THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 6, Issue of February 8, pp. 4128–4133, 2002

Printed in U.S.A.

Altered Thymidine Metabolism Due to Defects of Thymidine Phosphorylase*

Received for publication, November 16, 2001
Published, JBC Papers in Press, December 3, 2001, DOI 10.1074/jbc.M111028200

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Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is an autosomal recessive human disease due to mutations in the thymidine phosphorylase (TP) gene. TP enzyme catalyzes the reversible phosphorolysis of thymidine to thymine and 2-deoxy-D-ribose 1-phosphate. We present evidence that thymidine metabolism is altered in MNGIE. TP activities in buffy coats were reduced drastically in all 27 MNGIE patients compared with 19 controls. All MNGIE patients had much higher plasma levels of thymidine than normal individuals and asymptomatic TP mutation carriers. In two patients, the renal clearance of thymidine was ~20% that of creatinine, and because hemodialysis demonstrated that thymidine is ultrafiltratable, most of the filtered thymidine is likely to be reabsorbed by the kidney. *In vitro*, fibroblasts from controls catabolized thymidine in medium; by contrast, MNGIE fibroblasts released thymidine. In MNGIE, severe impairment of TP enzyme activity leads to increased plasma thymidine. In patients who are suspected of having MNGIE, determination of TP activity in buffy coats and thymidine levels in plasma are diagnostic. We hypothesize that excess thymidine alters mitochondrial nucleoside and nucleotide pools leading to impaired mitochondrial DNA replication, repair, or both. Therapies to reduce thymidine levels may be beneficial to MNGIE patients.

Mitochondrial encephalomyopathies encompass a diverse and expanding group of human disorders due to defects of the mitochondrial respiratory chain (1). Because subunits of the electron transport chain are the products of two genomes, nDNA and mtDNA, mitochondrial encephalomyopathies can be caused by defects in either genome or in cross-talk between the genomes that regulate mtDNA integrity and quantity. For more than a decade, investigators have focused primarily upon mtDNA and have identified more than 100 distinct point mutations as well as large scale molecular rearrangement (2). Since 1995, a growing number of nDNA mutations have been identified as causes of respiratory chain monoenzymopathies (3, 4). By contrast, mutations in nDNA causing defects of intergenomic signals, mtDNA depletion or multiple deletions, are just beginning to be defined.

Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE)[1] is an autosomal recessive disease associated with multiple deletions and depletion of mtDNA in skeletal muscle (5, 6). Clinically, MNGIE presents between the 1st and the 5th decades with ptosis, ophthalmoparesis, or both; skeletal myopathy; peripheral neuropathy; gastrointestinal dysmotility, manifesting as diarrhea and pseudo-obstruction; and cachexia (7). Clinical diagnostic tests typically reveal leukodystrophy on brain magnetic resonance imaging scans, lactic acidosis, and peripheral neuropathy with demyelination, axonal degeneration, or both. 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.

We identified mutations in the thymidine phosphorylase (TP) gene, located on chromosome 22q13.32-qter, as the cause of MNGIE (8, 9). Normally, TP catabolizes thymidine to thymine and 2-deoxy-D-ribose 1-phosphate (10). In MNGIE patients, mutations in TP cause severe reductions of the enzyme activity (9). We report enzymatic TP activity and plasma thymidine levels from MNGIE patients and urine thymidine excretion in two patients studied at our center.

EXPERIMENTAL PROCEDURES

Subjects—We studied 27 probands with MNGIE, 29 asymptomatic relatives, and 23 normal controls. The clinical diagnosis of MNGIE required the presence of six features as follows: 1) severe gastrointestinal dysmotility; 2) cachexia; 3) ptosis, ophthalmoparesis, or both; 4) peripheral neuropathy; 5) leukodystrophy on brain magnetic resonance imaging scan; and 6) laboratory evidence of mitochondrial dysfunction. As reported previously, we identified homozygous or compound heterozygous mutations in the TP gene in all 27 probands and heterozygous mutations in 22 relatives (7, 9). Samples from three patients with diagnosis of autosomal dominant progressive external ophthalmoplegia, another disorder associated with multiple deletions of mtDNA, were also studied.

