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Elevated plasma deoxyuridine in patients with thymidine phosphorylase deficiency☆

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Abstract

Mutations in the nuclear gene encoding thymidine phosphorylase (TP) cause mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), an autosomal recessive disease with mitochondrial dysfunction and mitochondrial DNA abnormalities. We have demonstrated alterations of thymidine (dThd) metabolism in MNGIE patients. Here, we report the accumulation of another substrate of TP, deoxyuridine (dUrd), whose circulating levels ranged from 5.5 to 24.4 μM (average 14.2) in MNGIE and were undetectable (<0.05 μM) in both TP mutation carriers and controls. The dramatic accumulation of dUrd may contribute to nucleotide pool imbalances and, together with the increased levels of dThd, is likely to contribute to the pathogenesis of MNGIE.

Keywords: Deoxyuridine; Thymidine phosphorylase; MNGIE; Mitochondria; Nucleoside; Nucleotide; DNA; Depletion

Thymidine phosphorylase (TP) catalyzes the first step in the catabolism of the thymidine (dThd) by converting this nucleoside to thymine and 2′-deoxyribose 1-phosphate [1]. Although the enzyme can catalyze both forward and reverse reactions, only phosphorolysis is important under physiological conditions. Therefore, as a catabolic enzyme, TP plays an important role in nucleoside homeostasis.

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive disorder caused by mutations in the TP gene, which produce loss of function of TP activity in patients with this disease [2,3]. The clinical manifestations of MNGIE are ptosis, ophthalmoparesis, skeletal myopathy, peripheral neuropathy, gastrointestinal dysmotility, and cachexia [4]. Clinical diagnostic tests typically reveal leukoencephalopathy, lactic acidosis, and peripheral neuropathy. Skeletal muscle biopsies show neurogenic changes, together with mitochondrial abnormalities such as ragged-red-fibers, ultrastructurally abnormal mitochondria, and decreased cytochrome *c* oxidase activity, either in isolation or in association with multiple respiratory chain enzyme defects. In addition to these signs of mitochondrial impairment, studies of mitochondrial DNA (mtDNA) from skeletal muscle have shown depletion and multiple deletions [5,6], and recently we have demonstrated the presence of site-specific somatic mtDNA point mutations in tissues and cultured cells from MNGIE patients (Nishigaki et al., submitted).

We have reported that dThd metabolism is severely altered in MNGIE patients [3]. Given the clear abnormalities of mitochondrial function in these patients and, specifically, defects of mtDNA, we hypothesized that excessive accumulation dThd can produce imbalances of mitochondrial deoxynucleoside triphosphate (dNTP) pools, compromising the normal replication of mtDNA [3,4]. Here, we report another important alteration in nucleoside metabolism in MNGIE, the accumulation of high levels of circulating deoxyuridine (dUrd). This novel result provides further insight into the mechanisms linking nucleoside alterations to the specific mtDNA alterations.

☆ *Abbreviations:* dNTP, deoxynucleoside triphosphate; dThd, thymidine; dUrd, deoxyuridine; MNGIE, mitochondrial neurogastrointestinal encephalomyopathy; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; Pol γ, polymerase γ; TS, thymidylate synthase; TP, thymidine phosphorylase; UP, uridine phophorylase.

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Experimental procedures

Subjects. We studied 25 patients with MNGIE, 14 asymptomatic carriers, and 20 normal controls. All patients fulfilled the clinical criteria for MNGIE: (1) severe gastrointestinal dysmotility; (2) cachexia; (3) ptosis, ophthalmoparesis, or both; (4) peripheral neuro-pathy; (5) leukodystrophy on brain MRI scan; and (6) laboratory evidence of mitochondrial dysfunction [4]. As previously reported, we identified homozygous or compound heterozygous mutations in the TP gene in all 25 probands and heterozygous mutations in 14 relatives [2,4].

