Cultured lymphoblasts	*HPRT*	1×10^{-6}	[Chu et al., 1988]
	Lymphocytes	*HPRT*	3×10^{-6}	[Albertini et al., 1990]
	Kidney epithelium	*HPRT*	2×10^{-4}	[Colgin et al., 2002]

Hprt). In fact, a recent study in *Caenorhabditis elegans* suggests a mutation rate 10-fold higher than those estimated using selectable mutation targets [Denver et al., 2004]. Even so, considering that each cell contains approximately 25,000 genes, we estimate that each cell accumulates one or two mutant genes during the life span of an individual [Loeb, 1991]. This apparently low mutation frequency must also be considered in the context of what is currently understood about the biology of cancer development. First, cancers arise in multipotential stem cells, and detailed studies using mouse embryonic fibroblasts indicate that mutation frequencies in stem cells are 100-fold lower than those observed in cultured fibroblasts derived from adult tissues [Cervantes et al., 2002]. If one can equate mutation frequencies in embryonic stem cells to mutation frequencies in stem cells, then mutation rates in cells that give rise to malignancies are much lower than mutation rates in normal somatic cells. In contrast, the mutation data in kidney epithelial cells indicate a much higher frequency of mutagenesisis (Table I) [Colgin et al., 2002] and most cancers are of epithelial origin. Also, it is likely that somatic mutation rates in human cells are lower than in rodent cells, and this may contribute to the resistance of human cells to transformation in vitro [Bohr et al., 1985; Mellon et al., 1987]. If this disparity between humans and rodents is manifested in cancer precursor cells, it suggests that carcinogen testing in rodents is not an adequate model for human susceptibility, even though it is currently the most efficacious available. Based on the assumption that the accuracy of DNA replication in stem cells is similar to that in human somatic cells, we estimated that each stem cell accumulates one to two mutant genes (assuming 100 cell divisions during a human life span) [Jackson and Loeb, 1998; Jackson et al., 1998]. However, based on a Poisson probability distribution, there would be a few stem cells that would contain as many as 12 mutations [Jackson and Loeb, 1998].

HOW MANY MUTATIONS ARE PRESENT IN HUMAN TUMORS?

In contrast to the rarity of mutations in normal cells, cancer cells contain multiple mutations. Even though chromosome rearrangements are only occasionally diagnostic of specific tumors, they are frequently observed in most human cancers. Studies on DNA copy number and loss of heterozygosity (LOH) have established that individual tumors can contain as many as 40 alterations [Kallioniemi et al., 1992]. These changes involve the repositioning of DNA segments that encompass millions of nucleotides. From these data, we surmised that one might be sampling the tip of an iceberg and that there may be many more changes involving small segments of DNA or single nucleotide substitutions.

The extensiveness of changes in the nucleotide sequence of cancer genomes was first heralded by findings in human hereditary colon cancer [Peinado et al., 1992]. In this disease, manifested by mutations in genes encoding enzymes that correct errors in DNA synthesis, the associated colon cancers contain tens and perhaps hundreds of thousands of changes in the length of repetitive nucleotide sequences between genes (microsatellites) [Stoler et al., 1999]. Mutations in microsatellites should frequently result in frameshift mutations and therefore inactivate adjacent downstream genes. Also, repetitive sequences are present within many genes that function in maintaining genetic stability and changes in the length of these repeats are likely to destabilize the genome further during subsequent divisions in tumor cells [Eshleman et al., 1995]. In addition to hereditary nonpolyposis colorectal cancer (HNPCC), microsatellite instability has been reported in a large variety of tumors, many of which do not encode mutations in mismatch repair genes. Changes in the length of repetitive nucleotide sequences are believed to result from DNA polymerase slippage and the lack of correction by the mismatch repair system. Our analysis of the literature suggests that microsatellites are hot spots for mutagenesis and thus could serve as a signature of genomes that conceal a large number of other types of mutations.

A considerable number of genes are mutated in human cancers and have the potential to disregulate cell homeostatic mechanisms causing further mutations and/or changes in gene expression. Based on a few reports showing mutations in these genes in primary human tumors, it has been proposed that more than 1% of genes contribute to human cancers [Futreal et al., 2004]. Many of these genes are involved in ensuring the stability of the

