Serum Deprivation, but not Inhibition of Growth per se, Induces a Hypermutable State in Chinese Hamster G12 Cells

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ABSTRACT

Spontaneous mutagenesis is thought to play a crucial role in spontaneous carcinogenesis. We recently described a new mathematical model for estimation of the spontaneous mutation rate (mutation/gene/generations) based on the assumption that mutations are fixed in the S-phase of the cell cycle. With this definition, the spontaneous mutation rate should be independent of the growth rate. In the present study, we tested this hypothesis, using cell line G12, a transgenic Chinese hamster V79 derivative, which contains a single copy of the Escherichia coli gpt gene as a target for mutagenesis. The growth rate was modulated by varying the serum concentration or the seeding density, or by addition of suramin, transforming growth factor β, or dichlorobenzimidazole riboside to the medium. Significant increases in the spontaneous mutation rate occurred when cell proliferation was blocked by serum deprivation. Density-dependent inhibition of growth and inhibition of growth by suramin, transforming growth factor β, or dichlorobenzimidazole riboside did not result in significant increases in spontaneous mutation rates. The level of oxidants in cells cultivated in the presence of low concentrations of serum was higher compared to control cells, suggesting that the increases in the spontaneous mutation rates under low serum conditions may be partly a result of oxidative stress due to a lack of serum antioxidants. This was shown to be the case, because spontaneous mutation rates were significantly reduced in serum-depleted cells when antioxidants were added to the medium. We suggest that during carcinogenesis, when tumors are in a quiescent state, the spontaneous mutation rate may be elevated, and this process may contribute to the genetic instability of the tumor cells.

INTRODUCTION

Mutations that can activate proto-oncogenes or inactivate tumor suppressor genes may arise either as a consequence of endogenous processes (spontaneous mutagenesis) or as a result of exposure to environmental agents (induced mutagenesis). The effects of environmental mutagens are always superimposed upon the preexisting spontaneous background. The importance of spontaneous mutagenesis in carcinogenesis can be illustrated by the increased spontaneous mutagenesis in HNPCC patients (1). Yet little is known about the cellular processes which control spontaneous mutagenesis. It has been argued that an important precursor of spontaneous mutagenesis is oxidative damage to DNA by free radicals formed, for example, in Fenton reactions or during lipid peroxidation (2). Spontaneous mutagenesis is also thought to result from such processes as depurination, deamination, and tautomerization of bases, errors in DNA replication and repair, and aberrant methylation of DNA (3–6).

Research on factors controlling spontaneous mutagenesis has been hindered by the lack of exact methods to evaluate the spontaneous mutation rate. Recently, we described a new experimental method and mathematical model for estimation of the spontaneous mutation rate (7). The method is simple and suggests user-friendly formulas for estimating the mutation rate and the SD of this estimate. Our model is based on the assumption that mutagenesis occurs only in dividing cells. This model assumes that the mutation rate should be independent of the growth rate. It was of interest to check this prediction by modulating the growth rate using various approaches.

Normal mammalian cells possess a mechanism which allows them to be either proliferative or quiescent. This is the so-called restriction point (R) in G1 (8) where cells pause when external conditions (e.g., nutritional deprivation) restrict their progress through the cycle. They can reenter the cycle when supplied with complete medium and serum. There are a number of techniques to block proliferation of cells in culture. Fibroblasts and epithelial cells stop dividing when they form a confluent monolayer in a phenomenon known as density-dependent inhibition of cell division (9, 10). When most cells are deprived of serum, they stop growing and accumulate in Gt or G0 (11). TGF-β inhibits the proliferation of many cultured cells (12, 13), inducing growth arrest at late G1, close to R (14). Suramin is a nontargeted growth factor antagonist used in chemotherapy (15) and has been shown to induce an antiproliferative effect in cultured cells (16–19). DRB is a reversible inhibitor of RNA synthesis (20), and should block cell cycling.