Biochemical methods. Plasma dThd and dUrd were measured using a previously described HPLC method [7,8] with modifications. Anticoagulated blood samples were centrifuged and plasma was removed and processed for HPLC analysis. Plasma was treated with perchloric acid (0.5 M, final concentration) and centrifuged to remove the precipitated protein. Fifty μl of the supernatant was injected into an Alliance HPLC apparatus from Waters Corporation (Milford, Massachussets), using an Alltima C18 NUC 100 Å, 5 μm, 250 × 4.6 mm, Alltech reverse phase column preceded by a guard column (Alltima C18 5 μM, 7.5 × 4.6 mm, Alltech, Deerfield, IL). Injected samples were eluted over 110 min through a methanol-gradient buffered mobile phase. Eluent A: potassium phosphate 20 mM, pH 5.6. Eluent B: potassium phosphate 20 mM, 60% methanol, pH 5.6. The elution was performed with a constant flow rate of 1.5 ml/min as follows: 0–5 min, 100% eluent A; 5–92 min, 100–0% eluent A; 92–93 min, 0–100% eluent A; and 93–110 min, constant 100% eluent A. The absorption of the eluate was measured at 267 nm. Quantitation of dThd and dUrd was based on external standards. Identification of peaks was based upon retention time and always confirmed by treatment of a second aliquot of each sample with a large excess of purified thymidine phosphorylase (Sigma–Aldrich, Milwaukee, Wisconsin) to eliminate dThd and dUrd.

Results and discussion

MNGIE is one of the expanding number of diseases that affect intergenomic communication. In these disorders, defects in nuclear DNA (nDNA) impair normal mtDNA replication, repair or both. In MNGIE, mutations in TP gene cause loss of function of the enzyme, leading to a generalized disturbance on dThd metabolism [3]. Here, we report the systemic accumulation of another nucleoside, dUrd. Fig. 1 shows two representative chromatograms obtained from plasma of a MNGIE patient and a healthy control. Levels of both dThd and dUrd are undetectable in the controls ($n = 20$) and heterozygotes ($n = 14$), i.e., concentrations below 0.05 μM. As we have reported [3], it is important to confirm the identity of the peaks by treating each sample with purified TP. UV-absorbing substances that coelute with dThd and dUrd produce small peaks which can be easily misidentified as dUrd and dThd in plasma (see Fig. 1, bottom). Similar to our previous results [3], we found increased levels of dThd in MNGIE patients (8.6 ± 3.4; mean ± SD, range 3.9–17.7, $n = 25$). In addition, dUrd was present in the plasma of all patients analyzed (14.2 ± 4.4, range 5.5–24.4).

Interestingly, concentrations of dUrd in the blood of MNGIE patients are significantly higher than the concentrations of dThd. Fig. 2 illustrates the correlation observed between circulating dThd and dUrd in all patients studied. In all cases, dUrd was clearly higher than dThd. Despite moderate dispersion, the data indicate clearly that the differences between dUrd and dThd are constant rather than proportional. This observation is reinforced by the fact that the regression analysis of dUrd (dependent variable) vs dThd (independent) gives an equation with a statistically significant constant of 5.1 μM ($p = 0.001$), with a slope of 1.05 ($p < 0.001$), very close to 1.

Studies on substrate specificity of TP from humans, as well as from other species, demonstrate that dThd

