Genetic Instability Induced by the Tumor Microenvironment[1]

Toni Y. Reynolds, Sara Rockwell, and Peter M. Glazer[2]

Department of Therapeutic Radiology, Yale University School of Medicine, New Haven, Connecticut 06520-8040

ABSTRACT

The tumor microenvironment is characterized by regions of fluctuating hypoxia, low pH, and nutrient deprivation. To determine the genetic consequences of growth under these conditions, we used a tumorigenic cell line carrying a recoverable, chromosomally based λ phage shuttle vector designed to report mutations without the need for genetic selection of mutant cells. The cells were grown in parallel either in culture or as tumors in nude mice. The frequency of mutations arising in cells within the tumors was found to be 5-fold higher than that in otherwise identical cells grown in culture. A distinct pattern of mutation was also seen, with significantly more deletions and transversions in the tumors than in the cell cultures. Furthermore, exposure of the cultured cells to hypoxia produced an elevated mutation frequency and a mutation pattern similar to that seen in the tumors. These results indicate that the conditions within solid tumors are mutagenic and suggest that a fundamental mechanism of tumor progression *in vivo* is genetic instability induced by the tumor microenvironment.

INTRODUCTION

As cancers develop, they often become more malignant in their behavior, a process termed tumor progression. This phenomenon has been associated with genomic instability, as manifest in the evolution of tumor cell subpopulations with accumulating chromosomal aberrations (1). In fact, large numbers of mutations are found in malignant cells; these mutations cannot be accounted for by the low rate of mutation generally found among somatic cells (2). The basis for this genetic instability in cancer has not been fully established. There has been much recent work investigating the significance of specific gene defects that lead to genetic instability in mammalian cells and thereby accelerate malignant transformation. Examples include the DNA mismatch repair genes implicated in hereditary forms of colon carcinoma (3) and the *p53* gene associated with a variety of cancers (4, 5). In contrast, we have considered the possibility that the microenvironment of an incipient developing tumor might itself contribute to genomic instability and mutagenesis, leading to tumor progression and an evolution of the malignant phenotype.

One of the hallmarks of the tumor microenvironment is the severe, regional hypoxia occurring because of insufficiencies in the vasculature of the growing neoplasm (6, 7). Microenvironmental heterogeneity develops very early in the growth of solid tumors: significant numbers of hypoxic cells are already present when neoplasms are still microscopic in size, before the initiation of angiogenesis, and persist throughout the growth of the tumor (6). Hypoxia occurs in well-differentiated, slowly growing, nonmetastatic tumors as well as in rapidly growing, anaplastic, aggressive malignancies (6). Hypoxic areas of solid tumors typically have low pH and low levels of glucose and other nutrients, and it is known that hypoxic cells have numerous alterations in metabolism and enzyme activity (7–10).

Because the tumor microenvironment causes such profound metabolic changes in the cells, we hypothesized that it may lead to conditions that either cause increased damage to DNA or compromise DNA repair processes. Furthermore, because microenvironmental stress occurs early in tumor development, if it was a cause of genetic instability, it could well play a role in the evolution of the cell population to an increasingly malignant phenotype.

To test this hypothesis, we have used a tumorigenic cell line carrying a mutation reporter gene within a chromosomal shuttle vector. We report here that a higher frequency of mutations is produced in the cells when they are grown as tumors in nude mice than when they are grown in culture under standard conditions. Furthermore, exposure of cells in culture to transient hypoxia also resulted in an elevated mutation frequency. These results support the hypothesis that the tumor microenvironment is a cause of genetic instability, and they suggest that the hypoxia found within tumors may be responsible for this effect.

MATERIALS AND METHODS

Cells. The LN12 cells are a mouse L-cell-derived line carrying approximately 100 copies of the λsupF shuttle vector at a single chromosomal locus. Their design and construction have been described (11, 12). The cells were maintained in modified Eagle's medium supplemented with 10% fetal bovine serum in a humidified air incubator adjusted to contain 5% carbon dioxide as described previously (12).

