Chemotherapeutic deletion of CTG repeats in lymphoblast cells from DM1 patients

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ABSTRACT

Myotonic dystrophy type 1 (DM1) is caused by the expansion of a (CTG) (CAG) repeat in the DMPK gene on chromosome 19q13.3. At least 17 neurological diseases have similar genetic mutations, the expansion of DNA repeats. In most of these disorders, the disease severity is related to the length of the repeat expansion, and in DM1 the expanded repeat undergoes further elongation in somatic and germline tissues. At present, in this class of diseases, no therapeutic approach exists to prevent or slow the repeat expansion and thereby reduce disease severity or delay disease onset. We present initial results testing the hypothesis that repeat deletion may be mediated by various chemotherapeutic agents. Three lymphoblast cell lines derived from two DM1 patients treated with either ethylmethanesulfonate (EMS), mitomycin C, mitoxantrone or doxorubicin, at therapeutic concentrations, accumulated deletions following treatment. Treatment with EMS frequently prevented the repeat expansion observed during growth in culture. A significant reduction of CTG repeat length by 100–350 (CTG) (CAG) repeats often occurred in the cell population following treatment with these drugs. Potential mechanisms of drug-induced deletion are presented.

INTRODUCTION

The expansion of trinucleotide repeats and other short tandem DNA repeats is associated with an increasing number of inherited neurological diseases. These include myotonic dystrophy type 1 (DM1), DM2, Huntington’s disease (HD), HD-Like 2, dentatorubral pallidolysian atrophy, fragile X syndrome, FRAXE mental retardation, Kennedy’s disease, oculopharyngeal muscular dystrophy, Friedreich ataxia and several spinocerebellar ataxias (SCAs) (SCA1, 2, 3, 6, 7, 8, 10, 12 and 17) (1–5).

Several common features are shared by most tandem repeat diseases. First, patients with most of these diseases exhibit an earlier age of onset and increased severity in successive generations, a phenomenon termed anticipation. This phenomenon is generally correlated with increasing repeat length (6). Second, expanded repeats in most of these diseases show both somatic and germline instability usually with a bias toward expansion. In transgenic animal models of these diseases, the longer repeat in the transgene led to increased severity and earlier onset (7–11). Therefore, the repeat length is critical in the pathogenic mechanism and is one of the most important determinants of disease severity. Repeat expansion leads to the respective disease phenotypes through diverse mechanisms. For example, expanded CAG repeats encoding polyglutamine tracts lead to a gain of function of a protein as with HD, Kennedy’s disease and several of the SCAs. A large (CGG)-(CCG) repeat expansion in the 5′-untranslated region (UTR) of the FMR1 and FMR2 genes and an expansion of an intronic (GAA)-(TTC) repeat in the FRDA gene result in a loss of function of the protein products in fragile X syndrome, FRAXE mental retardation and Friedreich ataxia, respectively (1). In SCA12, an expansion of CAG repeat in the 5′-UTR may cause enhanced transcription of the PPP2R2B gene (12). The pathogenic mechanisms of other repeat expansion diseases remain less well defined.

No treatment currently exists for DNA repeat expansion-associated neurological diseases. Because the molecular etiology is the repeat expansion, treatment with chemicals or drugs that would reduce the repeat size would target the very source of the problem and may be generally applicable to delay onset or reduce the severity of diseases associated with repeat expansion. This may be especially important in diseases where somatic expansion during life may be associated with disease onset and severity.

DM1 is a multiscystem disorder, characterized by muscle weakness, wasting and myotonia. DM1 results from an

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expansion of a CTG repeat in the 3′-UTR of the myotonic dystrophy protein kinase gene (DMPK). The expanded CTG repeat is unstable with a bias toward further expansion in germline and somatic tissues, giving rise to anticipation, and repeat size increases in tissues during life (13,14). The molecular mechanism downstream of the mutation in DM1 is complex and is associated with partial loss of DMPK, decreased transcription of a neighboring gene, Six5, in addition to binding of CUG-binding proteins by the expanded CUG mRNA (15). Thus, DM1 may be considered as a representative disease model to test the feasibility of chemical or drug treatments that decrease the repeat size.