Two MNGIE patients underwent hemodialysis. Patient A underwent a single hemodialysis, and patient B underwent three consecutive weekly treatments. For each hemodialysis treatment, blood samples were collected immediately before the dialysis, pre-filtered and post-

* This work was supported by National Institutes of Health Grant HD37529 and a grant from the Muscular Dystrophy Association. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: MNGIE, mitochondrial neurogastrointestinal encephalomyopathy; TP, thymidine phosphorylase; dTTP, thymidine triphosphate; dNTP, deoxynucleoside triphosphate; HPLC, high pressure liquid chromatography.

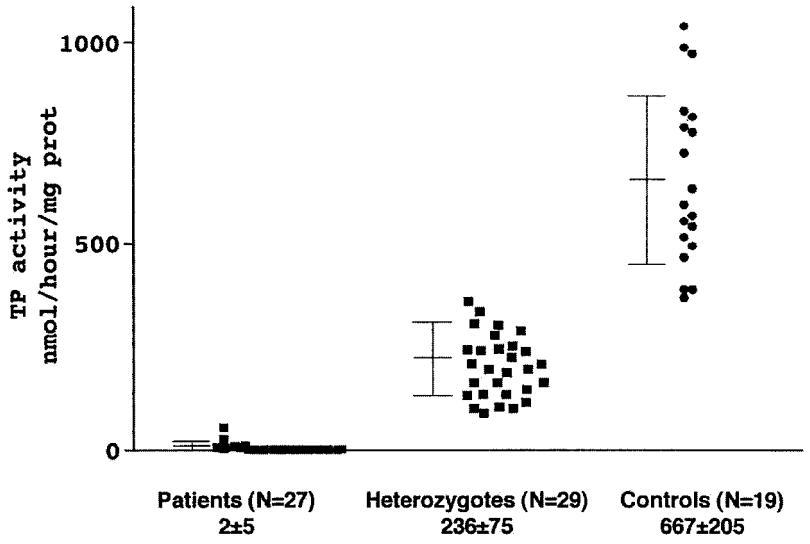

FIG. 1. **TP activity in buffy coats.** *Lines* represent average TP activity expressed as nanomoles of thymine produced over 1 h per mg of protein (*middle horizontal line*) and 1 S.D. above and 1 S.D. below the average. Numbers under each group are average ± S.D. (nmol/h/mg protein).

filtered blood 15 min after initiation of the treatment, immediately after completion, and 3 h, 19 h, 1, 2, and 3 days after dialysis. We measured thymidine concentration in these samples. The study was performed under a protocol approved by the Columbia University Institutional Review Board.

Biochemical Methods—The rate of conversion of thymidine to thymine was measured spectrophotometrically using modifications of methods described previously (11–13). Buffy coats were homogenized in lysis buffer (50 mM Tris-HCl, pH 7.2, containing 1% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and 0.02% 2-mercaptoethanol) and subjected to brief sonication. Samples were centrifuged at 20,000 × *g* for 30 min at 4 °C, and the supernatants were used for the enzyme assay. The protein content was determined through the bicinchoninic acid method (14). From each supernatant, 100–250 μg of protein was incubated with 0.1 M Tris arsenate buffer, pH 6.5, and 10 mM thymidine in a total volume of 0.1 ml. After incubation for 1 h at 37 °C. The reaction was terminated by addition of 1 ml of 0.3 M NaOH. Absorbance at 300 nm was measured, and the amount of thymine formed was determined using the 3.4×10^3 liter/mol/cm difference in the molar extinction coefficient between thymidine and thymine at alkaline pH. Enzyme activity was expressed as the amount of thymine (nanomoles) generated per h per mg of protein. Results were obtained from two independent experiments performed in duplicate.

Plasma, cell culture medium, and urine thymidine concentration was measured by an isocratic HPLC method. Anticoagulated blood samples were centrifuged and plasma removed and processed for HPLC analysis. In two patients, urine samples were collected for 24 h. Aliquots of urine were used to measure thymidine and creatinine levels. Simultaneous serum creatinine and plasma thymidine levels were measured. Urine samples were diluted 1:20 in phosphate-buffered saline prior to thymidine measurement. Plasma, cell culture medium, and diluted urine were treated with perchloric acid (0.5 M, final concentration) and centrifuged to remove the precipitated protein. Fifty μl of the supernatant were injected in an Alliance HPLC apparatus from Waters Associates, using an Alltima C18 NUC 100-Å, 5-μm, 250 × 4.6-mm, Alltech reverse phase column from Alltech, (Deerfield, IL). Injected samples were eluted with 50 mM potassium phosphate buffer, pH 4.0, containing 10% methanol at a flow rate of 1.5 ml/min. The absorption of the eluted materials was measured at 267 nm. Quantitation of thymidine was based on external standards. Definitive identification of the thymidine peak was based upon retention time and by treatment of a 2nd aliquot of the sample with a large excess of purified thymidine phosphorylase (from Sigma) to eliminate the thymidine.