Establishment of Experimental Tumors. Experimental tumors were established by s.c. injection of the LN12 cells into the flanks of the mice as described previously (13). The athymic nude mice (*nu/nu*) in the BALB/c background were obtained from Life Sciences, Inc. (St. Petersburg, FL). Measurements of intratumor oxygen tension were carried out as described elsewhere (14).

Hypoxia. Hypoxic culture conditions were established as described previously (15).

Shuttle Vector Rescue and Analysis. Preparation of high molecular weight DNA from the tumor samples and the cultured cells, preparation of λ *in vitro* packaging extracts, rescue of λ vectors from the mouse cell genomic DNA, and detection of *supF* gene mutations in the rescued phage were carried out as described elsewhere (11, 12, 16, 17).

Analysis of *supF* Mutations. PCR amplification and DNA sequence analysis of the *supF* locus in the mutant phage was performed as described previously (12, 17–19). Deletions were confirmed by four separate PCR reactions using two different sets of primers (17).

RESULTS

To investigate mutagenesis in the tumor microenvironment, we used a tumorigenic mouse cell line, LN12 (Fig. 1), carrying a chromosomally based λ phage shuttle vector (11, 12, 18, 19). The vector, λsupF, contains the *supF* tRNA suppressor gene as a mutation reporter gene (11). Using λ *in vitro* packaging extracts, the λ vector DNA can be identified, cut out, and packaged from within the LN12 cell DNA into viable phage particles for genetic analysis in bacteria (16). In this way, mutations that have occurred in the LN12 cells grown under different conditions can be detected using bacterial genetic techniques, obviating the need to transplant the cells into culture and grow them under selective conditions after the experimental manipulations to detect mutations. Hence, the shuttle vector pro-
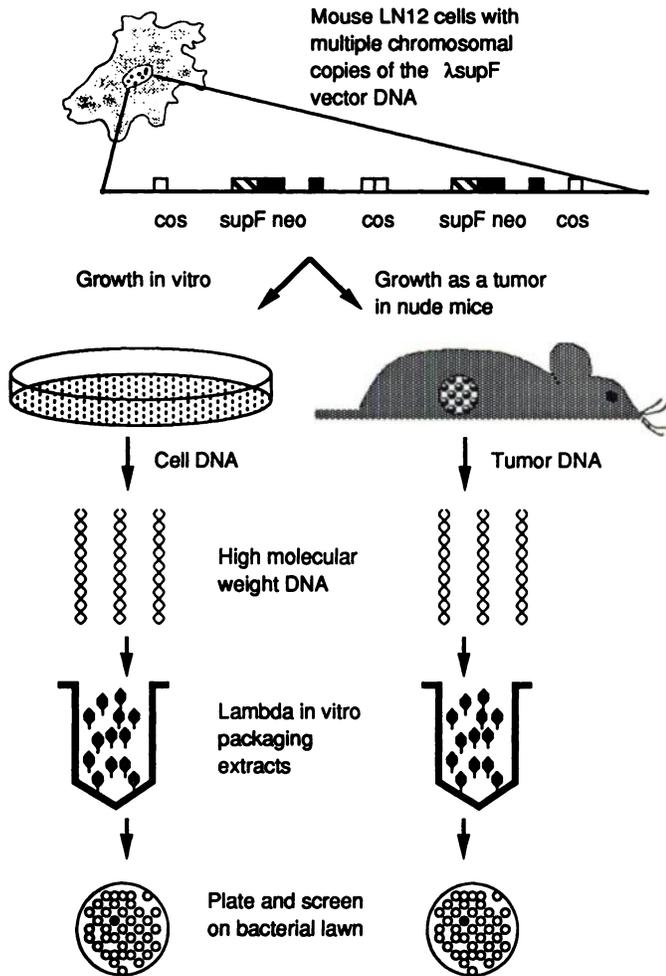
Fig. 1. Experimental protocol to examine mutagenesis in the tumor microenvironment. LN12 cells, carrying in their genome multiple copies of a recoverable λ phage shuttle vector (λsupF), were grown either as s.c. tumors in mice or in culture under standard conditions. At various times, the tumors and cell cultures were collected for preparation of genomic DNA, which was used as a substrate in λ *in vitro* packaging extracts to rescue the λ vector DNA from within the mouse cell DNA into viable phage particles. Mutations in the *supF* gene were detected by growth of the rescued phage on an indicator host.