The actual mechanism of tandem repeat expansion remains unclear. Several lines of evidence implicate various processes, including DNA replication, repair and recombination (2,16–18). One common feature of the repeats that may contribute to their instability is their ability to form various DNA secondary structures (2). CTG, CAG, CCG and CGG repeats form stable hairpins (19–21); (CTG)-(CAG) and (CGG)-(CCG) repeats form slipped-strand DNA structures caused by out-of-register misalignment within the repeat tract (22); (GGA)-(GGC) repeats form quadruplex DNA structures (23,24); and (GAA)-(TTC) repeats can form triplex structures and parallel stranded DNA (25–32). (ATTCT)-(AGAAT) repeats can form stable unpaired regions in DNA (33). A reasonable hypothesis remains that the formation of an alternative DNA secondary structure elicits repeat expansion during replication, repair, or recombination (2) or promotes aberrant DNA replication (33).

While mechanisms of repeat expansion are uncertain, mechanisms leading to (CTG)-(CAG) repeat deletion have been generally well accepted. A large body of evidence suggests that the formation of a single-stranded region containing the (CTG)ₙ strand [and to a lesser extent in the (CAG)ₙ-containing strand] leads to the formation of hairpins that fail to be copied during DNA replication leading to large repeat deletions (17,34–38). Deletions have been thought to result from replication slippage across CTG hairpins formed in the lagging template strand when single-stranded during replication (2,17,34,39,40). Hairpins stabilize misaligned intermediates preferentially in the lagging strand and promote misalignment (41,42). Recent results, however, show that functional RecA and RecB are required for a high rate of repeat deletion, and it is difficult to incorporate these proteins into a simple replication slippage model for repeat deletion (43). An alternative model suggests that repeat deletion occurs from errors occurring during replication restart following the collapse of the replication fork during synthesis of the repeats via a pathway that involves a double-strand break (43). Unrepaired DNA damage will also stop the replication fork, causing fork collapse. In bacteria and yeast, replication through repeats can cause replication fork blockage (44–46). In yeast, repeats are associated with chromosome "fragility", presumably a double-strand break that initiates recombination (37,45,47–52). Thus, DNA damage can block replication fork progression and lead to double-strand breaks in bacterial, yeast and human cells, where repair occurs by a variety of homologous and non-homologous based mechanisms (53–56). Moreover, many DNA damage repair processes involve the generation of a single-stranded gap in the DNA as a damaged base is removed (57). Thus, we hypothesize that the introduction of DNA damage to cells harboring (CTG)-(CAG) triplet repeats could lead to an increase in the rate of triplet repeat deletion both by creating single-strand regions and by causing replication fork collapse. Our results in a model bacterial system confirmed this hypothesis, showing that the DNA-damaging agents ethylmethanesulfonate (EMS), ethynitrosourea, and ultraviolet (UV) light, and chemotherapeutic agents mitomycin C, cisplatin and X-rays increased the rate of deletion of (CTG)-(CAG) repeats (58). Moreover, oxidative damage to DNA also increased the rate of deletion of triplet repeats (58). These results provide the molecular basis to suggest that a chemotherapeutic approach to triplet repeat deletion may offer one rationale to slow, stop or reverse the progression of these diseases. In fact, as discussed below, recent experiments in several mammalian cell systems support our basic hypothesis (59–62).

Here, we treat lymphoblast cell lines derived from DM1 patients with DNA-damaging agents, including EMS, a monofunctional alkylating agent, and several chemotherapeutic agents, including mitomycin C, which introduces interstrand crosslinks; mitoxantrone, an inhibitor of topoisomerase II, which intercalates into DNA causing crosslinks and strand breaks; and doxorubicin, which intercalates into DNA and inhibits DNA synthesis. Mitomycin C, mitoxantrone and doxorubicin are currently used in the treatment of cancer and mitoxantrone is also used to treat multiple sclerosis. Our results indicate that drug treatment can prevent expansion commonly observed in lymphoblastoid DM1 cells. In many cases, a significant reduction of (CTG)-(CAG) repeat length occurred in the entire cell population following drug treatment.