Cell Culture Study—We analyzed the thymidine contents of cell culture medium over 10 days of culture of fibroblasts from three MNGIE patients and three controls. We plated 10^5 fibroblasts from every patient or control in 10 35 × 10-mm Petri dishes (day 0) with 3 ml of minimum Eagle's medium from Invitrogen, plus 15% fetal bovine serum, 1 mM pyruvate, 100 μM non-essential amino acids, 2 mM L-glutamine, and 1× minimum Eagle's medium vitamin solution from Invitrogen. We grew the cells at 37 °C in 5% CO_2. We trypsinized three plates of each cell line on the 2nd day, two plates on the 4th, 6th, and 8th day, and one plate on the 10th day, and we counted the number of cells in every plate. Prior to trypsinization, we saved 1 aliquot of medium from all of the plates, as well as from one plate of medium without cells as a blank to assess thymidine concentration by HPLC.

Statistical Methods—Statistical analysis of the data was performed with SPSS 10.0 software. Distributions of values were tested for normality, and we used Student's *t* test to find differences between groups of normal distributions. We used nonparametric tests for non-normal distributions or when $n \leq 10$.

RESULTS

TP activities in buffy coats were severely reduced in all 27 patients (2 ± 5 nmol/h/mg) compared with 19 controls (667 ± 205 nmol/h/mg) ($p < 0.001$) (Fig. 1). Enzymatic activities in all patients were <5% of the normal control mean, and they were partially reduced in heterozygotes (236 ± 75 nmol/h/mg) compared with those of the controls ($p < 0.001$). The difference between patients and heterozygotes was also statistically significant ($p < 0.001$). There was no relationship between TP mutations and severity of the enzymatic defect or between enzymatic activity and clinical phenotype.

Thymidine levels were dramatically increased in plasma from MNGIE patients (8.68 ± 5.23 μM) (range 3.5–24.7; $n = 16$), compared with normal controls or heterozygous TP mutation carriers, who did not show detectable levels of thymidine ($n = 23$ and 14, respectively). The detection limit of our method (0.05 μM) was established by repeating measurements ($n = 10$) of control plasma samples with decreasing concentrations of thymidine. We defined the detection limit as the lowest concentration that we were able to determine with a coefficient of variation (standard deviation divided by average and expressed as percentage) ≤20% (data not shown).

Table I shows plasma and urinary concentrations of thymidine in two patients and control subjects. Thymidine was undetectable in the urine of the controls, whereas it was high in the two patients. Both patients showed normal renal clearances of creatinine, whereas their clearances of thymidine were ~20% that of creatinine.

Hemodialysis reduced circulating concentrations of thymidine in two patients; patient A underwent one dialysis, and patient B underwent three consecutive weekly dialysis treatments. Fig. 2 shows the concentrations of plasma thymidine before, during, and after dialysis of both patients. The reduction in the plasma level of the nucleoside immediately after dialysis was about 50%, indicating that thymidine is ultrafiltratable. However, 3 h after dialysis, levels of the nucleoside had returned to pretreatment values.

Over 10 days of culture, control fibroblasts gradually reduced thymidine concentration in the medium; by contrast, MNGIE

TABLE I
Urinary excretion of thymidine in MNGIE

The abbreviations used are: Thd, thymidine; Creat, creatinine; Pl thd, concentration of thymidine in plasma; U thd, concentration of thymidine in urine; Cl, clearance.

	n	Pl thd	U thd	U thd	Thd renal Cl
		μM		*μmol Thd/mg Creat*	*% Creat Cl*
MNGIE (I)	1	4.8	96	0.18	18.9
MNGIE (II)	1	10.3	412	0.49	19.1
Controls	6	<0.05	<1		