genome in normal cells and thus are potential targets for producing a mutator phenotype in cancer cells. The list of cancer-associated genes includes genes involved in the recombination activating complex of V(D)J recombination, DNA replication, repair, signal transduction, chromosomal segregation, and mitosis. It will soon be feasible to sequence entire human genomes from normal and tumor cells. At this time, however, extensive DNA sequencing projects are just beginning to be reported [Wang and Taylor, 1992; Futreal et al., 2004]. These studies have enumerated nucleotide sequence changes in exons and adjacent junctional sites using DNA obtained from tumors cell lines or from the early passage of primary tumor cells. Prior to considering these results, it is important to analyze some of the limitations inherent in this approach. First, cells passaged in culture are subjected to selection and thus one would not detect the initial heterogeneity of the population of cells within a tumor. Second, changes that were not clonally present in the population would not be discernible unless one sequenced individual DNA molecules [Loeb et al., 2003] or DNA isolated from single cells. Thus, any random mutations introduced after the last round of clonal proliferation would not be detected. Third, mutations in introns have not been reported even though these would provide a more pertinent measure of mutation accumulation since they would be less subject to selection and expansion. Nevertheless, these studies are of importance for they have provided our first view of mutated genes in tumors and they catalog the types of nucleotide sequence changes found in malignancies.

In a comprehensive analysis of sporadic colon cancer cell lines, Wang et al. [2002] sequenced 3.2 megabases from 12 sporadic colon cancer cell lines. They detected a total of 320 substitutions in coding sequences. They first eliminated 90 single nucleotide polymorphisms (SNPs) that were previously detected in the general population. It may have been inappropriate to eliminate all of these as SNPs could represent hot spots for mutagenesis. After subtracting an additional 227 substitutions present in DNA from normal cells from the same patients, there remained only three new tumor-specific coding mutations. The authors suggest that their data fail to support a mutator phenotype in colorectal cancers. They surveyed 504 genes in 12 cancer cell lines and identified only three mutations. If one assumes that the changes in nucleotide sequence were randomly spread throughout the genome, then each tumor cell would contain 3,000 mutations, a number much greater than that which would be predicted on the basis of mutation frequencies in normal cells.

An extension of this analysis to 100 potential instability genes in 24 colorectal cancers yielded mutations in the exons of five genes [Wang et al., 2004a]. Analysis of the five genes in a larger panel of 168 tumor cell lines yielded 19 somatic mutations. Eight of these occurred in the *MRE11* gene, a gene involved in double-strand break repair [Paull and Gellert, 1998]. These results indicate that mutations are more frequent when one restricts the search to genes involved in genetic instability. In other studies, the coding regions and intron-exon junctions of BFAF, a serine/threonine kinase involved in the *ras* kinase pathway, was sequenced in 530 cancer cell lines from a variety of tumors [Futreal et al., 2004]. Mutations were found in 66% of malignant melanoma cell lines and at a lower frequency in a variety of other human cancer cell lines. The fact that mutations in this gene were found at a low frequency in a large number of tumors indicates that in many tumors mutations and/or changes in the expression of other genes can substitute for deficiencies in this pathway. Using a similar protocol, 83 somatic mutations were identified in the tyrosine phosphatase gene superfamily in a variety of human cancers [Wang et al., 2004b]. Since these mutations were detected by PCR amplification of genomic DNA, they were present in the majority of cells within each tumor and thus are clonal. They either imparted a selective growth advantage or "hitchhiked" with other mutations that afforded enhanced proliferation. Random mutations arising late in tumorigenesis would not be detected since they would be present in only a small fraction of cells within a tumor.

IS A MUTATOR PHENOTYPE AN EARLY EVENT IN TUMOR PROGRESSION?

Intuitively, for the expression of a mutator phenotype to be a major factor in tumor progression, it must be an early event. Mutations that occur early and that impart a selective growth advantage are more likely to enable the mutated cells to repopulate the tumor than those that occur at a later time. Mutations that occur at a later time would be limited to a fraction of the cells within a tumor. Considering that it takes 20 years from the time an individual is exposed to a carcinogen to the time that a tumor is manifested, it will be difficult to identify very early steps in the evolution of a tumor. As a result, evidence to document the time of expression of a mutator phenotype is likely to be indirect.

Genetically determined cancer-prone conditions, however, directly reveal the consequence of a mutator phenotype early in carcinogenesis. Several rare inherited disorders that are associated with an increased predisposition to cancer development exhibit DNA repair deficiencies and increased chromosomal fragility [Fearon, 1997]. For example, patients with the recessively transmitted disease, xeroderma pigmentosum, are defective in excision repair and develop multiple skin tumors when exposed to ultraviolet light [Cleaver and Kraemer 1989; van Steeg et al., 2000]. Fanconi's anemia [Strathdee and Buchwald, 1992] and ataxia telangiectasia [Swift et al., 1991] are

inherited human diseases associated with a predisposition to cancer, chromosomal instability, and DNA repair defects. Diseases such as Bloom [Ellis et al., 1995] and Werner [Shen and Loeb, 2000] syndromes encode mutations in DNA helicases, which are likely to be involved in resolving DNA-blocking lesions and/or alternative DNA structures and are associated with increases in the incidence of specific human cancers. These rare human diseases establish that deficits in DNA repair can result in cancer. The question is whether similar mutations occur in the more common human cancers.