MATERIALS AND METHODS

Cell growth

Clonal lymphoblast cell lines derived from DM1 patients have been described previously (63,64). Cell lines 313-1-15, 313-1-20 and 428 initially contained about 770, 740 and 440 (CAG) repeats, respectively (63). DM1 lymphoblast cells were grown between 2 × 10⁶ and 2 × 10⁶ cells/ml at 37°C with 5% CO₂ in RPMI (Hi-Clone) media supplemented with 10% calf serum (Hi-Clone) and 1 μM insulin. Cells were grown for a defined number of generations for comparison. Generations is calculated as [(DF × log N₀) − log N₀]/log₂, where DF is a dilution factor (calculated from the total number of times cells were diluted), N₀ is the total number of cells at the time of sampling, and N₀ is the number of cells at the beginning of the experiment. Previously, growth has been reported as ‘passages’ (63). A passage represents 2–4 generations, when cells are diluted 1:4 or 1:8 for subsequent growth. The calculation of generations in our analysis does not take into account cell death in the population (only population doubling), and thus may represent a minimum or underestimate of the actual number of cell divisions at the time of harvest.

Treatment with drugs

DM1 lymphoblast cell lines were treated with drugs in growth media at ~1 × 10⁶ cells/ml for various times. Treatment with 500 or 700 μg/ml EMS or 0.1 or 0.2 μg/ml of mitomycin C was for 14–16 h, treatment with 50 or 100 nM mitoxantrone was
for 1 h, while treatment with 0.1–5 μg/ml doxorubicin was for 30 min. Drug exposures were adjusted to reduce survival to 10–30%, except for treatment with mitoxantrone, which reduced survival to ~30–50%. Following treatment, the cells were pelleted, washed once with media and resuspended in media at ~1 × 10⁶ cells/ml and incubated. Growth was generally continued for up to 10–30 generations. At selected passages aliquots of 1 × 10⁷ cells were harvested by centrifugation for 5 min and frozen at −20°C until the genomic DNA was isolated for small pool PCR. In certain experiments, a second dose of the drug was administered in the same manner as above after recovery of the culture.

DNA purification and PCR analysis

For allele length analyses, genomic DNA was purified from ~5–10 × 10⁶ lymphoblast cells using a DNeasy Tissue Kit (Qiagen) followed by one or two ethanol precipitations. For small pool PCR, reactions contained 50–200 pg of HindIII-digested DNA corresponding to ~1–50 diploid genome equivalents per reaction. Two different conditions were used with identical results. First, PCR was performed according to the procedure described by Monckton et al. (13). In addition, PCR was performed as follows: 0.5 U Platinum Taq DNA Polymerase High Fidelity (Invitrogen), 0.2 μM primer DM-DR (dCAGGCCTGACAGTTTGCCCATC) and 0.2 μM primer DM-H (dTTCGCCGCCAGCTCAGTC), 2 mM MgSO₄, 0.2 mM dNTPs, in a total volume of 10 μL. PCR was performed in a Perkin–Elmer 9700 PCR machine with the following conditions: the DNA was denatured at 94°C for 1 min and then 30–35 cycles of 94°C for 45 s, 72°C for 45 s, 70°C for 2 min 40 s, with a final extension step at 70°C for 35 min.

Southern hybridization

PCR products were separated by electrophoresis through 1.8% agarose gels in 0.5x TBE buffer (1x TBE: 90 mM Tris, 90 mM Borate, 2.5 mM EDTA, pH 8.3), transferred to a Hybond N membrane (Amersham), the DNA fixed by UV irradiation and then the membrane was hybridized with an (AGC)₁₀ oligonucleotide end-labeled with [γ-³²P]ATP. Hybridization was performed for 16 h at 42°C in 1x SSC buffer. Membranes were then washed twice for 30 min at 49°C in 0.5x SSC, 0.1% SDS, rinsed in H₂O and then exposed for 1–2 days to a Molecular Dynamics PhosphorImager plate. Alleles were sized by comparison with molecular weight markers. Lambda/HindIII/ pUC19/TaqI/Sau3A1 molecular weight markers (Invitrogen) were labeled with ³²P as follows: 1 μg of DNA was added to 10 U Klenow (New England Biolabs), 1 mM dGTP, 1 mM dCTP, 1 mM dTTP and 50 μCi (3000 Ci/mM) [α-³²P]dATP, in 20 μl Klenow buffer and the mixture was incubated 15 min at 20°C and then 15 min at 65°C. The number of repeats in individual alleles was calculated from the length of the PCR product and the DNA sequence flanking the (CTG)-(CAG) repeats.