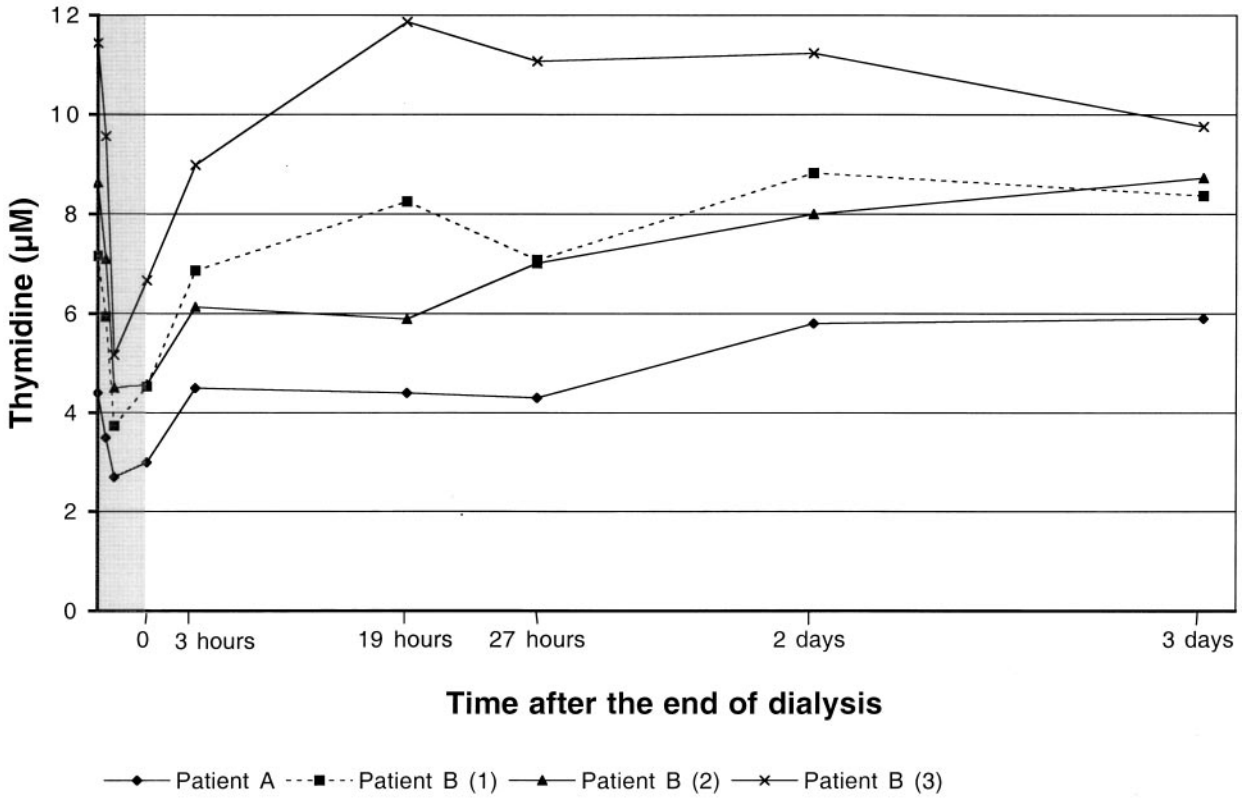

FIG. 2. **Effects of dialysis on circulating thymidine.** *Shaded area* represents period of dialysis. *y* axis is plasma thymidine concentration. *x* axis is time relative to completion of dialysis.

fibroblasts from all patients tested released thymidine (Fig. 3). We determined the linear regression of thymidine concentration in the medium over the time of culture of fibroblasts from patients and controls (Table II). The growth curves of the fibroblasts varied considerably. Fibroblasts from two MNGIE patients grew poorly compared with the controls; however, growth of fibroblasts from the third patient was comparable with that of the controls (Fig. 4). There was no correlation between number of the cells and thymidine concentration in the culture medium of the MNGIE fibroblasts; in fact, we found a significant negative correlation between these variables in the control group (-0.714; $p = 0.001$; $n = 18$), which disappeared when we adjusted the analysis by the time of the culture.

