Growth and DNA Synthesis of Folate- and Methionine-Depleted L1210 Mouse Leukemia Cells in Culture

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Summary The growth and DNA synthesis of L1210 mouse leukemia cells were examined under folate- and methionine-deficient conditions. Cell proliferation was dependent on methionine supplementation rather than on folate concentration. The UdR suppression value was abnormally high in the folate-deficient condition. However, it was also high when the methionine was low, despite folate supplementation. In accordance with this, UdR incorporation was significantly improved with various folates by cells grown in low-methionine conditions. Methionine depletion resulted in marked impairment of UdR incorporation regardless of folate concentration. These findings indicate close metabolic interrelations between folate and methionine, which may be relevant to the pathological biochemistry of human megaloblastic anemia.

Key Words folate deficiency, methionine deficiency, megaloblastic anemia, deoxyuridine suppression test, DNA synthesis, cell culture

Folate and methionine play an important role in cell metabolism. Folate derivatives are required for purine and pyrimidine synthesis and amino acid interconversions. Methionine is involved in the transfer reaction of the methyl group to various acceptors after being converted to its active form, *S*-adenosyl-L-methionine (SAM) (1). The majority of naturally occurring folates, when ingested, is converted to 5-methyl-tetrahydrofolate (CH₃-THF) upon absorption through the intestinal epithelia and is stored in the liver (2). CH₃-THF is converted to tetrahydrofolate (THF) by the cobalamin-dependent methionine synthetase [EC 2.1.1.13] reaction before entering the cellular pool of active folates. Through this reaction, the methyl group of CH₃-THF is transferred to homocysteine, generating methionine and THF (Fig. 1) (3).

Cobalamin deficiency leads to disturbed metabolism of both folate and methionine. In this regard these three are closely interrelated in the cell metabolism,

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Fig. 1. Metabolic pathways relating folate, methionine and vitamin B₁₂. C₁, active one-carbon; Hcy, homocysteine; Met, methionine; CH-THF, methenyl-tetrahydrofolate; dUMP, deoxyuridine monophosphate; dTMP, deoxythymidine monophosphate. For other abbreviations see text.

although the precise mechanism has been a matter of conjecture(3). We recently observed that in bone marrow cells from patients with megaloblastic anemia due to cobalamin deficiency, *in vitro* addition of methionine significantly improved the deranged DNA synthesis that is characteristic of this condition (4).

In order to elucidate this point further, we investigated the effects of folate and methionine deprivation on the cell growth and DNA synthesis of L1210 mouse leukemia cells in culture.

EXPERIMENTAL

A special deficient medium was prepared by omitting pteroylglutamic acid (PGA), methionine, vitamin B₁₂ (B₁₂) and bactopectone from the standard formula of modified McCoy's 5A medium (Kyokuto Seiyaku Co.). This was supplemented with 10% fetal calf serum (FCS, Flow Lab.), 100 units/ml of penicillin and 50 µg/ml of streptomycin (Gibco Lab.) ("basal medium"). The B₁₂ and folate concentrations of undiluted FCS were 280 pg/ml and 6.8 ng/ml, respectively, as determined by radioassay. Stock L1210 cells were maintained at 37°C in a humidified atmosphere (5% CO₂, 95% air) in the basal medium supplemented with L-methionine (15 µg/ml, Sigma) and PGA (1,000 ng/ml, Takeda Chemical Co.), and the medium was changed every 3–4 days. Cell number was determined by a Coulter counter model D. Prior to the experiment, L1210 cells maintained as above were placed in basal medium supplemented only with 15 µg/ml of methionine, and then transferred 3–4

times in order to render the cells folate depleted. Such cells were then washed once with the deficient modified McCoy's 5A medium, before being transferred to the experimental medium as specified in the text. After 24 to 48 h when growing in the logarithmic phase, the cells were placed in duplicate tubes in 1 ml of suspensions containing $0.5\text{--}1.0 \times 10^6$ cells.

For the deoxyuridine (UdR) suppression test according to our modified method (5), $1.0 \mu\text{Ci}$ (50 pmol) of $[^3\text{H}]$ thymidine (TdR) and $10 \mu\text{g}$ ($0.044 \mu\text{mol}$) of unlabeled UdR (Sigma) were added to each tube simultaneously. Likewise, for the assessment of UdR incorporation into DNA, $1.0 \mu\text{Ci}$ (48 pmol) of $[^3\text{H}]$ UdR and either one of various additives such as PGA (5 or $50 \mu\text{g}/\text{ml}$), $\text{CH}_3\text{-THF}$ ($5 \mu\text{g}/\text{ml}$, Sigma), 5-formyl-tetrahydrofolate (CHO-THF, $5 \mu\text{g}/\text{ml}$, Lederlie) or L-methionine (1.5 or $15.0 \mu\text{g}/\text{ml}$) were added to each tube simultaneously immediately before incubation. After being incubated at 37°C for 1 or 2 h in a water bath, the cells were washed once by centrifugation with 5 ml of ice-cold Hanks' balanced salt solution. To each cell pellet was added 3 ml of ice-cold 5% trichloroacetic acid (TCA). Two small drops of 1% bovine serum albumin (Sigma) were added as carrier and the tubes were left in ice for 20 min. Acid-insoluble material was obtained by washing three times with ice-cold 5% TCA. Radioactivity was measured using a NCS solubilizer (Amersham) and a toluene scintillation system using a liquid scintillation spectrometer (Aloka LSC-700). The results were expressed as percentages of the respective controls without unlabeled UdR or various additives. The radioactive compounds were purchased from New England Nuclear Co.