Statistical analysis

For statistical analysis, chi-square tests were calculated for each set of experiments. The numbers of original-length alleles and expansions and deletions were compared between control, non-treated cells and drug-treated cells. Statistical results for a single regimen of drug treatment for both cell lines were then analyzed using a modified Bonferroni comparison to control for type 1 error. The significance following this comparison is reported in the results section.

RESULTS

Repeat instability in lymphoblast cell lines 428 and 313-1-15

Growth of lymphoblast cell lines through many generation is accompanied by expansion of the expanded (CTG)-(CAG) repeat, at a variable magnitude and frequency (63,64). (CTG)-(CAG) repeat lengths were analyzed by small pool PCR using a well-established procedure that can easily detect more than 1000 (CTG)-(CAG) repeats (13,14,63–67). We have analyzed the effect of treatment with a common alkylating agent and three chemotherapeutic drugs on repeat instability. Two of these agents, EMS and mitomycin C, increased the rates of (CTG)-(CAG) deletion in a bacterial model system (17). Cell line 313-1-15 contains about 770 (CTG)-(CAG) repeats that remain relatively stable but do show a slight and gradual increase in the repeat size during growth (data not shown). The initial culture of cell line 428 showed a distribution of allele sizes of about 440 ± 100 repeats (Figure 1A).

After growth for 27 generations in one experiment (experiment A), two allele sizes dominated the cell population: 440 and 770 repeats (Figure 1A, experiment A). In a subsequent experiment, which was started from a sub culture of experiment A after five generations growth, the 770 repeat allele dominated the population following an additional 17 generations of growth (experiment B). Figure 1B shows the percentage of alleles plotted as a function of the number of (CTG)-(CAG) repeats for many repetitions of the PCR reactions as shown in Figure 1A. To confirm the rapid expansion in cell line 428, cells from the initial distribution population shown in Figure 1A were sub-cloned by growth of an initial 100–300 cells for 18–20 generations. All populations analyzed showed predominantly the larger 770 repeat allele. Six of nine sub-clones analyzed showed only the 770 repeat allele, while three showed 94–97% the 770 repeat allele (data not shown).

Clearly, during these drug experiments cell line 428 was prone to repeat expansion in control, non-treated cultures. Control, of non-treated cultures of cell line 428 was repeated more than 10 times. At later passages, after the repeat had expanded to >700 repeats, spontaneous reductions in allele sizes in the population were never observed without drug treatment. The behavior of this cell line mimics events occurring in certain DM1 tissues, where repeat expansion continues throughout life (13).

The rapid change in repeat lengths is presumably due to cells with larger (CTG)-(CAG) repeats having a growth advantage over those with smaller expanded alleles (64). Given the potential for rapid change in cell line 428, it seemed that it may be sensitive to the effects of DNA-damaging agents. On the other hand, cell line 313-1-15 was used for analysis because it is relatively stable and the accumulation of small deletions may be more easily observed in this line. Following growth of this cell line in 17 independent experiments, deletions comprised <2% of the allele population in the absence of drug treatment, and a large deletions of the repeat allele in the entire population were never observed.

Figure 1. Expansion of (CTG)(CAG) repeats in cell line 428. (A) Representative small pool PCR reactions with 300 pg DNA are shown for cell line 428 for an initial early passage culture and following growth. In experiment A, the cells were grown for 27 generations. Experiment B was started from sub culture of cells from experiment A after five generations. Experiment B was grown for an additional 17 generations. Size markers are indicated relative to the number of (CTG) repeats in the PCR products. Under the PCR conditions used, 300 pg corresponds to ~6–12 genome equivalents per reaction. (B) Allele spectra for 428 cultures at the time of initial growth (panel a) \( n = 151 \); experiment A after five generations growth (panel b), \( n = 320 \); and experiment B after 17 generations growth (panel c), \( n = 515 \). \( n \) = the number of alleles analyzed.

