Deoxyribonucleotide Pool Imbalance Stimulates Deletions in HeLa Cell Mitochondrial DNA*  

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Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive disorder associated with multiple mutations in mitochondrial DNA, both deletions and point mutations, and mutations in the nuclear gene for thymidine phosphorylase. Spinazzola et al. (Spinazzola, A., Marti, R., Nishino, I., Andreu, A., Naini, A., Tadesse, S., Pela, I., Zammarchi, E., Donati, M., Oliver, J., and Hirano, M. (2001) J. Biol. Chem. 277, 4128–4133) showed that MNGIE patients have elevated circulating thymidine levels and they hypothesized that this generates imbalanced mitochondrial deoxyribonucleoside triphosphate (dNTP) pools, which in turn are responsible for mitochondrial (mt) DNA mutagenesis. We tested this hypothesis by culturing HeLa cells in medium supplemented with 50 μM thymidine. After 8-month growth, mtDNA in the thymidine-treated culture, but not the control, showed multiple deletions, as detected both by Southern blotting and by long extension polymerase chain reaction. After 4-h growth in thymidine-supplemented medium, we found the mitochondrial dTTP and dGTP pools to expand significantly, the dCTP pool to drop significantly, and the dATP pool to drop slightly. In whole-cell extracts, dTTP and dGTP pools also expanded, but somewhat less than in mitochondria. The dCTP pool shrank by about 50%, and the dATP pool was essentially unchanged. These results are discussed in terms of the recent report by Nishigaki et al. (Nishigaki, Y., Marti, R., Nishino, I., Andreu, A., Naini, A., Tadesse, S., Pela, I., Zammarchi, E., Donati, M., Oliver, J., and Hirano, M. (2003) J. Clin. Invest. 111, 1913–1921) that most mitochondrial point mutations in MNGIE patients involve T → C transitions in sequences containing two As to the 5′ side of a T residue. Our finding of dTTP and dGTP elevations and dATP depletion in mitochondrial dNTP pools are consistent with a mutagenic mechanism involving T-G mispairing followed by a next-nucleotide effect involving T insertion opposite A.

The human mitochondrial genome is a 16.6-kbp double-stranded circular DNA molecule that encodes 22 tRNAs, 2 rRNAs, and 13 polypeptides, all of which are essential proteins of the respiratory chain enzyme complexes (1). Mitochondrial DNA (mtDNA), which is usually present in 2–10 copies per mitochondrion and 10^5 to 10^6 copies per cell, comprises ~1% of total cellular DNA (2, 3). Since mtDNA replication is continuous, even in post-mitotic cells (4), a constant supply of nucleotides and balanced nucleotide pools are required for replication and maintenance of the mitochondrial genome. It has been shown that dNTPs within mitochondria comprise a metabolically and physically distinct pool (5–7), but it is not clear how dNTPs arise within mitochondria nor how mitochondrial dNTP pools are regulated. Evidence supports the existence of four different pathways by which dNTPs could arise within mitochondria. First, dNTPs could be synthesized in the cytosol and taken up by specific transport systems as the triphosphates (8). Second, cytosolic dNTPs could be dephosphorylated and then the corresponding deoxyribonucleoside diphosphates (dNDPs) be taken up by the deoxynucleotide transporter (9) and converted to dNTPs within the mitochondrion. Third, ribonucleoside diphosphates could be taken up by the deoxynucleotide carrier (9), then reduced within the mitochondrion by mitochondrial ribonucleotide reductase (10). Fourth, mitochondria could directly import deoxyribonucleosides, then phosphorylate them within the organelle by thymidine kinase 2 and deoxyguanosine kinase (11, 12).

It is well established that dNTP pool imbalances are mutagenic to cells (13), with mutagenic mechanisms including both insertion errors and next-nucleotide effects (14, 15). In vitro studies have demonstrated that dNTP imbalances can induce frameshift mutations as well (16). Recent evidence has suggested that an imbalance of mitochondrial nucleotide pools plays an important role in the pathogenesis of several human diseases, including mitochondrial neurogastrointestinal encephalomyopathy (MNGIE; Ref. 17), autosomal dominant progressive external ophthalmoplegia (18, 19), and Amish microcephaly (20). So far, however, there are no published data reporting direct analysis of mitochondrial dNTP pools in any of these conditions. Our laboratory described direct measurement of mitochondrial dNTP pools as long ago as 1982 (6). However, the measurements were laborious, and progress was slow. Recently we have modified our analytical procedures for dNTPs, allowing for reliable measurements of mitochondrial dNTPs from as few as 2 × 10^5 cultured cells. Using these modified techniques, we have explored, in HeLa cells, some of the predictions made from studies on one mitochondrial disorder, MNGIE.