The cell line G12, a transgenic line derived from Chinese hamster V79, contains a single copy of the Escherichia coli gpt gene which is used as a genetic target (21). The gpt gene in these cells is integrated subtelomeric in the largest chromosome 1a (22). In this study, we demonstrate that when the growth of G12 cells was suppressed by suramin, TGF-β, or density-dependent inhibition, there was no effect on the spontaneous mutation rate. However, when growth was modulated by serum deprivation, significant increases in the spontaneous mutation rate were observed. Levels of oxidative stress were found to be significantly higher in cells upon removal of serum compared to control cells. This effect can be blocked by the addition of antioxidants. The role of serum components in protecting against spontaneous mutagenesis is discussed.

MATERIALS AND METHODS

Chemicals. TGF-β was purchased from Sigma Chemical Co. (St. Louis, Mo). Suramin was purchased from CB Chemicals, Inc. (Woodbury, CT). Catalase from bovine liver (3400 units/mg protein), mannitol, and ascorbic acid were purchased from Sigma Chemical Co. DRB was purchased from Calbiochem-Novabiochem (San Diego, CA). Sterile stock solutions of chemicals were stored at −20°C.

Cell Culture. G12 cells were routinely grown in Ham’s medium F12 supplemented with 5% fetal bovine serum (GIBCO, Grand Island, NY), from lots of serum previously tested to give high cloning efficiencies, and 100 units/ml penicillin-streptomycin (GIBCO) at 37°C in an atmosphere of 5% CO2 and 95% air. Freshly cloned stocks of cells were stored frozen in liquid nitrogen in medium containing 10% DMSO.

Spontaneous Mutagenesis Assay. The mathematical modeling and method to determine the spontaneous mutation rate is described in Rossman...
et al. (7). In brief, cells are cleansed of preexisting mutants by growth in HAT medium (medium containing 0.2 mM hypoxanthine, 1.0 mM aminopterin, and 0.1 mM thymidine) for 2 weeks. Cells are seeded for growth expansion as indicated and at a lower density for cloning efficiency, which is used to correct the initial cell number. (The cloning efficiency of G12 cells is usually around 80%.) Mutants are then allowed to accumulate while the culture grows for 6 days, after which the cells are trypsinized and 10 dishes are reseeded at a density of 2 × 10^5 cells/100-mm dish into selection medium containing 6-thioguanine (10 µg/ml). The reseeding plating efficiency in nonselective medium is also determined for use in mutant fraction calculations. The selection medium is replenished after 1 week, and mutant colonies are stained and scored after 10–13 days. The mutant fraction is calculated by dividing the number M of mutant colonies by the number of cells seeded, corrected for the plating efficiency. The initial number of cells in the mutagenesis assay (N_0) is derived by dividing the number of cells plated for selection (M)/by the increase (fold) that the whole population experienced.

All measurements are computed after an approximated adjustment for plating efficiency. The number of generations (g) is approximated as the base 2 logarithm of the ratio of the final population size to the size of the initial population. The mutation rate (p) is estimated according to the formula:

\[
p = \frac{2F}{g}
\]

where F denotes the mutant fraction M/N_0^2g after generations of growth. The SE (σ) is calculated using the formula:

\[
σ = \frac{1}{2} \sqrt{\frac{2p}{N_0}}
\]

Measurement of Oxidative Stress. Intracellular peroxides and other reactive oxygen species were measured using DCF fluorescence, as described in the report by Huang et al. (23). In brief, the cells were seeded as described, and at different times after seeding were incubated with 500 µM dichlorofluorescein diacetate (Kodak, Rochester, NY) for an additional 30 min at 37°C. The cells were washed twice with ice-cold PBS, scraped from the plate, and resuspended at 10^6 cells/ml for fluorescence measurement. Fluorescence was analyzed using a Fluorescent Spectrophotometer (Perkin Elmer/Cetus, Norwalk, CT).