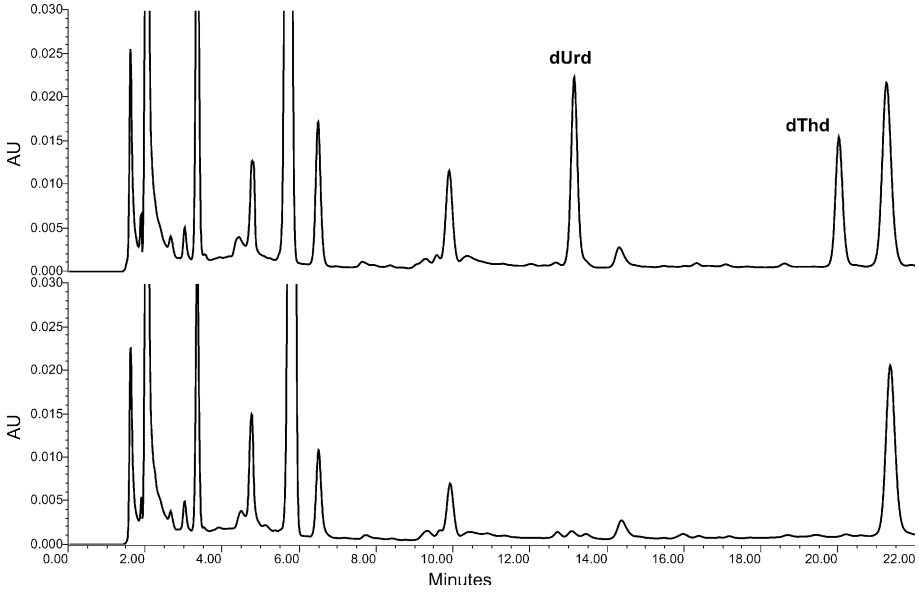

Fig. 1. Representative chromatograms of a MNGIE patient (top) and a control (bottom).

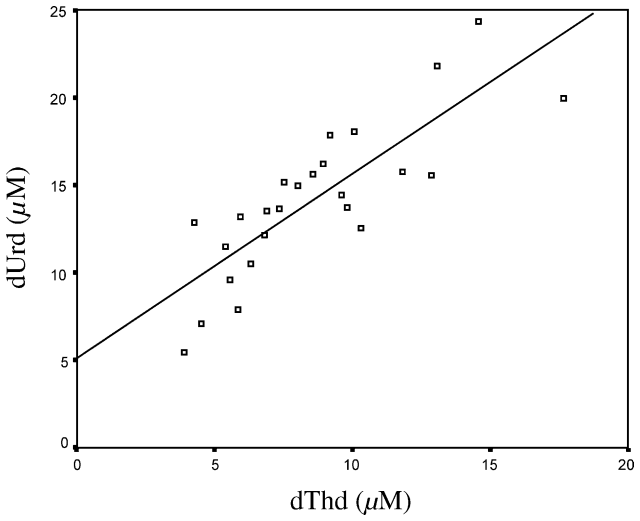

Fig. 2. Correlation between circulating dThd and dUrd in MNGIE patients.

and dUrd are the preferred substrates for the enzyme, although TP also catalyzes less efficiently the phosphorolysis of other natural and synthetic (deoxy)ribonucleosides [9–11]. Uridine phosphorylase (UP), the other enzyme with phosphorolytic activity against pyrimidine nucleosides, does not appear to participate significantly in the catabolism of dUrd [10]. Low efficiency of UP compared to TP for uracil deoxynucleoside in humans and mice strongly suggests that, at least in mammals, dUrd is mainly catabolized by TP [10]. Our results support this concept. In MNGIE, the absence of TP activity causes accumulation of both dThd and dUrd, leading to levels of dUrd in blood that are even higher than the corresponding levels of dThd. Since the physiological levels of both dThd and dUrd in plasma are below the detection limit of our method (~0.05 μM), we cannot identify which nucleoside is more elevated relative to baseline levels, but in all cases the increases are more than 70-fold for dThd and more than 100-fold for dUrd.

dThd and dUrd are precursors of their triphosphates, dTTP and dUTP. In the synthesis of DNA, polymerases cannot distinguish between both deoxynucleoside triphosphates [12], therefore, both dTTP and dUTP can be incorporated into the nascent strand, opposite to an adenine in the template strand, with the same efficiency. Cells avoid significant incorporation of uracil into the DNA by maintaining a high ratio dTTP/dUTP, so that there is little dUTP available to compete with dTTP. Under physiological conditions with normal dTTP/dUTP ratio ($>10^5$), uracil is undetectable in DNA [13,14]. Uracil transiently present in DNA (either through uracil incorporation from dUTP or by deamination of cytosine present in the DNA) is recognized by specific enzymes and corrected by base excision repair mechanisms [12].