tocol eliminates biases that might be introduced by an assay dependent on the ultimate *ex vivo* cloning of the cells.

The LN12 cells were injected s.c. into the flanks of six mice to generate tumors (Fig. 1). Each mouse received 2.5×10^6 cells in 0.25 ml of medium (13). At the same time, equal numbers of LN12 cells were placed in culture and grown under standard cell culture conditions. To avoid misleading results arising from the prior accumulation of mutations in culture, the cells were cloned before injection into the nude mice, and we used cells from the same clonal population to establish the parallel *in vitro* cultures.

Tumors were collected for analysis when they reached a volume of 1–1.5 cm³, based on caliper measurements of the tumor diameters. There was some variability in tumor growth, with individual tumors requiring between 6 and 21 weeks to reach this size. Prior to collecting the tumor samples, we measured the oxygen tensions at 100 positions within each tumor using an Eppendorf pO_2 histograph (14). All six tumors contained numerous areas of hypoxia (Fig. 2).

The cells in culture were kept in logarithmic phase growth and were subcultured twice weekly to maintain subconfluent conditions. The doubling time of the cell cultures showed little variation and was determined by serial cell counts to average 25.6 h. The cell cultures were maintained for different periods of time ranging from 6 weeks

(39 doubling times) to 21 weeks (138 doubling times), and the cells were collected for analysis at several times over this range.

This protocol for the cell cultures was chosen to control for potential variability in the number of cell divisions within the tumors. The tumors represent a heterogeneous cell population, with mixtures of growing, nondividing, and dying cells. The cell cycle time is not uniform, and it is difficult to determine with precision the number of generations in the pedigrees of the cells within the tumors. We therefore propagated the cell cultures over a range of cell generations to provide benchmarks for comparison to the tumors.

To detect mutations, genomic DNA was prepared from each of the tumors and from the cell cultures. The DNA samples were incubated in λ *in vitro* packaging extracts to rescue the λ vector DNA and to produce viable λ particles for genetic analysis of the *supF* gene (Table 1). We found a total mutation frequency of 9.3×10^{-5} for the cells in the tumors *versus* 1.8×10^{-5} for the cultured cells. Moreover, every tumor sample had a mutation frequency significantly above that of any of the cell cultures. The overall 5-fold elevation in mutation frequency was highly significant ($P < 0.0001$, Fisher's exact test).

The mutation frequencies found in the cultured cells did not vary significantly with the length of time in culture and therefore with the number of cell divisions. This does not mean that the mutation frequency is independent of the number of cell divisions; rather, it indicates that the effect of cell division on mutagenesis within the range studied is small compared to the background mutation frequency in the assay. This background of 1.8×10^{-5} is likely due to the *ex vivo* manipulation of the genomic DNA in the process of vector rescue.

For comparison, also listed in Table 1 are the frequencies of mutations in phage rescued from the skin of transgenic mice carrying the same λ shuttle vector (17) and from fibroblasts explanted into culture from a skin biopsy from a transgenic mouse. The low frequency of mutations observed in the transgenic mice shows that multiple cell divisions *in vivo per se* (as in the growth of a mouse from a fertilized egg) do not necessarily produce the kind of elevation in mutation frequency seen in the tumors.