DISCUSSION

The identification of loss-of-function TP gene mutations has enabled us to study the pathogenesis of MNGIE. The functional significance of TP protein has been well characterized *in vitro* (10). TP is a homodimer with an important role in the homeostasis of cellular nucleotide pools. Normally, TP protein catalyzes the reversible phosphorolysis of thymidine to thymine and 2-deoxy-D-ribose 1-phosphate. The forward reaction, conversion of thymidine to thymine, is important in the catabolism of the nucleoside, whereas the reverse reaction may be important for the pyrimidine salvage pathway, which allows thymidine to be converted to thymidine triphosphate (TTP), an essential building block of DNA.

TP activity is drastically reduced in MNGIE patients compared with the controls, whereas the activities of the carriers are ~35% of the normal value. This result is consistent with the fact that TP is a homodimer (15, 16), as we would expect 25% of functional enzyme when only 50% of monomers is normal. The most direct consequence of this loss of activity seems to be the accumulation of thymidine. Plasma concentrations of thymidine are elevated more than 60-fold in MNGIE patients compared with normal controls and asymptomatic carriers of heterozygous TP mutations. Therefore, only complete or near-total reduction of TP activity has detectable consequences on thymidine metabolism, because partially reduced activities of TP in carriers are sufficient to maintain low levels of circulating thymidine.

We could not detect thymidine in plasma of normal controls or carriers, indicating that the concentration is less than 0.05 μM. This result contrasts with the higher concentrations previously reported (17, 18) ranging from <0.04 to 0.6 and 0.1 to 1.16 μM thymidine in normal human serum, respectively. Dudman *et al.* (17) obtained their results using an HPLC method with UV detection. We verified the specificity of the thymidine peak by treating each sample with purified thymidine phos-

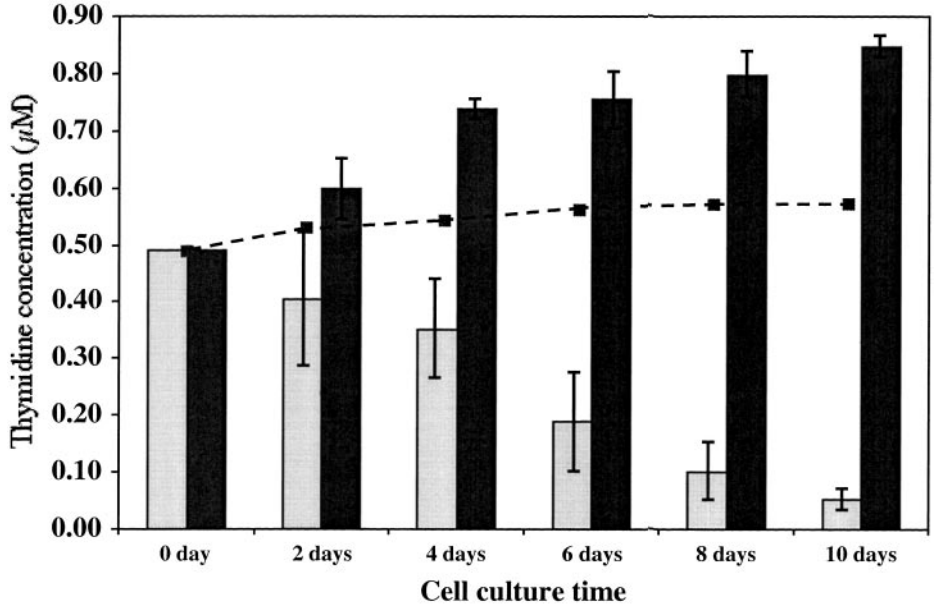

FIG. 3. **Thymidine in fibroblast culture medium.** *Bars* represent averages ($n = 3$). *Lines* represent 1 S.D. above and 1 S.D. below the average. *Dark bars*, MNGIE patients. *Gray bars*, controls. *Dashed line* represents levels of thymidine in culture medium without cells.

TABLE II
Linear regression of thymidine concentration in fibroblasts culture medium versus time
Values for Pearson's correlation coefficient are listed under *r*.

	Slope	*r*	*p*	*n*
	(μmol/l/day) × 10²			
Patient 1	3.7	0.959	0.003	6
Patient 2	3.2	0.946	0.004	6
Patient 3	3.6	0.901	0.037	6
Control 1	−4.8	−0.932	0.007	6
Control 2	−4.5	−0.976	0.001	5
Control 3	−4.9	−0.979	0.001	6

phorylase and comparing the result to those from untreated samples, in order to identify peaks, co-eluting at the same retention time as thymidine, that could account for falsely higher concentrations. Holden *et al.* (18) used a radioimmunoassay; therefore, nonspecific signals could also account for their overestimation of circulating thymidine concentrations.