RESULTS

Cell growth

Cell growth curves in the basal medium supplemented with $50 \mu\text{g}/\text{ml}$ of PGA and methionine at one of 6 different concentrations ranging from none to $15 \mu\text{g}/\text{ml}$ are shown in Fig. 2, panel A. The cell growth pattern was dependent on the

Fig. 2. Methionine-dependent growth of cells at two different PGA concentrations.

Fig. 3. Udr suppression tests in cells grown under different folate and methionine compositions. Each bar represents the mean and one standard deviation of three sets of separate experiments.

methionine concentration, attaining the highest plateau at 7.5 µg/ml. Doubling of methionine concentration (15 µg/ml) failed to improve the cell growth significantly. Such apparent dependence of cell growth on added methionine was essentially identical when the cells were grown in the presence of 1,000 ng/ml of PGA (Fig. 2, panel B).

Udr suppression test

The Udr suppression test was performed using cells grown for 24 h under 6 different medium compositions, *i.e.*, 3 different methionine concentrations (1.5, 4.5 or 15 µg/ml) with or without 1,000 ng/ml PGA. As shown in Fig. 3, Udr suppression values were equally high (average: 28.8%) for cells grown in the absence of added PGA (upper panel), irrespective of methionine concentration. On the other hand, for cells cultured in the presence of 1,000 ng/ml of PGA (lower panel), 1.5 µg/ml of methionine gave an average value of 25.9%, whereas 4.5 or 15.0 µg/ml methionine gave a value of 16.2% or 17.3%, respectively.

Effects of folates on Udr incorporation by folate-depleted or folate-repleted cells

Effects of various folate derivatives on Udr incorporation were examined using cells grown for 24 h under the identical culture conditions as above. At the start of the incorporation study, various folates were added to the cell suspension simultaneously with [³H]Udr. The results are summarized in Fig. 4. In the culture without PGA supplementation (panel A), the addition of any of three folates resulted in a significant increase in Udr incorporation in all instances. However it was evident that the percentage of improvement was most prominent when the methionine concentration was lowest (1.5 µg/ml). The relative efficacy among the different folates at the same concentration (5 µg/ml) was greatest with CHO-THF.

Fig. 4. Effects of added folates on Udr incorporation by cells grown under different methionine and folate concentrations. In panel A, PGA was not supplemented, while in panel B, PGA was supplemented at a concentration of 1,000 ng/ml. Each bar represents the mean and one standard deviation of three sets of separate experiments.

Five $\mu\text{g/ml}$ of CHO-THF gave better Udr incorporation than 50 $\mu\text{g/ml}$ of PGA. However, no further improvement was obtained by increasing the amount of CHO-THF above 5 $\mu\text{g/ml}$ (data not shown). On the other hand, when the cells were grown in the media supplemented with 1,000 ng/ml PGA for 24 h before the experiment

(panel B), a significant increase of UdR incorporation by added folates was observed only in cells grown with the least amount of methionine (1.5 $\mu\text{g/ml}$). When the cells were supplemented with more methionine (4.5 or 15 $\mu\text{g/ml}$) no improvement was observed in UdR incorporation upon the addition of any folate.

Effects of folates and methionine on methionine-depleted cells

The effects of PGA, $\text{CH}_3\text{-THF}$, CHO-THF or methionine were examined on cells that had been cultured for 48 h in media supplemented with 1.5 $\mu\text{g/ml}$ methionine and one of the following 3 different folate conditions: none, 1 $\mu\text{g/ml}$ of PGA or 1 $\mu\text{g/ml}$ of CHO-THF . The 48-h culture with low methionine concentration (1.5 $\mu\text{g/ml}$) resulted in a marked depression of UdR incorporation into DNA (Table 1). Under such conditions, addition of various folates at a concentration of 5 $\mu\text{g/ml}$

Table 1. Effects of added folates and methionine on UdR incorporation by cells which had been grown in methionine-depleted media for 48 h. Values in the table denote percent incorporation of [^3H]UdR to respective controls, and those in parentheses indicate the actual cpm/tube.