To elucidate the characteristics of the tumor microenvironment that might be responsible for the induction of genetic instability, we tested the effect of hypoxic conditions on the LN12 cells in culture. Subconfluent cells were incubated for 4 h in a 95% nitrogen and 5% carbon dioxide atmosphere with a partial pressure of oxygen of less than 1 mm Hg (15), a level of hypoxia similar to that found in the
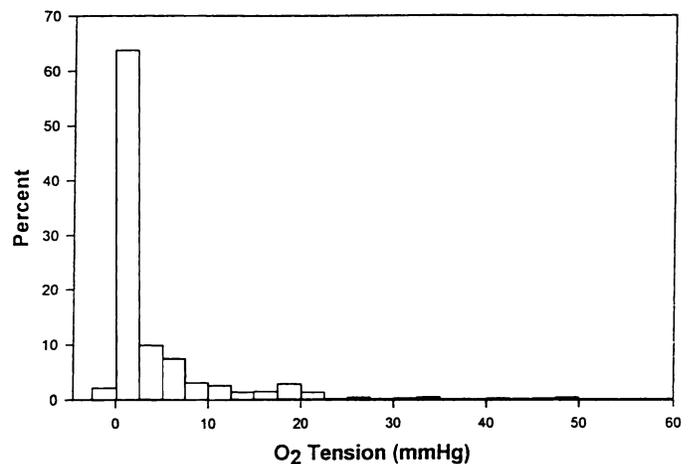

Fig. 2. Oxygen tension measurements in experimental tumors. pO_2 measurements were taken at 75–100 positions within each tumor using the Eppendorf pO_2 histograph system. The frequency distribution shown pools data from a total of 502 sampled positions in six different tumors. The graph indicates on a percentage basis the number of times each pO_2 value was obtained.

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experimental tumors (Fig. 2). The cells were returned to standard conditions for 5 days, after which genomic DNA was isolated for shuttle vector rescue and analysis. We also carried out a protocol of subacute exposure of the cells to hypoxia, with cells treated once a week for 2 and 3 weeks. Following a single exposure to hypoxia, we found a mutation frequency of 6.2×10^{-5} (Table 2), 3.4-fold above that for the untreated cells in culture (1.8×10^{-5}). The cells exposed multiple times to hypoxia showed even higher mutation frequencies, indicating a cumulative dose-response effect. Hence, transient exposure to levels of hypoxia that are found within tumors is mutagenic.

The *supF* mutations were examined by PCR amplification and DNA sequence analysis (Table 3). The mutations in the tumors consisted of 69% point mutations and 31% deletions. In contrast, all of the mutations in the cultured cells were point mutations; no deletions were seen. There was also a distinct spectrum among the point mutations in the tumors characterized by 62% transversions and only 38% transitions. In the cultured cells, only 39% of the point mutations were transversions and 61% were transitions. The transversions in the tumors consisted of mostly C:G to A:T changes, but T:A to G:C mutations were also overrepresented. The elevated frequency of deletions in the tumors is highly statistically significant ($P < 0.0001$), as is the increase in transversions ($P = 0.0069$). These findings indicate that there are both qualitative and quantitative abnormalities in the maintenance of genomic integrity by cells growing in the microenvironments within solid tumors.

In addition, analysis of the hypoxia-induced mutations (Table 3) revealed more frequent transversions and deletions than those seen in the cells grown under standard conditions, constituting an overall mutation pattern similar to that found in the experimental tumors. This similarity suggests that there is a common mechanism causing mutagenesis in the hypoxic cells and in the tumors, suggesting that hypoxia is a critical factor producing genetic instability in the tumor microenvironment.