Treatment with the alkylating agent EMS results in the accumulation of the 440 repeat allele in cell line 428

Lymphoblast cell line 428 was treated with 500 and 700 \( \mu g/ml \) EMS and grown for \( \sim 10–20 \) generations. Small pool PCR analysis of alleles shows that the treatment resulted in the accumulation of much smaller allele sizes than present in the non-treated cells grown relatively for the same number of generations (Figure 2). Treatment with 500 \( \mu g/ml \) EMS generally resulted in the accumulation of two or three repeat sizes (440, 550 and 770 repeats) (Figure 3). When cells were treated with a second dose of EMS, the accumulation of the smaller sized alleles was more pronounced (Figure 3A, panel b and Figure 3B, panel c). Treatment with 700 \( \mu g/ml \) EMS resulted in the accumulation of the 440 repeat allele size, a reduction in size of 330 repeats relative to the expansion observed in non-treated cells. In the experiment shown in Figure 3A, the difference in the allele distributions for both drug-treated samples relative to the non-treated distribution was significant (\( P \leq 0.001 \)) using a modified Bonferroni comparison. In the experiment shown in Figure 3B, while the single treatment of 500 \( \mu g/ml \) EMS resulted in the accumulation of many shorter sized deletions (panel b), the distribution was not significantly different from the control, non-treated distribution comparing the numbers of 440 repeat alleles and other-size alleles (\( P = 0.072 \)). In this experiment, distributions following the second dose of 500 \( \mu g/ml \) EMS treatment and the single dose of 700 \( \mu g/ml \) EMS treatment (panels c and d) were significantly different (\( P \leq 0.001 \)). Experiments involving cell growth following treatment with EMS were repeated six times for this cell line with essentially identical results. Occasionally (in \(<10\%\) of drug-treated samples) an EMS-treated sample with predominantly the expanded (770 repeat) allele was observed. It is important to reiterate that, from independent analyses of the growth of many cell lines and many individual growth analyses, large deletions, especially reductions in the repeat tract in the entire population, were not observed in this, or previous studies (63,64). Thus, the large repeat reductions observed in the population are highly significant and drug dependent.

Treatment with mitomycin C results in the accumulation of (CTG)(CAG) deletions

Lymphoblast cell line 428 and 313-1-15 were treated with 0.1 or 0.2 \( \mu g/ml \) mitomycin C and grown following drug exposure. A dramatic effect was observed in both cell lines following treatment with mitomycin C. In the experiment shown in Figures 2 and 4A for cell line 428, a broad range of repeat sizes accumulated following drug treatment. Little of the original 440 repeat size allele was observed in cells treated once or twice with 0.1 \( \mu g/ml \) mitomycin C (Figure 4A, panels b and c). Following the second treatment (Figure 4A, panel c), a broad distribution of allele sizes was observed (from 300 to 675 repeats), corresponding to reductions of 100–470 repeats relative to non-treated cells. Compared with the control non-treated distribution, the allele spectra for cells treated twice with mitomycin C were significantly different (Figure 4A, panel b, \( P \leq 0.001 \); panel c, \( P = 0.002 \)). The allele distribution for cells treated once with 0.2 \( \mu g/ml \) mitomycin C was not significantly different from control considering two populations of alleles (the initial 440 repeat size and all other expansions or deletions). Considering three populations of alleles (440 repeats, 770 repeats, and all other expansions and deletions), the allele spectra were significantly different (\( P \leq 0.001 \)). This data is representative of five independent experiments with cell line 428.
with 0.2 μg/ml mitomycin C observed in line 313-1-20, a large deletion of about 350 repeats was observed in some samples in this cell line, and alleles with 100 repeats, or fewer, were observed. Following a single exposure to 0.1 μg/ml mitomycin C (Figure 4B, panel b), the frequency of deletions was not significantly different from that in control cells. However, following a second treatment of 0.1 μg/ml mitomycin C (Figure 4B, panel c), the allele spectra were significantly different, after only 4–5 generations growth, with a P-value of <0.001. Following a single treatment with 0.2 μg/ml mitomycin C (Figure 4B, panel d), the difference in allele spectra was not significantly different (P = 0.052). In a separate experiment, a deletion from 770 to 675 repeats was observed in the entire population of 313-1-15 cells following treatment with 0.2 μg/ml mitomycin C (Figure 5). In this experiment, the allele spectra following 17 and 19 generations growth showed a reduction to 675 repeats (Figure 5, panels b and c). Both allele distributions were significantly different from non-treated, control cells (P < 0.001). Experiments with cell line 313-1-15 and mitomycin C treatment were repeated 12 times. In another experiment using cell line 313-1-20, a large deletion of about 350 repeats was observed in ~10–15% of the culture following treatment with 0.2 μg/ml mitomycin C (data not shown). Experiments with cell line 313-1-20 and mitomycin C treatment were repeated four times.