RESULTS

Previously, we found that there was no significant difference in the spontaneous mutation rates of G12 cells grown in 5% or 2.5% serum, although the latter condition slowed the rate of growth by about 20% (7). It was of interest to determine whether the independence of the spontaneous mutation rate on the growth rate applies to situations in which the growth rate is dramatically reduced. G12 cells were plated in normal medium (containing 5% serum) until attached (4 h), rinsed with PBS, and the medium was replaced with medium containing various concentrations of serum. After 5 days of incubation, significant differences in the growth rates were observed to be inversely related to serum concentration, as expected (Fig. 1). Five days of growth in 5% or 10% serum resulted in 5.16 and 5.68 generations of growth, respectively, while cells grown in 0.025% or 0.05% serum went through less than two doublings. The mutant fractions of all cell populations were assayed at the end of the growth period, and the mutation rate was calculated (see "Materials and Methods"). Incubation of cells in serum concentrations higher than 0.25% did not result in any significant difference in the spontaneous mutation rate. However, an increase in the mutation rate (up to almost 5-fold) was observed when cells were grown in the presence of <0.25% serum (Fig. 1).

To determine whether the observed effect is unique for blockage of growth by serum deprivation, a number of other approaches which allow reduction of growth were used. Fig. 2 demonstrates the effects on growth and the mutation rate of seeding density. It was possible to obtain reductions in the growth rate similar to those seen with serum deprivation by seeding cells at high densities. Cells seeded at 5.58 × 10^5/plate (number corrected for plating efficiency) underwent 2.33 generations after 5 days. Cells seeded at the lowest density (0.09 × 10^5/plate) had a much higher growth rate (6.8 generations after 5 days). However, reducing the growth rates by this method had little effect on the mutation rate (Fig. 2). There are no significant differences between the mutation rates in any of the populations.

TGF-β, a well-known inhibitor of cell growth, is highly effective in serum-free medium (24). However, for this study it was inappropriate to combine the effect of serum-free medium with the effect of TGF-β. Cells were therefore plated in medium containing 5% serum, allowed to attach for 4 h, followed by a medium change to medium containing either 1% serum or 1% serum and TGF-β. As discussed above, the growth of cells in 1% serum did not change the mutation rate compared to control conditions (5% serum; Fig. 1). It was not possible to achieve a dose-dependent inhibition of growth with TGF-β (1, 5, and 10 ng/ml). Growth was reduced by approximately 30% by all concentrations used. The differences between spontaneous mutation rates of cells grown in the presence of TGF-β and control cells were not statistically significant (data not shown).

When cells were cultivated in medium containing 5% serum and suramin, inhibition (up to 40%) of the growth rate was observed at concentrations of 400 and 500 µg/ml. Concentrations of 600 µg/ml and higher were too toxic to perform mutagenesis experiments. Again, no significant differences in spontaneous mutation rates were observed when cell growth was inhibited by suramin compared with control cells (data not shown).

When cells were cultivated in medium containing 5% serum and DRB, an inhibitor of RNA synthesis (20), 50% inhibition of growth
SERUM DEPRIVATION INCREASES MUTAGENESIS

We recently described a new mathematical model for the estimation of the spontaneous mutation rate (7). Here, we used this method to test the hypothesis that the mutation rate is independent of the growth rate. Our results demonstrate that when G12 cells were grown in very low concentrations of serum, their spontaneous mutation rate was elevated (Fig. 1). Thus, under these conditions, the mutation rate was not independent of the growth rate. Do these results then invalidate our mathematical model which was based on the assumption that mutations are fixed during DNA replication? To answer this question, spontaneous mutagenesis was assayed under conditions other than serum deprivation which inhibit cell proliferation.

When cells form a confluent monolayer, they undergo density-dependent inhibition of cell division, originally called “contact inhibition” (9). It was later demonstrated that the final population density can be increased by increasing the concentration of growth factors in the medium (24). Cells appear to stop dividing not because they are in contact, but because the growth factors present in the medium are limiting. Polypeptide growth factors in serum play a crucial role in stimulating the proliferation of cells and in maintaining their viability (reviewed in Refs. 26 on July 13, 2017. © 1996 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from 26 was observed at a concentration of 40 μM. Higher concentrations were too toxic to use. No statistically significant differences in the spontaneous mutation rate were seen in cells grown with DRB compared with control cells (data not shown).