HNPCC results from mutations in genes encoding mismatch repair proteins. Heterozygotes have a single mutation in one of the mismatch repair genes in all tissues, yet only develop tumors in a limited number of organs, particularly in the large intestine. These tumors usually contain a deletion mutation in the other allele. Mutations in the adenomatous polyposis coli (*APC*) gene are dominant and occur with exceptionally high frequency in both HNPCC and sporadic colon cancers. An interesting and controversial question is whether mutations in *APC* precede mutations in mismatch repair genes, or whether mutations in mismatch repair genes cause mutations throughout the genome, including those in *APC*. The fact that mutations in mismatch repair genes have a characteristic signature, i.e., changes in the length of repetitive sequences, allows one to approach this question. If mismatch repair gene mutations result in *APC* mutations, then there should be evidence for this in the *APC* mutation spectrum, which in fact has been reported [Huang et al., 1996].

Benign tumors are encapsulated and fail to invade or metastasize. These tumors are often envisioned as early lesions that have not fully acquired a malignant phenotype. Many of these tumors exhibit chromosomal alterations, microsatellite instability, and mutations in many oncogenes and tumor suppressor genes [Tamura et al., 1995; Tarafa et al., 2003]. Presumably, benign tumors originate early during tumor progression from cells that harbor mutations, allowing them to divide when they should not, yet these cells do not possess or express the changes required for progression to a malignant phenotype.

For some tumors, there is evidence that a field of cytologically normal cells, which already contain multiple genetic changes, surround cancer cells. This idea of a field of premalignant cells from which tumors can arise is supported by studies on chronic inflammatory diseases, such as ulcerative colitis [Brentnall et al., 1996] and Barrett's esophagitis. In these diseases, cells are repetitively stimulated to undergo proliferation by acid reflux from the stomach [Barratt et al., 1999].

Lastly, it is important to state that direct measurements of spontaneous mutation rates in cells have been inconclusive. In some studies, there is no difference in the mutant frequency between normal and tumor cells in culture [Elmore et al., 1983]. In other studies, the difference is marked [Lin et al., 2004]. It is likely that enhanced mutagenesis could be disadvantageous during the later stages of tumor progression and thus cells that harbor too many mutations may be eliminated from the population. Studies on random mutagenesis of a human repair protein indicate that 34% of single amino acid substitutions will lead to proteins that are functionally inactive [Guo et al., 2004]. Thus, the accumulation of multiple mutations throughout the genome is likely to result in mutations that functionally inactivate proteins required for cellular metabolism. These detrimental mutations could account for the large numbers of apoptotic cells and abnormal mitotic figures present in high-grade tumors and thus may limit the size and growth of tumors. Even though the most highly malignant tumors may fail to exhibit a high mutation rate in the late stages of tumor development, the imprints of the mutator phenotype should still be imbedded in the DNA sequence of these tumors and should be evidenced by their elevated mutation frequency (Fig. 1).

PROPENSITY TO METASTASIZE

Most cancer-associated deaths result from metastasis. Until recently, the acquisition of metastatic potential was considered a late or even the final event in tumor progression. It has been argued that changes that enable tumors to metastasize need not confer a selective proliferative advantage on the primary tumor and could already be present in rare tumor cells early during tumor progression [Bernards and Weinberg, 2002]. Support for this concept is the frequent finding of multiple single metastatic breast carcinoma cells in bone marrow in conjunction with small-localized breast carcinomas [Klein et al., 1999]. In addition, the profile of RNA expression in metastatic tumors is similar to the primary tumors from which they were derived. Since expression profiles are indicative of cell clones that constitute the major fraction of cells in a population, the data indicate that the metastatic cells are similar to the majority of cells in the primary tumor and that they do not represent rare cells in the tumor that have acquired genetic or expression patterns that conferred metastatic potential. Klein et al. [1999] compared single isolated bone marrow cells to cells from primary tumors with respect to chromosomal exchanges, regions with loss of heterozygosity, and regions exhibiting differences in comparative genomic hybridization. They observed that most chromosomal aberrations in metastatic cells are similar to those in the primary tumor, implying a common origin. Additional changes were present in the metastatic cells that presumably occurred later during proliferation of the metastasis. In addition, there have been no mutant genes that confer metastatic potential when expressed in appropriate tumors models. Thus, it seems reasonable to propose that mutations are required in multiple genes to