In vitro studies of fibroblasts provides further evidence of altered thymidine metabolism in MNGIE patients. Normal fibroblasts gradually catabolize thymidine in the culture medium. By contrast, not only are MNGIE fibroblasts unable to degrade thymidine that is initially present in the medium, they even release thymidine. We did not identify a correlation between the number of MNGIE fibroblasts in culture and thymidine levels in the culture medium. These variables, cell number and thymidine level, seemed to show a clear negative correlation in the control group, which was actually due to the effect of the covariate, time, because the correlation disappeared when we adjusted for the time of culture. The lack of correlation between cell number and thymidine levels in the control group prevented us from identifying any quantitative relationship derived from the fact that more cells contains more TP. Fibroblasts from MNGIE patients did not show a homogeneous growing behavior; two cell lines of patients grew very poorly, but the third one showed a growing curve comparable with those of the control fibroblasts. The observation that one MNGIE cell line grew well indicates that loss of TP function and levels of thymidine, ≤ 0.90 μM, do not interfere with fibroblast growth in culture.

Urinary excretion of thymidine is not completely effective in MNGIE patients. Normal controls do not eliminate thymidine through urine because they have absent or very low circulating concentrations of this nucleoside. By contrast, two analyzed MNGIE patients excreted high concentrations of thymidine in urine (96 and 412 μM), but the renal clearances of this nucleoside were very low compared with their clearance of creatinine. Plasma thymidine was reduced by hemodialysis in the patients, similarly to freely dialyzable molecules. However, after 3 h, the levels of the nucleoside returned to pre-dialysis values. Because thymidine is ultrafiltratable, the low renal clearance, as compared with that of creatinine, indicates that most of the filtered nucleoside is reabsorbed. A Na^+/thymidine transporter is present in the brush border of the proximal tubule, likely responsible for the reabsorption (19). Interestingly, a previous study (20) reported low renal clearance of thymidine in humans when this compound was infused intravenously to induce steady-state blood concentrations around 1.5 μM. By contrast, other papers have reported high renal clearances when thymidine was infused to elevate steady-state blood levels to millimolar concentrations in humans (21) and in monkeys (22). MNGIE patients have increased blood thymidine in the micromolar range, but renal clearance for this nucleoside is low. Millimolar concentrations of thymidine probably saturate the reabsorption mechanism, accounting for the higher clearances.

Our results indicate that determination of TP activity in buffy coats and circulating thymidine are powerful diagnostic tools to identify MNGIE patients. We are currently performing these tests in our laboratory and look for TP gene mutations only in those patients whose thymidine metabolism is demonstrated to be disturbed by the biochemical assays.

The facts that MNGIE patients lack TP activity and accumulate thymidine, together with the previous observations of mtDNA alterations and mitochondrial dysfunction-like symptoms, suggest that high levels of thymidine causes mitochondrial DNA impairment. In MNGIE patients, alterations of the thymidine salvage pathway seem to affect deoxynucleoside triphosphate (dNTP) pools more severely in mitochondria than in nuclei. One possible reason for this selective effect is that mitochondria are thought to have separate and independently regulated dNTP pools (23–25). Bogenhagen and Clayton (26) showed that mitochondrial DNA replication in mouse L cells is