DISCUSSION

We have used a tumorigenic cell line carrying the λsupF shuttle vector to compare the frequency and types of mutations arising when

Table 1 *Mutagenesis induced by the tumor microenvironment*

Growth condition	Growth period (wk)	Mutation frequency ($\times 10^{-5}$)[a]	Mutants/total[a]
LN12 cells grown in culture			
LN12A	6	1.7	6/359,224
LN12B	6	2.3	7/302,538
LN12C	9	1.5	5/340,806
LN12D	12	2.4	4/167,025
LN12E	15	1.6	9/572,316
LN12F	18	1.6	4/253,714
LN12G	21	2.3	6/258,947
Total		1.8	41/2,254,570
LN12 cells grown as tumors in nude mice			
Tum1	16	6.8	40/587,672
Tum2	19	14.8	28/189,566
Tum3	21	11.8	22/186,560
Tum4	6	10.8	38/353,088
Tum5	8	7.7	40/519,026
Tum6	6	11.3	10/88,108
Total		9.3	178/1,924,020
Transgenic mouse skin (λsupF mice)		1.3	10/761,144
Transgenic mouse fibroblasts in culture		1.7	3/176,471

[a] The values in the third and fourth columns give the frequencies of *supF* gene mutations detected in the λsupF vectors rescued from the LN12 cells either grown in culture under normal conditions or grown as tumors in nude mice, as indicated. The frequencies of mutations obtained from the skin of transgenic mice carrying the λsupF vector in their genome (17) and from primary fibroblasts explanted from the mice are also presented for comparison. Construction of transgenic mice carrying the λsupF shuttle vector has been described (17). The data presented were obtained from transgenic mouse 1054.

Table 2 *Mutagenesis induced by hypoxia*

Growth conditions in culture[a]	Mutation frequency ($\times 10^{-5}$)[b]	Mutants/total[b]
Standard atmosphere	1.8	41/2,254,570
Hypoxia × 1	6.2	24/386,259
Hypoxia × 2	13.6	9/66,200
Hypoxia × 3	10.1	7/69,315

[a] LN12 cells were grown in culture and received multiple transient (4-h) exposures to hypoxia, as indicated. The values for the cells grown under a standard atmosphere only are extracted from Table 1.

[b] The values represent the frequencies of *supF* gene mutations detected in the λsupF vectors rescued from the LN12 cells grown in culture under the conditions indicated.

Table 3 *Spectrum of mutations induced within the tumor microenvironment*

Mutation[a]	Growth of mouse LN12 cells		
	Tumors	Cells in culture: standard conditions	Cells in culture: hypoxia
C:G → T:A	16	19	4
T:A → C:G	1	3	1
C:G → A:T	14	8	4
C:G → G:C	4	2	4
T:A → A:T	5	2	0
T:A → G:C	6	3	3
+1 insertion	1	0	0
−1 deletion	0	1	1
Deletions >200 bp	21	0	3
Total	68	38	20

the cells are grown as tumors in nude mice *versus* when they are grown in culture. In comparing multiple tumor samples and multiple cell culture replicates, we consistently observed an increase in mutation frequency in the tumors. Furthermore, the tumor-derived mutations were characterized by high proportions of deletions and transversions. Since identical, clonally derived, isogenic cells were aliquoted to establish the tumors and the parallel cell cultures, the only variable between the two experimental groups was the condition of cell growth (in tumors *versus* in culture). Consequently, the differences between the tumors and the cell cultures in mutation frequencies and in mutation spectra can be attributed to the growth conditions.

A potential complicating factor is clonal variation. It is possible that during the process of tumor development, mutations accumulate not only in the *supF* reporter gene but also occasionally in cellular genes whose function may be important in DNA repair, cell cycle regulation, or apoptosis. In such cases, the frequency of mutations detected in the *supF* gene within that tumor sample may be influenced not only by the direct effect of the tumor environment but also by indirect pathways reflecting these other genetic changes. However, any mutations in these cellular genes are likely to vary from tumor to tumor. Moreover, within each tumor there is also likely to be heterogeneity, since multiple mutations could occur during tumor growth. (Of course, genetic defects could accumulate in the LN12 cells grown in culture, as well.) This potential variability is accounted for in our experimental design, in which we examined multiple replicates for each experimental condition. It should be recalled that the LN12 cell line is already a transformed tumorigenic line; the LN12 cells therefore do not require additional genetic changes to enable them to form tumors in the nude mice. Hence, there is no selective pressure for disruption of additional genes during the experiment.