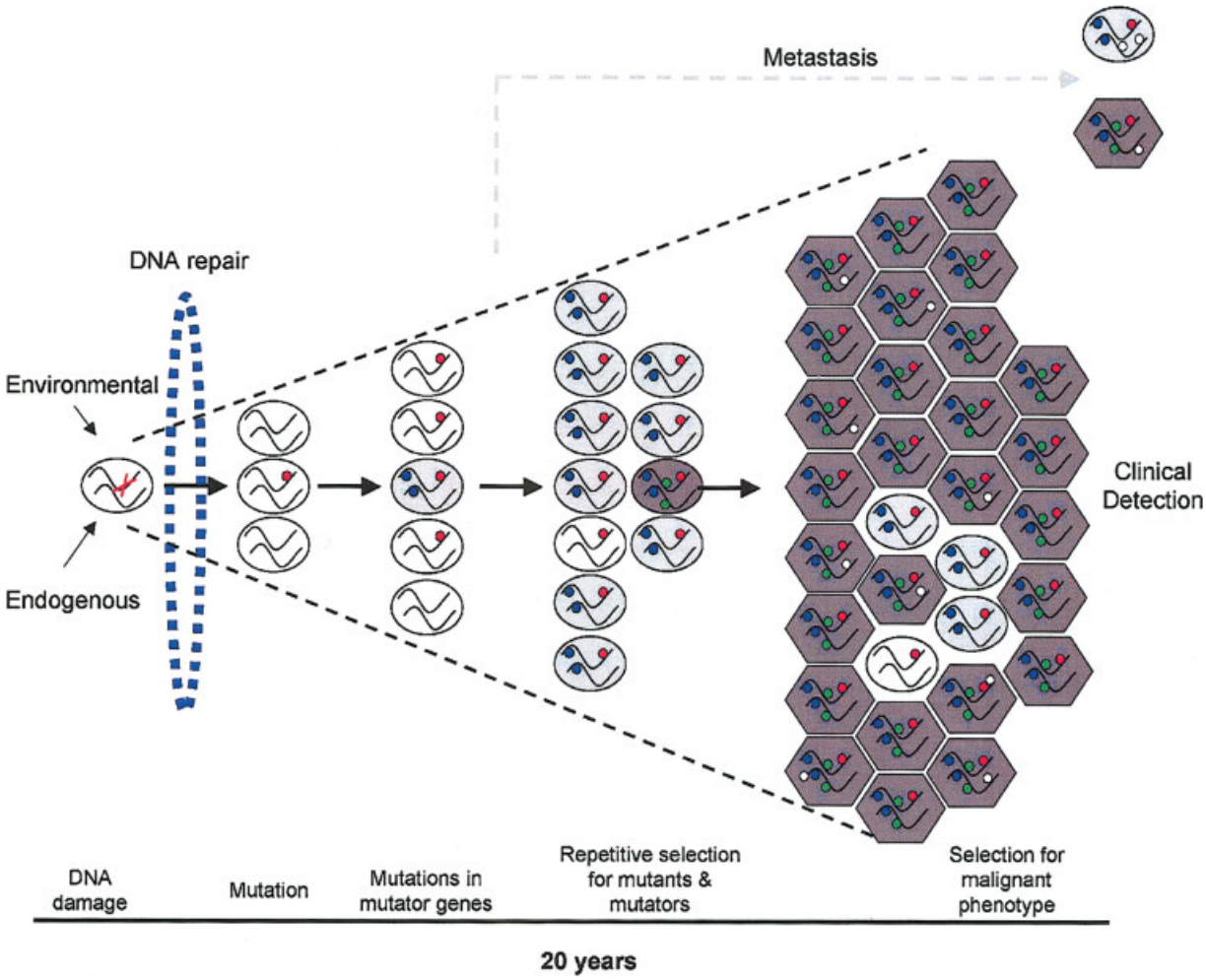

Fig. 1. Mutation accumulation during tumor progression. Random mutations result when environmental and endogenous DNA damage exceed the cell's DNA repair capacity. Mutations in mutator genes (red circles) can elevate the rate of mutagenesis and allow for clonal expansion. Repetitive rounds of selection for mutants yield coselection of mutations in mutator genes (blue and green circles); some cells may acquire a metastatic potential, as only a few mutations may be necessary for this [Bernards and Weinberg, 2002]. Continued rounds of selection for cells that escape the host's regulatory mechanism result in a tumor composed mostly of cells that are phenotypically malignant (hexagonal) where random point mutations accumulate (white circles). During this later stage of tumorigenesis, selection for cells with lower mutation rates is likely and may limit tumor growth. The imprints of the mutator phenotype, however, should still be imbedded in the DNA sequence of these tumors. Unfortunately. the measurement of random nonclonal point mutations has not been feasible with current technology.

confer on tumor cells the ability to metastasize and that this occurs early during tumor progression and results from an increase in random mutations throughout the genome (Fig. 1).