Accumulation of dUrd in MNGIE patients can have different effects on the dNTP pools in cytosol and mitochondria. Several studies seem to demonstrate that mitochondrial dNTP originates from the nucleotide

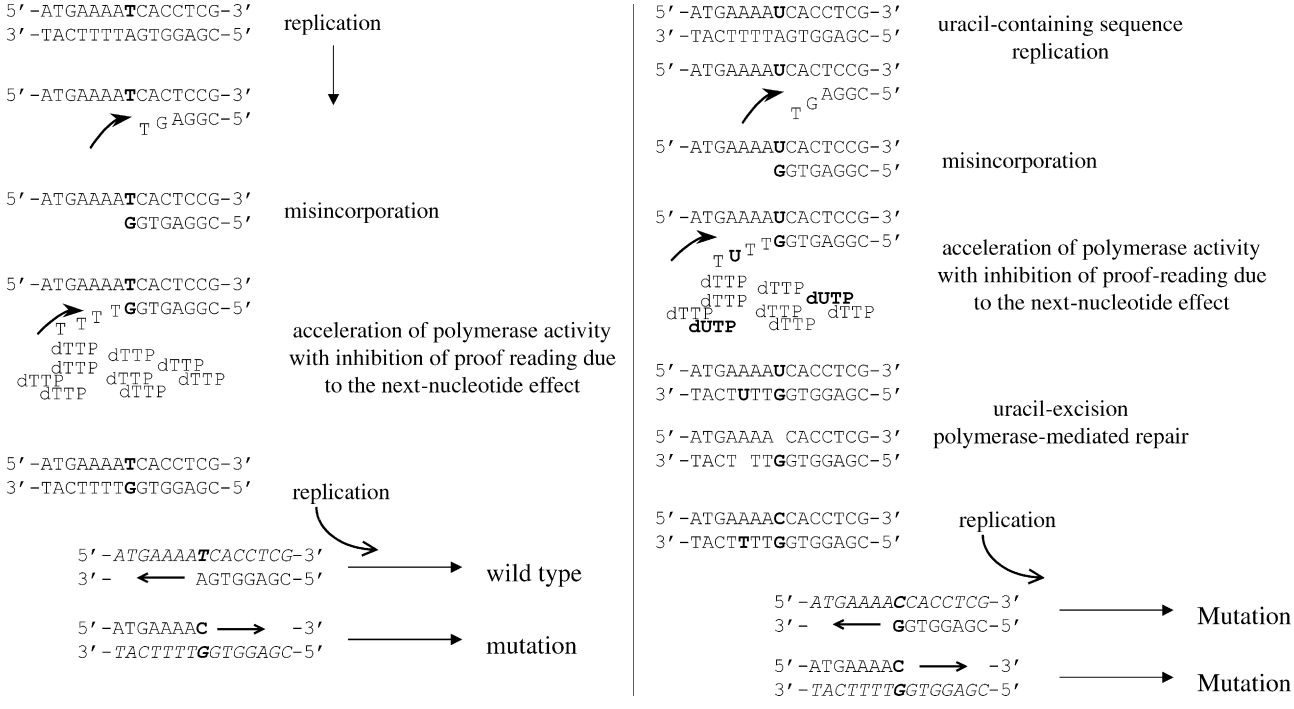

Fig. 3. Hypothetical mechanism for the involvement of uracil in the expansion of the mtDNA point mutations. The representative mutation in this figure is T5814C (Nishigaki et al., submitted).

salvage pathway more than from de novo synthesis [15,16]. In fact, there is a consensus that mitochondria lack de novo enzymatic machinery, although there is biochemical evidence of the de novo pathway enzyme ribonucleotide reductase activity in mitochondria [17]. dUrd accumulated in the cytosol can be phosphorylated by the cytosolic thymidine kinase, TK1, and the resultant dUMP can be metabolized in two ways: further phosphorylated to dUDP and dUTP, which, in turn, can be transformed back into dUMP by the nuclear dUTPase [18], or transformed into dTMP, via the de novo synthesis enzyme thymidylate synthase (TS). Consequently, accumulation of dUTP in the cytosol is unlikely. The absence of TS in mitochondria obligates mitochondrial dUrd to be fully phosphorylated (to dUTP). The existence of a mitochondrial dUTPase [18] prevents the accumulation of dUTP, but only by cycling it back to dUMP, hence large increases of dUrd in mitochondria ultimately could elevate the concentration of dUTP and decrease the dTTP/dUTP ratio, thereby favoring the incorporation of uracil into mtDNA. As reported, excess of uracil in the DNA has negative consequences for the cell, most likely due to the cumulative effects of base excision repair initiated by uracil-DNA glycosylase, which can compromise the stability of the DNA [19]. In addition, uracil incorporation has been reported to be the mechanism responsible for thymineless death produced by TS inhibitors that decrease the cytosolic dTTP/dUTP ratio [14,19,20].