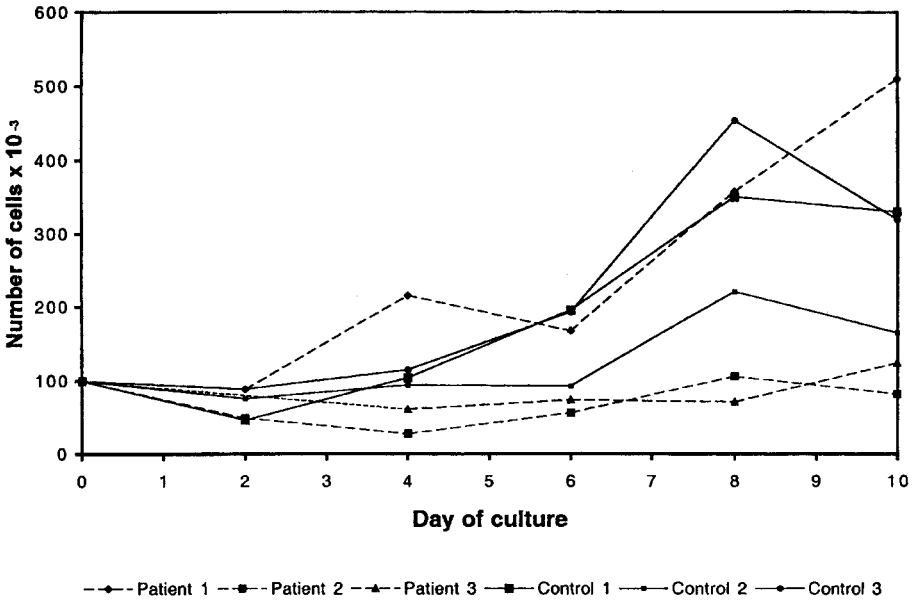

FIG. 4. **Growth curve of MNGIE fibroblasts.** *x* axis is day of culture. *y* axis is number of cells $\times 10^{-3}$. 1×10^5 cells were plated in 35 $\times$ 10-mm dishes and grown in a standard culture medium (see "Experimental Procedures") at 37 °C in 5% CO_2.

resistant to inhibition by methotrexate or 5-flurodeoxyuridine; both agents block *de novo* thymidine synthesis by inhibiting thymidylate synthetase that converts dUMP to thymidylate (deoxythymidine monophosphate or dTMP). Subsequently, Bestwick and colleagues (23, 24) demonstrated an expansion of the mitochondrial nucleoside triphosphate pools of the cells in response to those anti-metabolites. These results indicate that mitochondria depend more on the thymidine salvage pathway than on the *de novo* synthetic pathway. Because of this dependence on thymidine salvage pathway, mitochondrial dTTP pools could be perturbed by alterations of thymidine levels. The existence of a mitochondrial specific thymidine kinase 2, which converts thymidine to thymidine monophosphate, probably contributes to the alterations of the mitochondrial dNTP pool (24, 25, 27, 28). The mitochondrial thymidine kinase 2 is expressed constitutively, in contrast to the cytosolic thymidine kinase 1 that is up-regulated during cell division (28). The constant activity of thymidine kinase 2 is likely to contribute to the susceptibility of mitochondrial dNTP pools to altered thymidine levels. We hypothesize that the mitochondrial dNTP pools are altered and lead to mtDNA depletion and multiple deletions in patients with MNGIE.

If high concentrations of thymidine are the cause of mitochondrial DNA impairment, one possible therapy for MNGIE will be to reduce or, if possible, to virtually eliminate thymidine from blood. Hemodialysis does not seem to be a very effective strategy, because the rate of production of thymidine in MNGIE patients may exceed the ability of the dialysis to eliminate the molecule from the blood, restoring the predialysis concentrations <3 h after the end of the treatment. Alternatively, it is possible that large body stores of thymidine are rapidly released into blood after hemodialysis and that multiple dialysis treatments may ultimately be effective in removing accumulated thymidine in MNGIE patients. Another treatment strategy is blocking the reabsorption of the nucleoside by the kidney, thereby increasing the urinary elimination of thymidine. Several descriptions of the kinetics and pharmacological properties of nucleoside transporters in the cell have been published (19, 29–31). Multiple drugs can inhibit the function of these carriers (32, 33). Decreasing the rate of renal reabsorption of thymidine by chemically inhibiting nucleoside carriers could lead to a significant decrease in thymidine concentrations in blood of MNGIE patients. If such a strategy is effective, it is probably necessary to treat the patients as early as possible, in order to avoid probable irreversible consequences of thymidine toxicity on cells.

In summary, as a consequence of loss of TP activity, MNGIE patients accumulate thymidine, which cannot be efficiently eliminated from blood. TP activity measurement in buffy coats and thymidine in plasma are of diagnostic value for this disease. We hypothesize that, in MNGIE patients, persistently elevated levels of thymidine unbalances nucleotide pools in mitochondria, leading to mtDNA abnormalities; therefore, therapeutic strategies to reduce thymidine concentration in blood of these patients may be effective.

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J. Biol. Chem. 2002, 277:4128-4133.
doi: 10.1074/jbc.M111028200 originally published online December 3, 2001