We present the following mechanism for the emergence of a mutator phenotype prior to the clinical appearance of a tumor. DNA damage in human cells is produced by both environmental and endogenous processes. It has been estimated that as many as 10^5 lesions are introduced in genomic DNA per cell per day [Ames et al., 1995]. Most of these lesions are removed by cellular repair mechanisms and only a small fraction escape and cause mutations at the time of DNA replication. Among these mutations would be those that occur in genes involved in maintaining genetic stability. As a result, there would ensue a cascade of mutations, many of which would involve other genetic stability genes. Prior to the clinical detection of a tumor, mutations could also occur in genes that result in an increased ability to metastasize (Fig. 1).

IMPLICATIONS FOR CANCER TREATMENT

Even if the presence of multiple mutations in cancers is only a manifestation of a mutator phenotype, it has important implications for the treatment of human cancers. The presence of thousands of random mutations within individual cells indicates that within any tumor

there are billions of mutations. Thus, among the 10^8 cells that populate a clinically detected tumor, there will be many cancer cells that harbor mutant genes, rendering them resistant to any chemotherapeutic agent directed against the tumor. In the presence of chemotherapy, these mutant cells would have a selective advantage and would enable them to proliferate and repopulate the tumor. The presence of preexisting mutant cells could account for the efficacy of combinational chemotherapy, since it would be unlikely that any single cell would have mutations in multiple genes, each rendering them resistant to a different chemotherapeutic agent.

Tumors may be stratified based on the number of random mutations per cell and on the number of damaged nucleotides that have the potential to cause mutations during subsequent rounds of DNA replication. The accumulation of single-base substitutions provides a measure of errors in DNA replication that escape mismatch repair. The accumulation of modified nucleotides could provide a measure of the deficiency of DNA repair. The accumulation of chromosomal alterations provides evidence of deficits in chromosomal segregation guided by genes that are only beginning to be characterized. Thus, the characterization of the spectrum of mutations might provide clues to the mechanism of formation and the response to therapy.

It is likely that there will be many types of mutations within each cancer cell, and, as a result, the stage of a tumor and the response to therapy will be correlated with the overall frequency of changes in the nucleotide sequence. Tumors with small numbers of mutations might harbor fewer drug-resistant cells. Those with a large number of mutations would be more likely to have metastasized and would contain a greater fraction of drug-resistant cells. Similar considerations apply to immunotherapy. New tumor clones that encode mutant proteins expressed on the cell surface have already demonstrated their ability to evade the host's immunological defenses. Thus, immunotherapy directed against tumors that are already expressing these antigens are unlikely to be effective. In contrast, immunotherapy has proven to be effective against some cancer cells that overexpress normal cell surface antigens [Bohen et al., 2003]. An important area for investigation would be immunotherapy against individual tumors that express a variety of mutant proteins [Nelson et al., 1996].

With increasing evidence that cancer cells contain large numbers of mutations, it becomes important to determine if these mutations drive the progression of tumors from cells that resemble surrounding tissue to those that are increasingly malignant. If mutagenesis is rate-limiting for the phenotypic changes that are progressively manifested during tumor growth, it becomes important to determine if one can inhibit mutation accumulation, perhaps even prior to the clinical appearance of the tumor. Since cancer is a chronic disease characterized by a 20-year latent period in the case of adult solid tumors, and a disease that occurs predominantly in older individuals, even a twofold delay in cancer progression could result in a significant reduction in cancer mortality. For example, chronic infection with either hepatitis B or C virus results in hepatocellular carcinoma 20–40 years after the initial infection. A twofold delay in progression would delay the clinical manifestation of the disease from a peak incidence of 56 years to the seventh, eighth, or ninth decade.

Epidemiological studies suggest that diet plays a role in the etiology of cancer. Caloric restriction (CR) reduces tumor incidence in laboratory animals and may confer protection from invasive breast cancer [Michels and Ekbom, 2004]. CR rats exhibit a decreased level of somatic mutation and tumor incidence thought to result from a reduced level of reactive oxygen species (ROS) [Aidoo et al., 2003]. Moreover, CR in mice resulted in a protective effect against MNU-induced mutation, although an altered balance between cell proliferation and apoptosis by CR is believed to be the mechanism for mutation reduction [Shima et al., 2000]. Nevertheless, the correlation between reduced mutation and cancer is a strong indicator that prevention by CR is a viable means that might reduce cancer mortality.