Levels of oxidative stress were measured in cells grown in normal medium (5% serum) and in low serum (0.05%; Fig. 3). Growth of cells for 24 h in low serum resulted in a >2-fold increase of the level of oxidants compared to the level of control cells. After 48 h, the level of oxidants was reduced in the serum-depleted cells, but was still higher than that in control cells. Levels of oxidative stress were also measured in cells seeded at high cell density (6 × 10^6 cells/100-mm plate) and at low density (1 × 10^6/100-mm plate). No differences in the levels of oxidants were observed between the two groups (data not shown).

Spontaneous mutation rates were calculated in cells grown in low serum (0.025%) in the presence of the antioxidants mannitol and/or ascorbic acid. Similar to results presented earlier (Fig. 1), growth in low serum resulted in approximately 70% inhibition of growth and a >2-fold increase in the mutation rate (Fig. 4). The mutation rate was reduced when cells were grown in low serum in the presence of mannitol (100 and 500 μg/ml) in a dose-dependent manner. Ascorbic acid was more effective than mannitol in reducing the mutation rate, since 50 μg/ml gave a reduction in mutagenesis equivalent to 500 μg/ml mannitol. Ascorbic acid in combination with 100 μg/ml mannitol was as effective as ascorbic acid alone. The mutation rates of cells grown in low serum in the presence of antioxidants (except 100 μg/ml mannitol) were significantly lower than in cells grown in 5% serum (Fig. 4). Spontaneous mutation rates in cells grown in low serum in the presence of catalase (100 μg protein/ml) were reduced to control levels (mutation rate of cells grown in 5% serum; data not shown).

DISCUSSION

We recently described a new mathematical model for the estimation of the spontaneous mutation rate (7). Here, we used this method to test the hypothesis that the mutation rate is independent of the growth rate. Our results demonstrate that when G12 cells were grown in very low concentrations of serum, their spontaneous mutation rate was elevated (Fig. 1). Thus, under these conditions, the mutation rate was not independent of the growth rate. Do these results then invalidate our mathematical model which was based on the assumption that mutations are fixed during DNA replication? To answer this question, spontaneous mutagenesis was assayed under conditions other than serum deprivation which inhibit cell proliferation.

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and 27). In the present study, when cells were grown in very low concentrations of serum or when they were seeded at very high densities, similar levels of growth inhibition occurred. Yet, significant increases in the spontaneous mutation rate occurred only during serum deprivation and not during crowding (Figs. 1 and 2).

Suramin was originally shown to dissociate platelet-derived growth factor from its receptor (28), but was later demonstrated also to inhibit the binding of other growth factors to their specific receptors (29-31). DRB causes the inhibition of RNA synthesis, possibly by blocking a step catalyzed by RNA polymerase II (20). We show that proliferation of G12 cells was partially blocked by suramin and DRB. However, suramin and DRB, like density-dependent inhibition, did not cause any significant increase in the spontaneous mutation rate.

Cell proliferation can also be blocked by the action of negative growth regulators. An example of this class of molecules is TGF-β (reviewed in Ref. 14). The exact mechanism of growth inhibition is unknown, but TGF-β apparently inhibits cell growth through effects on multiple independent and synergistic pathways required for cell cycle progression (32-34). It was not possible to achieve >30% inhibition of proliferation in this study using TGF-β, due to the necessity to include 1% serum in the medium. It is known that serum interferes with the inhibitory effect of TGF-β (24). Inhibition of cell proliferation by TGF-β had no effect on the spontaneous mutation rate. Thus, only serum deprivation resulted in higher spontaneous mutation rates. Taken together, our results suggest that serum contains some crucial components which protect cells from spontaneous mutagenesis and without which cells quickly accumulate mutations even during growth inhibition.

Reactive oxygen species are constantly generated in vivo as a result of various metabolic processes. All cells possess antioxidant defense systems including such enzymes as superoxide dismutases, catalase, and glutathione peroxidase and low molecular mass antioxidants (glutathione, vitamin E, and ascorbic acid; reviewed in Refs. 35 and 36). FCS contains a number of compounds which serve as antioxidants, one of which (catalase) was recently identified as a growth-promoting factor (37). Our studies demonstrate that when cells were cultivated in the presence of very low concentrations of serum, the level of intracellular oxidants was elevated as measured by DCF fluorescence (Fig. 3). Previous studies have shown that various reactive species can oxidize DCF to a fluorescent product (38). At this time we do not know which specific oxygen species appear when cells are grown in serum-deficient medium.