Although the mutation frequencies in tumors and in culture are clearly different, calculation of true mutation rates (mutations per cell division) is complicated by differences between the proliferation patterns of malignant cells growing in cell culture and in solid tumors *in vivo*. (Data on mutation spectra are not affected by this problem, since the spectra would not be dependent on division number but instead would reflect mechanisms of mutagenesis.) We can count the number of doublings in culture, but it is impossible to determine

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precisely the number of doublings undergone by the cells in the tumors. However, it has been shown that, in experimental tumor models, the tumor cell populations consistently have longer mean cell cycle times than do parallel samples grown *in vitro* under optimal culture conditions (13). Consequently, the number of cell divisions over a given time period must be lower in the tumors than in the cultures. This means that the difference in the true mutation rates would be even greater than the observed difference in mutation frequencies.

The mutations in the experimental tumors are characterized by higher proportions of deletions and transversions than those seen in the cultured cells. However, these results should not be taken to imply that these two types of mutations are the most common ones to be found in cancers. In fact, a wide range of mutations have been associated with malignancy, such as the large variety of mutations in the *p53* gene (4). Rather, our findings suggest that the tumor micro-environment tends to produce these specific classes of mutations, representing one component of cancer-related mutagenesis.

The occurrence of deletions suggests that either excessive strand breaks are formed or repair of strand breaks is diminished. Such abnormal DNA metabolism could be due to impaired activity of enzymes such as topoisomerases, helicases, and ligases under the hypoxic acidic conditions within tumors. Furthermore, frequent or persistent strand breaks would predispose cells to the kinds of accu-mulated chromosomal rearrangements which typify tumor cells. This mechanism could also explain the loss of heterozygosity that is commonly seen at many critical loci in naturally occurring cancers.

The frequent C:G to A:T and T:A to G:C transversions may arise from errors in DNA repair and/or replication, perhaps due to reduced polymerase fidelity. Excessive metabolic damage to bases may also play a role. For example, the fluctuating perfusion in tumors may cause not only acidity and hypoxia during stasis and but also oxidative damage during reperfusion. Oxidative injury could generate excess levels of 8-oxoguanine, which has been shown to miscode for A and to lead to C:G to A:T transversions (20).

To begin to define the features of the tumor microenvironment that contribute to the observed mutagenesis, we tested the effect of transient, severe hypoxia on the cells. We detected a 3–4-fold elevation in mutation frequency induced by a 4-h incubation under severely hypoxic condi-tions. Higher levels of mutagenesis were seen in cells exposed mutiple times to hypoxia. The pattern of mutations induced by hypoxia was similar to that seen in the tumors, with transversions and deletions overrepresented. Although such *in vitro* hypoxic culture conditions do not exactly mimic the complex microenvironments in a tumor which vary both spatially and temporally, these results suggests that hypoxia is an important cause of genetic instability in developing tumors.

It has recently been shown that *p53* is induced in response to hypoxia (21). Because *p53* induction is associated with G_1 growth arrest and with apoptosis, cells with preexisting *p53* mutations are at a selective advantage under the hypoxic conditions within tumors (22). The *p53* mutant cells that preferentially survive hypoxia in tumors may therefore be particularly prone to genomic instability, both because of the role of *p53* in DNA repair (5, 23–26) and cell cycle regulation (27) and because of the direct mutagenic effect of the tumor microenvironment in which they are selected.

Hence, multiple factors may contribute to genetic instability in tumors, leading to tumor progression. On one hand, mutations in critical genes, such as *p53*, may predispose to genetic instability via alterations in the cell cycle and in DNA repair. On the other hand, the work presented here shows that the tumor microenvironment itself is mutagenic and may therefore be an important cause of tumor progression.

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