Additional protocols for prevention by delay would require additional knowledge of factors that are responsible for mutation accumulation. Some interventions would be immediately approachable while others could not be easily accomplished with our present state of knowledge. If enhanced mutagenesis is the result of increased damaged by ROS due to chronic infection, then antibiotics or drugs, which scavenge for ROS, can mediate the prevention of cancer by delay. If the underlying cause is alterations in nucleotide pools, then it is conceivable that these alternations can be recalibrated by dietary manipulation. However, new technologies may be required to prevent the enhanced misincorporation by altered DNA polymerases to prevent induction of error-prone DNA polymerases or to compensate for deficits in DNA repair.

SUMMARY

The slope of the exponential increase of cancer as a function of age suggests that there are 6–12 cancer-associated events [Armitage and Doll, 1954; Renan, 1993], each of which is rate-limiting for tumor progression. If one assumes that each of these mutations offers a growth advantage, then it is conceivable that normal mutation rates can account for the age increase in cancer. This, however, is unlikely, as these mutations must offer their clonal growth advantage by single allele mutation. Additionally, the mutation rate in normal cells cannot possibly account for the thousands of random mutations present in human cancers. The discrepancy between the nature and

rarity of mutations in normal cells and the large number and variety of mutations in cancer cells demonstrate that the manifestation of a mutator phenotype must be an early event in tumor progression.

ACKNOWLEDGMENTS

The authors thank Nicholas Griner and Mike Schmitt for carefully editing this manuscript. J.H.B. is supported by a postdoctoral fellowship from the Natural Sciences and Engineering Research Council of Canada.

REFERENCES

Aidoo A, Mittelstaedt RA, Bishop ME, Lyn-Cook LE, Chen YJ, Duffy P, Heflich RH. 2003. Effect of caloric restriction on Hprt lymphocyte mutation in aging rats. Mutat Res 527:57–66.

Albertini RJ, Nicklas JA, O'Neill JP, Robison SH. 1990. In vivo somatic mutations in humans: measurement and analysis. Annu Rev Genet 24:305–326.

Ames BN, Gold LS, Willet WC. 1995. The causes and prevention of cancer. Proc Natl Acad Sci USA 92:5258–5265.

Armitage P, Doll R. 1954. The age distribution of cancer and a multi-stage theory of carcinogenesis. Br J Cancer 8:1–12.

Barratt MT, Sanchez CA, Prevo LJ, Wong DJ, Galipeau PC, Paulson TG, Rabinovitch PS, Reid BJ. 1999. Evolution of neoplastic cell lineages in Barrett oesophagus. Nat Genet 22:106–109.

Bernards R, Weinberg RA. 2002. Metastasis genes: a progression puzzle. Nature 418:823.

Bielas JH, Heddle JA. 2003. Elevated mutagenesis and decreased DNA repair at a transgene are associated with proliferation but not apoptosis in p53-deficient cells. Proc Natl Acad Sci USA 100:12853–12858.

Bohen SP, Troyanskaya OG, Alter O, Warnke R, Botstein D, Brown PO, Levy R. 2003. Variation in gene expression patterns in follicular lymphoma and the response to rituximab. Proc Natl Acad Sci USA 100:1926–1930.

Bohr VA, Smith CA, Okumoto DS, Hanawalt PC. 1985. DNA repair in an active gene: removal of pyrimidine dimers from the DHFR gene of CHO cells is much more efficient than in the genome overall. Cell 40:359–369.

Boveri T. 1902. Über mehrpolige Mitosen als Mittel zur Analyse des Zellkerns. Verhandlungen der physicalisch-medizinischen Gesselschaft zu Würzburg. Neu Folge 35:67–90.

Brentnall TA, Haggitt RC, Rabinovitch PS, Kimmey MB, Bronner MP, Levine DS, Kowdley KV, Stevens AC, Crispin DA, Emond M, Rubin CE. 1996. Risk and natural history of colonic neoplastic progression of patients with primary sclerosing cholangitis and ulcerative colitis. Gastroenterology 110:331–338.

Cervantes RB, Stringer JR, Shao C, Tischfield JA, Stambrook PJ. 2002. Embryonic stem cells and somatic cells differ in mutation frequency and type. Proc Natl Acad Sci USA 99:3586–3590.