MNGIE is an autosomal recessive disorder associated with multiple deletions and depletion of mtDNA in skeletal muscle (21) as well as mtDNA point mutations (22). The disease is caused by loss-of-function mutations in the nuclear gene encoding thymidine phosphorylase (TP; Ref. 17). TP catalyzes phosphorylation of thymidine to thymine and deoxyribose 1-phosphate. The deficiency of TP leads to increased circulating levels of thymidine (dThd; Ref. 23) and deoxyuridine (24). It has been postulated...
that increased levels of dThd in MNGIE cause mitochondrial nucleotide pool imbalances, which, in turn, lead to mtDNA abnormalities (17, 23). We tested this hypothesis by culturing HeLa cells in medium supplemented with 50 μM thymidine and observing the effects upon dNTP pools within mitochondria and upon the generation of mtDNA deletions.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HeLa S3 cells were routinely grown at 37 °C with 95% air and 5% CO₂ in Dulbecco’s modified Eagle’s medium supplemented with 5% dialyzed fetal bovine serum (HyClone, Logan, UT), with or without 50 μM thymidine. When cultures became 80–90% confluent, cells were washed twice with phosphate-buffered saline and then collected after trypsin treatment. When needed for DNA extraction, 80% of those cells were pelleted, with the remaining cells being subcultured and the culture medium being changed every 3 days.

**Whole-cell dNTP Pool Extraction and Analysis**—HeLa S3 cells were cultured as described above. For each analysis, the cells in two 100-mm plates were washed with 5 ml each of cold phosphate-buffered saline. Extraction was carried out with 3 ml per plate of ice-cold 60% methanol. Plates were incubated at −20 °C for 1 h, following which the fluid was recovered and each plate was washed with an additional 1 ml of 60% methanol. Following this, all suspensions and washes were pooled. The pooled suspension was heated for 5 min in a boiling water bath, followed by centrifugation for 15 min at 17,000 × g. The supernatant was transferred to a fresh tube and dried under vacuum. The residue was dissolved in sterile water and stored at −20 °C for later analysis.

**Analysis of the dNTP pools in each extract was based upon the method of Sherman and Fyfe (25). Reaction mixtures (50 μl) contained 100 μM HEPES buffer, pH 7.5, 10 mM MgCl₂, 0.1 unit of Escherichia coli DNA polymerase I Klno fragment (United States Biochemical), 0.25 μM oligonucleotide template, and 0.67 μM [3H]dATP (30 Ci/mmol; 1.0 μCi per assay, Amersham Biosciences) or [3H]dTTP (PerkinElmer Life Sciences).**

**Mitochondrial dNTP Pool Extraction and Analysis**—To determine the effect of thymidine treatment on mitochondrial dNTP pools, HeLa S3 cells were grown in 150-mm tissue culture dishes in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum. Once the cultures had reached 70–80% confluence, thymidine was added to a final concentration of 50 μM. After a 4-h treatment, the medium was removed and mitochondria were prepared by a modification of the method of Trounce et al. (26). Briefly, ~2 × 10⁷ HeLa S3 cells were harvested from eight 150-mm tissue culture dishes by trypsin treatment and washed twice in ice-cold phosphate-buffered saline. The cell pellet was resuspended in 5 ml cold isolation buffer, consisting of 220 mM mannitol, 70 mM sucrose, 5 mM HEPES, pH 7.2, 1 mM EGTA, and 0.5% bovine serum albumin (fatty acid-free). Then 10% digitonin solution was added to the suspension to a final concentration of 0.3 mg/ml. After 5-min incubation on ice, the suspension was diluted by adding 20 ml of isolation buffer, and cells were pelleted by centrifugation. Then the cell pellet was resuspended in 10 ml of isolation buffer and the cells were disrupted with a motor-driven glass-Teflon homogenizer until about 80% of the cells were broken. The mitochondrial fraction was isolated by differential centrifugation and subsequently washed twice and resuspended in 2 ml of isolation buffer. Immediately after that, most of the mitochondrial suspension was centrifuged to pellet mitochondria and the remaining suspension was saved for later mitochondrial protein determination. The mitochondrial pellet was immediately resuspended in 1 ml of ice-cold 60% methanol and incubated at −20 °C for 1 h. The remaining procedures were the same as described for the whole-cell dNTP pool extraction procedures. Protein concentration was determined by the Bradford method (27).