The possible decrease of dTTP/dUTP in mitochondria might also account for the somatic specific point mutations observed in MNGIE (Nishigaki et al., submitted). The surprising abundance of T-to-C mutations when T is preceded by several A residues has been explained by misincorporation of one G opposite a template T base, followed by the inhibition of the 3′-exonuclease activity of polymerase γ (pol γ) due to the high concentration of dTTP which accelerates the incorporation of T opposite to the poly-A (next-nucleotide effect). In addition, elevated levels of dTMP may also inhibit the exonuclease activity of pol γ [21]. As illustrated in Fig. 3 (left), copies of the mutated strand replicate the base change, but the original AAAAT strand remains unmutated, and will produce daughter strands with the wild-type sequence (Nishigaki et al., submitted). The presence of an uracil residue in the template strand (AAAAU) doubles the rate of replication of the mutation, generated by original misincorporated G opposite U. Once the G:U mispair has been produced, base excision repair of U introduces the mutation in the original template strand (by inserting a C opposite to the G, see Fig. 3, right side).

The identification of dramatically increased levels of circulating dUrd in MNGIE has provided new insights into the disease pathogenesis. This nucleoside is likely to be an important factor contributing to nucleotide pool imbalances. In addition, the unique metabolism of dUrd and its derivatives may accelerate the accumulation of mtDNA point mutations.

Acknowledgments

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References

[1] N.S. Brown, R. Bicknell, Thymidine phosphorylase, 2-deoxy-D-ribose and angiogenesis, Biochem. J. 334 (1998) 1–8.
[2] I. Nishino, A. Spinazzola, M. Hirano, Thymidine phosphorylase gene mutations in MNGIE, a human mitochondrial disorder, Science 283 (1999) 689–692.
[3] A. Spinazzola, R. Marti, I. Nishino, A. Andreu, A. Naini, S. Tadesse, I. Pela, E. Zammarchi, M.A. Donati, J.A. Oliver, M. Hirano, Altered thymidine metabolism due to defects of thymidine phosphorylase, J. Biol. Chem. 277 (2002) 4128–4133.
[4] I. Nishino, A. Spinazzola, A. Papadimitriou, S. Hammans, I. Steiner, C.D. Hahn, A.M. Connolly, A. Verloes, J. Guimarães, I. Maillard, H. Hamano, M.A. Donati, C.E. Semrad, J.A. Russell, A.L. Andreu, G.M. Hadjigeorgiou, T.H. Vu, S. Tadesse, T.G. Nygaard, I. Nonaka, I. Hirano, E. Bonilla, L.P. Rowland, S. DiMauro, M. Hirano, MNGIE: an autosomal recessive disorder due to thymidine phosphorylase mutations, Ann. Neurol. 47 (2000) 792–800.
[5] M. Hirano, G. Silvestri, D.M. Blake, A. Lombes, C. Minetti, E. Bonilla, A.P. Hays, R.E. Lovelace, I. Butler, T.E. Bertorini, A.B. Threlkeld, H. Mitsumoto, L. Salberg, L.P. Rowland, S. DiMauro, Mitochondrial neurogastrointestinal encephalomyopathy MNGIE: clinical, biochemical, and genetic features of an autosomal recessive mitochondrial disorder, Neurology 44 (1994) 721–727.
[6] A. Papadimitriou, G.P. Comi, G.M. Hadjigeorgiou, A. Bordoni, M. Sciacco, L. Napoli, A. Prelle, M. Moggio, G. Fagiolari, N. Bresolin, S. Salani, I. Anastasopoulos, G. Giassakis, R. Divari, G. Scarlato, Partial depletion and multiple deletions of muscle mtDNA in familial MNGIE syndrome, Neurology 51 (1998) 1086–1092.
[7] R.A. Hartwick, S.P. Assenza, P.R. Brown, Identification and quantitation of nucleosides, bases and other UV-absorbing compounds in serum, using reversed-phase high-performance liquid chromatography. I. Chromatographic methodology, J. Chromatogr. 186 (1979) 647–658.
[8] R.A. Hartwick, A.M. Krstulovic, P.R. Brown, Identification and quantitation of nucleosides, bases and other UV-absorbing compounds in serum, using reversed-phase high-performance liquid chromatography. II. Evaluation of human sera, J. Chromatogr. 186 (1979) 659–676.
[9] C. Desgranges, G. Razaka, M. Rabaud, H. Bricaud, Catabolism of thymidine in human blood platelets: purification and properties of thymidine phosphorylase, Biochim. Biophys. Acta 654 (1981) 211–218.
[10] M.H. el Kouni, M.M. el Kouni, F.N. Naguib, Differences in activities and substrate specificity of human and murine pyrimidine nucleoside phosphorylases: implications for chemotherapy with 5-fluoropyrimidines, Cancer Res. 53 (1993) 3687–3693.
[11] A. Kono, Y. Hara, S. Sugata, Y. Matsushima, T. Ueda, Substrate specificity of a thymidine phosphorylase in human liver tumor, Chem. Pharm. Bull. (Tokyo) 32 (1984) 1919–1921.