Chu EHY, Boehnke M, Hanash SM, Kuick RD, Lamb BJ, Neel JV, Niezgoda W, Pivirotto S, Sundling G. 1988. Estimation of mutation rates based on the analysis of polypeptide constituents of cultured human lymphoblastoid cells. Genetics 119:693–703.

Cleaver JE, Kraemer KH. 1989. Xeroderma pigmentosum. In: Scriver CR, Beudet AL, Sktm WS, Valle D, editors. Metabolic basis of inherited disease. New York: McGraw-Hill. p 2949–2971.

Colgin LM, Hackmann AF, Emond MJ, Monnat RJJ. 2002. The unexpected landscape of in vivo somatic mutation in a human epithelial cell lineage. Proc Natl Acad Sci USA 99:1437–1442.

DeMars R, Held KR. 1972. The spontaneous azaguanine-resistant mutants of diploid human fibroblasts. Humangenetik 16:87–110.

Denver DR, Morris K, Lynch M, Thomas WK. 2004. High mutation rate and predominance of insertions in the *Caenorhabditis elegans* nuclear genome. Nature 430:679–682.

Ellis NA, Lennon DJ, Proytcheva M, Alhadeff B, Henderson EE, German J. 1995. Somatic intragenic recombination within the mutated locus BLM can correct the high sister-chromatid exchange phenotype of Bloom syndrome cells. Am J Hum Genet 57:994–997.

Elmore E, Kakunaga T, Barrett JC. 1983. Comparison of spontaneous mtation rates of normal and chemically transformed human skin fibroblasts. Cancer Res 43:1650–1655.

Eshleman JR, Lang EZ, Bowerfind GK, Parsons R, Vogelstein B, Wilson JKV, Veigl ML, Sedwick WD, Markowitz SD. 1995. Increased mutation rate at the *hprt* locus accompanies microsatellite instability in colon cancer. Oncogene 10:33–37.

Fearon ER. 1997. Human cancer syndromes: clues to the origin and nature of cancer. Science 278:1043–1050.

Foulds L. 1954. The experimental study of tumor progression: a review. Cancer Res 14:327–339.

Futreal PA, Coin L, Marshall M, Down T, Hubbard T, Wooster R, Rahman N, Stratton MR. 2004. A census of human cancer genes. Nat Rev Cancer 4:117–183.

Guo HH, Choe J, Loeb LA. 2004. Protein tolerance to random amino acid change. Proc Natl Acad Sci USA 101:9205–9210.

Huang J, Papadopoulos N, McKinley AJ, Farrington SM, Curtis LJ, Wyllie AH, Zheng S, Willson JKV, Markowitz SS, Morin P, Kinzler KW, Vogelstein B, Dunlop MG. 1996. *APC* mutations in colorectal tumors with mismatch repair deficiency. Proc Natl Acad Sci USA 93:9049–9054.

Jackson AL, Loeb LA. 1998. The mutation rate and cancer. Genetics 148:1483–1490.

Jackson AL, Newcomb TG, Loeb LA. 1998. Origin of multiple mutations in human cancers. Drug Metab Revp 287–305.

Kallioniemi A, Kallioniemi O-P, Sudar D, Rutovitz D, Gray JW, Waldman F, Pinkel D. 1992. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science 258:818–821.

Klein CA, Schmidt-Kittler O, Schardt JA, Pantel K, Speicher MR, Riethmuller G. 1999. Comparative genomic hybridization, loss of heterozygosity, and DNA sequence analysis of single cells. Proc Natl Acad Sci USA 96:4494–4499.

Kunkel TA. 1992. DNA replication fidelity. J Biol Chem 267:18251–18254.

Lin YW, Perkins JJ, Zhang Z, Aplan PD. 2004. Distinct mechanisms lead to HPRT gene mutations in leukemic cells. Genes Chromosomes Cancer 39:311–323.

Loeb LA. 1991. Mutator phenotype may be required for multistage carcinogenesis. Cancer Res 51:3075–3079.

Loeb LA, Springgate CF, Battula N. 1974. Errors in DNA replication as a basis of malignant change. Cancer Res 34:2311–2321.

Loeb LA, Loeb KR, Anderson JP. 2003. Multiple mutations and cancer. Proc Natl Acad Sci USA 100:776–781.

Mellon I, Spivak G, Hanawalt PC. 1987. Selective removal of transcription-blocking DNA damage from the transcribed strand of the mammalian DHFR gene. Cell 51:241–249.

Michels KB, Ekbom A. 2004. Caloric restriction and incidence of breast cancer. JAMA 291:1226–1230.

Muller HJ. 1951. Radiation damage to the genetic material. Sci Prog 7:93–165.