**Long Extension PCR**—Total DNA from HeLa cell samples was isolated by using the Wizard® genomic DNA purification kit (Promega). LX-PCR was performed to amplify the whole mtDNA genome using long extension PCR (Amersham Biosciences, Pittsburgh, PA). Primers were forward primer (L 15148–15174) and reverse primer (H 14842–14816). The PCR protocol consisted of initial 2-min denaturation at 94 °C, followed by 30 cycles of 94 °C for 15 s, 60 °C for 30 s, and 68 °C for 10 min, and a final extension of 7 min at 68 °C in a thermal cycler (PerkinElmer Life Sciences). The PCR products were subjected to electrophoresis on a 1% agarose gel at 100 V for 45 min and stained with 0.1 μg/ml ethidium bromide. To exclude the possibility that the low molecular weight bands were due to PCR artifacts, a second LX-PCR was performed using nested primers (L strand: 15148–15174) and (H strand: 13980–13956).

**Southern Blot Analysis**—Total DNA from HeLa cell samples was isolated as described above. Approximately 5 μg of total DNA was digested with restriction enzyme PvuII (Invitrogen) and the fragments were separated by electrophoresis through a 0.7% agarose gel. After electrophoresis the gel was blotted onto a nylon membrane (Hybond-N°, Amersham Biosciences). The membrane was hybridized with a mtDNA probe, which was generated by PCR using primers corresponding to mtDNA positions 8900–8922 and 9422–9400 and labeled with [α-32P]dCTP using random primers DNA labeling system (Invitrogen). Prehybridization, hybridization, and washing were carried out as described by Sambrook et al. (28). The washed membrane was exposed to an x-ray film at −70 °C with an intensifying screen.

**RESULTS AND DISCUSSION**

**Mitochondrial dNTP Extraction and Analysis**—A large body of literature exists regarding control of cellular dNTP pool sizes and the effects of pool imbalance on mutagenesis. In contrast, little information is available concerning mitochondrial dNTP metabolism, due to the technical difficulty of measuring these much smaller pools. Recent evidence indicates that an imbalance of mitochondrial nucleotide pools may play important roles in the pathogenesis of several human diseases, including MNGIE, autosomal dominant progressive external ophthalmoplegia, and Amish microcephaly (17–20). To investigate relationships between mitochondrial nucleotide metabolism and pathogenesis, it became necessary to increase the sensitivity of existing methods for dNTP measurement. By increasing the specific radioactivity of dNTPs used in the DNA polymerase-based method for dNTP analysis by some 3-fold, and by adopting the modifications to this assay of Sherman and Fyfe (25), we have increased the sensitivity of this method by nearly an order of magnitude. By adopting the methods of Trounce et al. (26), we have improved our yield of mitochondria with no loss in quality. With these improvements, we can determine as little as 0.05 pmol of dNTP in a cell or organelle extract. This means that we can prepare enough mitochondria for a complete dNTP assay from 2 × 10⁶ cells. Our earlier study (6) required more than 10⁷ cells for each analysis.

**Effects of Thymidine Treatment on dNTP Pools**—Spinazzola et al. (23) showed that MNGIE patients showed elevations in circulating thymidine levels, presumably a consequence of decreased thymidine catabolism resulting from the deficiency of thymidine phosphorylase. They speculated that thymidine could be salvaged to dTTP within mitochondria, generating a pool imbalance that could account for replication errors in the mitochondrial genome, leading both to deletions and point mutations. To test this hypothesis, we cultured HeLa cells in thymidine-supplemented medium and determined the effects, both upon dNTP pools, in short term experiments, and generation of mtDNA deletions, in a long term experiment. Thymidine supplementation was at 50 μM.

Fig. 1 shows the results of dNTP analyses in whole-cell and mitochondrial extracts. We found the mitochondrial dNTP pools to become imbalanced as a result of a 4-h exposure to 50 μM thymidine but not dramatically more so than the pools in whole-cell extracts. In whole-cell extracts dTTP and dGTP pools expanded by 2.2- and 1.5-fold, respectively; the dATP pool was unchanged, and the dCTP pool decreased to half its normal value. By comparison, the mitochondrial dATP and dGTP pools increased by 2.6- and 2.2-fold, respectively, while the mitochondrial dCTP pool decreased to 78 and 43%, respectively, of corresponding values in the untreated cells.

Several significant findings emerge from this experiment. First, as noted above, thymidine treatment did not unbalance the dNTP pools substantially more in mitochondria than in whole cells. However, factors such as a lack of mismatch repair in mitochondria (29, 30) may render mitochondrial DNA replication more susceptible than nuclear DNA replication to mod-
Fragments); lane 2, PCR product generated from cells grown in medium without thymidine supplement; lanes 3–5, PCR products generated from cells grown in medium with 50 μM thymidine supplement for 240, 247, and 254 days, respectively. The arrow indicates a 16.5-kbp full-length mtDNA product. The arrowheads indicate mtDNA molecules containing deletions.