Mitoxantrone induces deletions in DM1 lymphoblast cells

A culture of lymphoblast cell line 428, in which the repeat had expanded to 740 (CTG)-(CAG) repeats (>98% of alleles), was treated with 50 and 100 nM mitoxantrone. The allele spectra following treatment with 50 nM mitoxantrone were not significantly different from that for control, non-treated cells after 14 generations growth (Figure 6, panel b). However, after 14 generations a significant shift in the population to about 620 repeats was observed with the sample treated with 100 nM mitoxantrone (P < 0.001) (Figure 6, panel c). Eight independent cultures of 428 and 313-1-15 cells have been treated with mitoxantrone. The data shown in Figure 6 represent the most dramatic response.

Doxorubicin induces deletions in DM1 cells

DM1 lymphoblast cells were treated with several doses of doxorubicin and growth was continued for ~10 generations. Figure 7 (top) shows representative data from one of the four independent experiments in cell line 313-1-15. The rate of population doubling was reduced in a dose-dependent fashion following exposure to doxorubicin. Allele distributions, after 10–16 generations growth, showed the accumulation of deletion mutations in 10–20% of the allele population in a dose-dependent fashion that paralleled the initial decrease in survival and growth rate (Figure 7, bottom). In this experiment, the expanded allele measured ~840 repeats and most deleted alleles were between 350 and 650 repeats, or deletions of 200–500 repeats. The allele distributions following treatment with 1, 2 and 5 μg/ml doxorubicin were significantly different from that in the control distribution (P < 0.001).

DISCUSSION

Our results demonstrate that lymphoblast cells, derived from patients with DM1, which contain expanded (CTG)-(CAG) repeats in the DMPK gene, accumulate smaller (CTG)-(CAG) repeat alleles following treatment with EMS, mitomycin C, mitoxantrone or doxorubicin. Reductions in the length of the repeat from 770 to 100 or fewer (CTG)-(CAG) repeats were frequently observed following drug treatment. In many cases, the length of the expanded repeat was reduced from 770 to 650–440 (CTG)-(CAG) repeats in the entire population of cells. This is a significant result given the propensity of the DM1 (CTG)-(CAG) repeats to expand rather than contract in patients and in culture, as shown here and previously (13,14,64).

Multiple events may be contributing to the reductions in repeat tract length: drug-induced deletion and clonal selection.
Events associated with DNA repair or drug-induced replication fork collapse would be expected to result in deletions, and potential mechanisms are discussed below. The mixture of shorter alleles observed following treatment with mitomycin C or doxorubicin likely arise from multiple independent deletions within the population. Clonal selection after drug treatment may also be occurring within the population. As occurs in cancer chemotherapy, drugs may be selectively killing cells in the population with larger numbers of repeats, which have a more rapid rate of cell division than cells with shorter alleles (64). This would then select for the growth of cells with shorter alleles. Some evidence for clonal selection has been observed following exposure of mouse fibroblasts containing expanded CTG repeats to various chemicals (62).