Nelson EL, Li X, Hsu FJ, Kwak LW, Levy R, Clayberger C, Krensky AM. 1996. Tumor-specific cytotoxic T-lymphocyte response after

idiotpe vaccination for B-cell, non-Hodgkin's lymphoma. Blood 88:580–589.

Nowell PC. 1976. The clonal evolution of tumor cell populations. Science 194:23–28.

Paull TT, Gellert M. 1998. The 3′ to 5′ exonuclase activity of Mre 11 facilities repair of DNA double-strand breaks. Mol Cell 1:969–979.

Peinado MA, Malkhosyan S, Velazquez A, Perucho M. 1992. Isolation and characterization of allelic loss and gains in colorectal tumors by arbitrarily primed polymerase chain reaction. Proc Natl Acad Sci USA 89:10065–10069.

Renan MJ. 1993. How many mutations are required for tumorigenesis? implications from human cancer data. Mol Carc 7:139–146.

Shen J-C, Loeb LA. 2000. The Werner syndrome gene: the molecular basis of RecQ helicase-deficiency diseases. Trends Genet 16: 213–220.

Shima N, Swiger RR, Heddle JA. 2000. Dietary restriction during murine development provides protection against MNU-induced mutations. Mutat Res 470:189–200.

Stoler DL, Chen N, Basik M, Kahlenberg MS, Rodriguez-Bigas MA, Petrelli NJ, Anderson GR. 1999. The onset and extent of genomic instability in sporadic colorectal tumor progression. Proc Natl Acad Sci USA 96:15121–15126.

Strathdee CA, Buchwald M. 1992. Molecular and cellular biology of Fanconi anemia. Am J Pediatr Hematol Oncol 14:177–185.

Swift M, Morrell D, Massey RB, Chase CL. 1991. Incidence of cancer in 161 families affected by ataxia-telangiectasia. N Engl J Med 325:1831–1836.

Swiger RR, Cosentino L, Shima N, Bielas JH, Cruz-Munoz W, Heddle JA. 1999. The cII locus in the MutaMouse system. Environ Mol Mutagen 34:201–207.

Tamura G, Sakata K, Maesawa C, Suzuki Y, Terashima M, Satoh K, Sekiyama S, Suzuki A, Eda Y, Satodate R. 1995. Microsatellite alterations in adenoma and differentiated adenocarcinoma of the stomach. Cancer Res 55:1933–1936.

Tarafa G, Prat E, Risques R-A, Gonzalez S, Camps J, Grau M, Guino E, Moreno V, Esteller M, Herman JG, Germa J-R, Miro R, Peinado MA, Capella G. 2003. Common genetic evolutionary pathways in familial adenomatous polyposis tumors. Cancer Res 63:5731–5737.

van Steeg H, Mullenders LH, Vijg J. 2000. Mutagenesis and carcinogenesis in nucleotide excision repair-deficient XPA knock out mice. Mutat Res 450:167–180.

Wang CI, Taylor JS. 1992. In vitro evidence that UV-induced frameshift and substitution mutations at T tracts are the result of misalignment-mediated replication past a specific thymine dimer. Biochemistry 31:3671–3681.

Wang TL, Rago C, Silliman N, Ptak J, Markowitz S, Willson JKV, Parmigiani G, Kinzler KW, Vogelstein B, Velculescu VE. 2002. Prevalence of somatic alterations in the colorectal cancer cell genome. Proc Natl Acad Sci USA 99:3076–3080.

Wang Z, Cummins JM, Shen D, Cahill DP, Jallepalli PV, Wang T-L, Parsons DW, Traverso G, Awad M, Silliman N, Ptak J, Szabo S, Willson JKV, Markowitz SD, Goldberg ML, Karess R, Kinzler KW, Vogelstein B, Velculescu VE, Lengauer C. 2004a. Three classes of genes mutated in colorectal cancers with chromosomal instability. Cancer Res 64:2998–3001.

Wang Z, Shen D, Parsons DW, Bardelli A, Sager J, Szabo S, Ptak J, Stillman N, Peters BA, van der Heijden MS, Parmigiani G, Yan H, Wang T-L, Riggins G, Powell SM, Willson JKV, Markowitz S, Kinzler KW, Vogelstein B, Velculescu VE. 2004b. Mutational analysis of the tyrosine phosphatome in colorectal cancers. Science 304:1164–1166.

Wijnhoven SW, Van Sloun PP, Kool HJ, Weeda G, Slater R, Lohman PH, van Zeeland AA, Vrieling H. 1998. Carcinogen-induced loss of heterozygosity at the Aprt locus in somatic cells of the mouse. Proc Natl Acad Sci USA 95:13759–13764.