est dNTP asymmetries. Second, the composition of the mitochondrial pool is quite different from that of the much larger whole-cell pool. As we and others have observed repeatedly (31, 32), dGTP represents only 5–10% of the total dNTP pool in whole-cell extracts of cultured eukaryotic cells. However, in HeLa cell mitochondria, dGTP is considerably more abundant than either dATP or dCTP and is nearly as abundant as dTTP. Third, the effect of thymidine upon the mitochondrial dNTP pools suggests that ribonucleotide reductase plays a significant role in regulating dNTP pool sizes in the organelle. dTTP acts as an allosteric activator of GDP reduction by ribonucleotide reductase and an inhibitor of CDP reduction (33). In recent experiments we have detected in liver mitochondria a ribonucleotide reductase activity that is similar to the major cell ribonucleotide reductase in its response to allosteric effectors. The dGTP accumulation and dCTP depletion seen in mitochondria from thymidine-treated cells are consistent with a regulatory role for mitochondrial ribonucleotide reductase.

Of greatest interest, however, is the fact that the pool changes that we observe can account for the mutation spectrum reported by Nishigaki et al. (22) in mitochondrial DNA from MNGIE patients. These authors reported that most mitochondrial point mutations in MNGIE patients involve T → C transitions in sequences containing at least two As to the 5' side of a T residue. Our findings of dTTP and dGTP elevations and dATP depletion in mitochondrial dNTP pools are consistent with a mutagenic mechanism involving competition between dGTP and dATP for incorporation opposite template T, followed by a next-nucleotide effect, in which T from the expanded dTTP pool is incorporated opposite the two A residues on the 5' side of the mispairing.

Detection Mutagenesis Caused by dNTP Imbalance—Mitochondrial gene mutations in MNGIE patients include both point mutations and deletions (21, 22). As noted earlier, there are other disease states in which abnormalities in deoxyribonucleotide metabolism are associated with the generation of deletions in mitochondrial DNA (18, 19). Although large dNTP asymmetries are known to stimulate frameshift mutations in vitro (16), it is not immediately apparent how modest pool asymmetries, of the type seen in this study, could stimulate the formation of long deletions. Nevertheless, we carried out an experiment to determine whether mitochondrial deletions could, in fact, be generated by long term exposure to pool-imbalanced conditions.

To do this we cultured HeLa cells for 8 months in medium supplemented with 50 μM thymidine or without thymidine supplement. We then analyzed mitochondrial DNA from both cultures. Using a LX-PCR method, as shown in Fig. 2, we observed multiple deletion-containing mtDNA molecules ranging in size from 1 to 3.3 kbp in cells grown in thymidine-supplemented medium but not in cells grown for the same period in the same medium but without thymidine supplement.

4 S. Song and C. K. Mathews, unpublished results.
To exclude the possibility that the low molecular weight bands were due to PCR artifacts, a second LX-PCR was performed using nested primers. In all cases analyzed we obtained a pattern consistent with that observed in the first PCR (data not shown).

To further confirm that long term thymidine treatment induces mtDNA deletions, we analyzed mtDNA by Southern blotting with a mtDNA-specific probe. As shown in Fig. 3, we detected a deletion-containing mtDNA molecule about 3 kbp in size in cells grown in the thymidine-treated culture but not in the control culture.

Although these data clearly show a relationship between unbalanced dNTP pools and the creation of mitochondrial DNA deletions, they do not shed light on possible mechanisms. Several models, including a slip-replication model (34) and an illegitimate elongation model (35), have been proposed. Both of these require movement of single-stranded mtDNA and have been described (36) as compatible with the concept that dNTP pool imbalances can induce multiple deletions of mtDNA. In that study Hirano et al. (36) proposed that in the presence of unbalanced nucleotide pools, relatively low levels of one particular dNTP may lead to stalling of mtDNA replication, with the stalled replication intermediate containing a single-stranded region that would be susceptible to migration and to inappropriate annealing, particularly at direct sequence repeats. The data of Fig. 1 show that dCTP after short term thymidine exposure represents only two percent of the total mitochondrial dNTP pool. It seems reasonable to suggest that long term exposure to such conditions could generate deletions as a result of replication fork stalling, as proposed by Hirano et al. (36). A prediction from this model is that long term culturing of cells under conditions that prevent dCTP depletion should not result in the generation of deletions.

The significance of this study is 3-fold. First, by improvement in methods for mitochondrial dNTP extraction and analysis, we have shown that cell culture represents a suitable model for investigating mitochondrial diseases that involve abnormalities in nucleotide metabolism. Second, although it is well known that dNTP asymmetries stimulate mutagenesis, this may be the first study to relate specific pool changes to a mutagenic mechanism that can account for actual mutations seen in affected humans. Third, even though this study does not test specific mechanisms, it strengthens the predicted causal relationship between deoxyribonucleotide pool imbalance and deletion mutagenesis.

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