Oxidative stress induces DNA damage that may lead to mutations and chromosomal aberrations (reviewed in Refs. 2 and 6), and may play a role in p53 mutations in such tumors as smoking-related lung carcinoma, hepatocellular carcinoma, colorectal carcinoma, and skin tumors (reviewed in Ref. 39). Oxidative stress also results in the activation of gene expression. NFκB was shown to be an oxidative stress-responsive transcription factor in higher eukaryotes (reviewed in Ref. 40) and may induce expression of proto-oncogenes, thus participating in tumor promotion and progression. The striking result of this study is the blocking by antioxidants of the increased spontaneous mutation rate of serum-deprived cells. These data support the hypothesis that reactive oxygen species play an important role in mutagenesis and suggest that antioxidants in serum provide a strong defense against oxidative stress and therefore mutagenesis.

It has been suggested that the normal mutation rate cannot account for the number of mutational changes required for transformation of normal cells into tumor cells but can account for only about two mutations per cell per lifetime in humans (41, 42). This calculation is based on the assumption that mutagenesis occurs only in dividing cells.) Tumor cells possess more than two, and perhaps as many as six mutations (43). At least three mutations are needed for mouse cells to become tumorigenic (44). To explain this discrepancy, a number of hypotheses concerning spontaneous mutagenesis and carcinogenesis might be put forth (more than one of which may be true):

1. “Environmental” mutagens (in the broadest sense of the term) are crucial for carcinogenesis. Some individuals cannot escape being exposed to exogenous mutagens to such an extent that the induced mutation rate allows a sufficient number of mutations to occur to produce the malignant phenotype.

2. Human variability in spontaneous mutation rates could account for some individuals accumulating enough mutations per cell in a lifetime to give rise to the malignant phenotype even in the absence of exogenous mutagens. It has been established that there is interindividual variability in the mutant frequency (fraction) in human lymphocytes at the hprt locus (45). However, it is not known if this variability is due to variability in the mutation rate. Bloom syndrome patients, who are at increased risk of developing cancers, do show elevated cellular spontaneous mutation rates (46). Individuals differ in their abilities to detoxify electrophilic intermediates (47), and it is expected that individuals will show different levels of endogenous DNA damage and spontaneous mutation rates (4). Inherited mutations in mismatch repair genes were found in families with HNPPC (48, 49). These families also have an elevated incidence of cancers of the urinary tract, stomach, endometrium, and pancreas (1). Dramatically increased mutation rates were recently found in the endogenous hprt gene in HNPPC tumors (50).

3. It has been proposed that an early step in tumor progression might be the appearance of a mutator phenotype (i.e., a cell with an increased spontaneous mutation rate; Ref. 41). However, with the exception of HNPPC, there is no simple correlation between the spontaneous mutation rates in human cells and their malignancy (50-52). Nor do extracts from tumor cells show reduced fidelities of DNA replication compared with extracts from normal cells (53).

4. Strauss (42) proposes that spontaneous mutagenesis may not
always be cell generation dependent, but rather, under some conditions, may be time dependent. This argument is based on an analogy with the controversial proposal of directed mutagenesis in bacteria (54, 55). Without entering into this controversy, it can be noted that under some conditions which permit cell survival but where growth is inhibited, E. coli can apparently mutate in a time-dependent manner.

Based on the results of this study, it is possible to suggest another hypothesis which could account for elevated spontaneous mutagenesis under some conditions. During the development of tumors, the blood supply may be limiting due to lack of angiogenesis for a prolonged period of time, during which time the size of the tumor usually does not exceed a few mm$^3$ (56, 57). Cells grown in low serum may be analogous to prevacular tumors in that in the absence of some components, a hypermutagenic state can be created. Although oxygen may also be limiting in such tumors, the balance between oxidative species and serum antioxidants is not known.

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