Figure 3. Accumulation of deletions in 428 cells following EMS treatment. (A) Experiment started with 428 cells using the population of alleles shown in Figure 1B, panel a, at the time of treatment. Panel a, allele distribution following 27 generations growth in control, non-treated cells. Panel b, allele distribution following a second treatment with 500 μg/ml EMS and 16 generations growth (n = 362). Panel c, allele distribution following a single treatment with 700 μg/ml EMS and 18 generations growth (n = 323). In both drug-treated samples, the allele distribution was significantly different from the control, non-treated sample using a chi-square test (P < 0.001). (B) Experiment started with 428 cells (as described in text). Panel a, allele distribution following an additional 17 generations growth in control, non-treated cells. Panel b, allele distribution following a single treatment with 500 μg/ml EMS, 18 generations growth (n = 196). Panel c, allele distribution following two treatments with 500 μg/ml EMS, 12 generations growth (n = 269). Panel d, allele distribution following a single treatment with 700 μg/ml EMS, 16 generations growth (n = 603). The allele distribution in panel b was not significantly different from the control (P = 0.071). In panels c and d, the allele distributions were significantly different from the control, non-treated sample using a chi-square test (P ≤ 0.001).
the time one would expect population outgrowth of a shorter sized allele from a single clonal cell. From a single cell, 24–25 population doublings are required to generate $10^7$ cells. In the mitoxantrone experiment (Figure 6), in which the starting culture had $>98\%$ alleles with 740 repeats, the shorter 620 repeat allele would only generate $8.2 \times 10^3$ cells after 14 generations growth ($0.1\%$ of the cell population considering analysis of $10^7$ cells) and by this time a complete shift in the population had occurred. The $>300$ repeat reduction observed in the entire population in cell line 428 in earlier passages when a mixture of allele sizes was present may represent clonal selection. It is important to mention, however, that due to the strong drive toward expansion in the 428 cells, the larger alleles will naturally dominate the population.
Mixing experiments using cell lines with different allele sizes have shown that the cell line with the longer allele will dominate the population (64). We also showed that when the 'initial culture' of 428 shown in Figure 1 was sub-cloned, all populations analyzed showed predominantly the full-length expanded allele of ~770 repeats after 19 generations growth (n = 229). Panel b, allele distribution following 17 generations growth (n = 135). Panel c, allele distribution following 19 generations growth (n = 178). The differences in allele distribution in panels b and c were significantly different from that in the control sample (P < 0.001).

The data do not allow us, at present, to establish the mechanism(s) responsible for repeat deletion. Several phenomena may be contributing to the observed repeat deletion, and work is in progress to further understand the molecular basis for the drug-induced deletion. The working hypothesis is that DNA damage and/or replication fork collapse at or near the repeats elicits deletion. Deletions may arise through several different pathways. First, results using bacteria suggest that spontaneous deletions occur during a RecA and RecBC pathway of replication restart, in which the introduction of a double-strand break is associated with a high rate of repeat deletion (17,43). As shown in Figure 8A, replication fork collapse resulting from a DNA damage in the leading strand could lead to deletions. Evidence in yeast strongly supports the frequent introduction of double-strand breaks during DNA replication through (CTG)-(CAG) repeats (37,45,47–52). The addition of DNA damage would be expected to increase
allowing the formation of a (CTG)\(_n\) chance of deletion at this DNA repeat hotspot for mutation by DNA damage by an excision repair pathway could increase the probability of deletion in different steps of many repair pathways or during replication (17). Single-stranded DNA is generated by the generation of single-stranded DNA greatly increases the probability of deletion (Figure 8B). In bacterial cells, the generation of single-stranded DNA is believed to occur in bacterial model systems (2,43,58) with replication restart may also promote repeat deletion, but not in mammalian cells is associated with repeat deletion (75). An additional possibility is that treatment with DNA damage acts in a non-targeted fashion by inducing a generalized state of instability in cells, which results in deletion at the expanded (CTG)-(CAG) repeats. Persistent genomic instability occurring more than 30 generations following exposure to ionizing radiation is a well-documented phenomenon in cultured cells (76–78). In fact, transgenerational genomic instability following exposure to X-rays has been observed in mice (79).

The results presented here represent a ‘proof of principle’ that a chemotherapeutic approach to reduction in repeat length is feasible, although several issues must be addressed before this approach can be considered for clinical use. The concentrations of chemotherapeutic agents used in these experiments, 50–100 nM for mitoxantrone, 0.1–0.2 \(\mu\)g/ml for mitomycin C and 0.1–5 \(\mu\)g/ml doxorubicin, are within the therapeutic ranges, although EMS, a potent mutagen, cannot be used clinically. Our data are limited to (CTG)-(CAG) repeat expansion at the DMI locus. The drugs used may have varying effects with repeats associated with other repeat diseases. The drug effect may differ depending on repeat unit motifs, the repeat length, \(cis\) elements adjacent to the repeat and \(trans\) factors that may influence instability. In fact, in a similar study of lymphoblast cells containing 124 (CTG)-(CAG) repeats, mitomycin C treatment resulted in an increase in repeat length (59). This result may be due to the large differences in repeat length [124 compared with 440–770 (CTG)\(_n\)/CAG] repeats in mammalian cells (76–78). In fact, transgenerational genomic instability following exposure to X-rays has been observed in mice (79).