[12] R.G. Richards, L.C. Sowers, J. Laszlo, W.D. Sedwick, The occurrence and consequences of deoxyuridine in DNA, Adv. Enzyme Regul. 22 (1984) 157–185.
[13] M. Goulian, B. Bleile, B.Y. Tseng, The effect of methotrexate on levels of dUTP in animal cells, J. Biol. Chem. 255 (1980) 10630–10637.
[14] M. Goulian, B. Bleile, B.Y. Tseng, Methotrexate-induced misincorporation of uracil into DNA, Proc. Natl. Acad. Sci. USA 77 (1980) 1956–1960.
[15] R.K. Bestwick, C.K. Mathews, Unusual compartment of precursors for nuclear and mitochondrial DNA in mouse L cells, J. Biol. Chem. 257 (1982) 9305–9308.
[16] R.K. Bestwick, G.L. Moffett, C.K. Mathews, Selective expansion of mitochondrial nucleoside triphosphate pools in antimetabolite-treated HeLa cells, J. Biol. Chem. 257 (1982) 9300–9304.
[17] P. Young, J.M. Leeds, M.B. Slabaugh, C.K. Mathews, Ribonucleotide reductase: evidence for specific association with HeLa cell mitochondria, Biochem. Biophys. Res. Commun. 203 (1994) 46–52.
[18] R.D. Ladner, S.J. Caradonna, The human dUTPase gene encodes both nuclear and mitochondrial isoforms. Differential expression of the isoforms and characterization of a cDNA encoding the mitochondrial species, J. Biol. Chem. 272 (1997) 19072–19080.
[19] S. Melnyk, M. Pogribna, B.J. Miller, A.G. Basnakian, I.P. Pogribny, S.J. James, Uracil misincorporation, DNA strand breaks, and gene amplification are associated with tumorigenic cell transformation in folate deficient/repleted Chinese hamster ovary cells, Cancer Lett. 146 (1999) 35–44.
[20] W.D. Sedwick, M. Kutler, O.E. Brown, Antifolate-induced misincorporation of deoxyuridine monophosphate into DNA: inhibition of high molecular weight DNA synthesis in human lymphoblastoid cells, Proc. Natl. Acad. Sci. USA 78 (1981) 917–921.
[21] S.E. Lim, W.C. Copeland, Differential incorporation and removal of antiviral deoxynucleotides by human DNA polymerase γ, J. Biol. Chem. 276 (2001) 23616–23623.