Whether the same chemotherapeutic agents promote deletions in other repeats at different loci remains to be shown. Nevertheless, the ‘proof of principle’ shown in this study raises a possibility that similar approaches might work for repeat expansions of other diseases.

Our results agree favorably with several other studies showing that DNA-damaging agents, various chemicals and replication inhibitors can influence repeat instability (59–62). Two studies have shown that agents that influence DNA replication or repair can influence repeat instability. Gorbunova et al. (60) showed that the DNA replication inhibitor aphidicolin increased the rate of deletions in CHO cells containing 95 but not 61 (CTG)-(CAG) repeats, while Yang et al. (61) showed that replication inhibitors aphidicolin and emetine, but not mimosine, increased the rate of CTG expansion in DM fibroblast cells. Both studies are consistent with hypothesis that DNA replication is involved in repeat expansion. Gomes-Pereia and Monckton (62) showed that long-term
exposure of kidney cells from a transgenic mouse carrying 160 CTG repeats to cytosine arabinoside, ethidium bromide, 5-azacytidine and aspirin reduced the rate of repeat expansion, while exposure to caffeine increased the rate of expansion. Our approach is a bit different in that we tested the effect of chemotherapeutic agents known to damage DNA on the instability of CTG repeats in human lymphoblast cells from DM1 patients. While we saw dramatic reductions in repeat tract length and the accumulation of deletions in the cell population, our results with mitomycin C were in contrast with that of Pineiro et al. (59). This difference may result from the short allele size examined, the potential for repeat expansion in their cell line or other differences with experimental protocols. Thus, at present a general consensus on the effects of chemotherapeutic agents, DNA-damaging agents, or replication inhibitors on repeat instability is not yet available from these independent studies with natural DM1 alleles in human lymphoblast or fibroblast cells, or transgenic (CTG)\(_n\)/(CAG)\(_n\) repeats in CHO and mouse cells. However, the basic idea that the length of expanded DNA repeats associated with human genetic disease can be modulated by various treatments seems well established.

While currently used chemotherapeutic regimes may provide a viable rationale for treatment, drugs that react with high specificity to expanding repeats may be designed based on the structure and characteristics of the DNA helix or the unusual properties of these repeats. CTG and CGG tracts exist as an unusually flexible double helix (80–82), and they can form mismatched hairpins (20,21) and slipped strand DNA structures (83–85). GAA repeats can form intramolecular triplex DNA (25–32) and a bimolecular triplex structure (29). Thus, it is possible that drugs that selectively bind to these sequences and mediate deletion in long repeat tracts may eventually be identified, increasing the selectivity of this basic approach. An additional approach would be to couple a DNA-damaging agent to an oligonucleotide or a PNA (peptide nucleic acid) molecule complementary to a repeat since these can bind very tightly to DNA. A targeted approach to repeat damage may also be important in tissue where cell division may be limited, e.g. muscle or brain. Most model systems examined to date have analyzed repeat lengths in dividing cells [this report and (59–62)]. However, it is important to note that expansions are observed in the brain of HD patients (90), and one report has suggested that expansion can occur in sperm during maturation (91). Thus,
repeat deletion may be expected to occur in non-dividing cells during chromosome repair and maintenance. Moreover, many of the late onset and complex characteristics of DM1 may result from, or be aggravated by, somatic expansion that occurs throughout life (13,14).

In conclusion, DNA repeat expansion diseases form a large class of genetic disorders linked mechanistically by a single type of mutation, the expansion of (CTG) (CAG), (CGG) (CCG), (GAA) (TTC), (CTG) (CAGG) or (ATTCT) (AGAAT) repeats. All known repeat expansion diseases cause progressive neurological manifestations that often lead to total disability and death. At present, no treatment can delay, halt or reverse the progression of these diseases. The application of a chemotherapeutic drug approach may open a gate to a new therapy for the underlying etiology of these diseases.

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