Medium composition	Additives			
	$\text{CH}_3\text{-THF}$ (5 $\mu\text{g/ml}$)	PGA (5 $\mu\text{g/ml}$)	PGA (50 $\mu\text{g/ml}$)	CHO-THF (5 $\mu\text{g/ml}$)
Met (1.5 $\mu\text{g/ml}$)	74.0	85.7	276.0	200.0
PGA (—)	(57)	(66)	(213)	(154)
Met (1.5 $\mu\text{g/ml}$)	120.9	115.0	168.7	252.2
PGA (1 $\mu\text{g/ml}$)	(81)	(77)	(113)	(169)
Met (1.5 $\mu\text{g/ml}$)	79.3	106.5	73.9	90.2
CHO-THF (1 $\mu\text{g/ml}$)	(73)	(98)	(68)	(83)

Table 1. (continued)

Medium composition	Additives		
	Met (1.5 $\mu\text{g/ml}$)	Met (4.5 $\mu\text{g/ml}$)	Met (15.0 $\mu\text{g/ml}$)
Met (1.5 $\mu\text{g/ml}$)	1980.5	1929.9	1913.0
PGA (—)	(1525)	(1486)	(1473)
Met (1.5 $\mu\text{g/ml}$)	3282.1	3382.1	2980.6
PGA (1 $\mu\text{g/ml}$)	(2131)	(2266)	(1997)
Met (1.5 $\mu\text{g/ml}$)	2090.2	2195.2	2066.3
CHO-THF (1 $\mu\text{g/ml}$)	(1923)	(2020)	(1901)

failed to improve the UdR incorporation to any appreciable degree. In sharp contrast, however, even the addition of the smallest amount (1.5 $\mu\text{g/ml}$) methionine to such cultures promptly increased UdR incorporation to approximately 20–30 times the control value.

DISCUSSION

Blood cells of human megaloblastic anemia either due to vitamin B₁₂ or folate deficiency demonstrate abnormally high UdR suppression values as well as significant improvement of UdR incorporation after *in vitro* addition of the respective deficient vitamins (6, 7). These features are so characteristic that these tests are frequently used as a laboratory diagnostic aid for such conditions (5–7). We have recently demonstrated that bone marrow cells from human subjects with megaloblastic anemia due to vitamin B₁₂ deficiency showed significant improvement both in UdR suppression and UdR incorporation upon the *in vitro* addition of limited concentrations of methionine (4). Since it is known that folate addition results in similar improvement, methionine may be considered to exert a folate-like action under such circumstances. These observations also appear to suggest that the blood cells of human megaloblastic anemia may suffer from a relative deficiency of methionine at the cellular level. This prompted us to investigate the metabolic interrelations between folate and methionine by utilizing the L1210 cells in culture as an experimental model.

L1210 cells that were cultivated in media with low folate concentrations developed metabolic abnormalities characteristically found in human megaloblastic bone marrow cells, *i.e.*, elevated values of the UdR suppression test and improvement of UdR incorporation by folate addition. Such abnormalities became evident even when the medium was supplemented with sufficient methionine. Thus the cells were thought to have become folate deficient. It is unlikely that these cells were rendered cobalamin-deficient, since it has been shown that a small amount of cobalamin present in FCS is sufficient for the growth of L1210 cells (8). Simple omission of cobalamin from medium did not result in cobalamin deficiency, while pretreatment of whole medium with N₂O produced such condition, as has been reported elsewhere (9).

Under the culture conditions employed, the growth of L1210 cells was more heavily dependent on methionine than on folate (Fig. 2). Replacement of methionine with homocysteine, cobalamin and CH₃-THF is not sufficient to support the optimal growth of many cultured cell lines, particularly those derived from malignant tumors (10–12). The latter three are factors involved in the cellular synthesis of methionine (Fig. 1). Furthermore, cells grown under methionine-deficient conditions showed characteristic features which are essentially identical to those demonstrated by folate-deficient cells. This was substantiated by the improved UdR incorporation upon addition of several forms of folate (Fig. 4B). These observations clearly point to the close metabolic interrelations between folate and

methionine. Thus, methionine deprivation appeared to increase the cellular folate requirement and to aggravate marginal folate deficiency to a state of overt deficiency. In this regard, methionine was thought to behave like a folate under the experimental conditions employed in this study. Conversely, it has been reported by others that the abnormal metabolism seen in folate-deficient animals, such as an elevated urinary excretion of formiminoglutamic acid, is significantly corrected by simple methionine supplementation (13–15).

Folate-methionine relationships in the cell metabolism may be explained, at least partially, through the action of *S*-adenosyl-methionine (SAM). Methionine is quickly converted to SAM within the cell (16). Besides being a methyl donor, SAM is known to suppress the activity of 5,10-methylene-THF (CH_2 -THF) reductase [EC 1.1.1.68] (17). Methionine deprivation results in a decreased SAM level, which in turn activates the methionine-generating folate cycle (CH_3 -THF $\rightarrow$ THF $\rightarrow$ CH_2 -THF $\rightarrow$ CH_3 -THF, Fig. 1) through the activation of CH_2 -THF reductase due to removal of the suppression exerted by SAM on CH_2 -THF reductase. This cycle may convert cellular folates to CH_3 -THF independent of DNA synthesis. On the other hand, when methionine is repleted, the elevated SAM will channel the cellular folates toward the DNA-synthesizing cycle (THF $\rightarrow$ CH_2 -THF $\rightarrow$ DHF $\rightarrow$ THF) (18). In addition, it is known that SAM acts as a cofactor for the methionine synthetase reaction that converts CH_3 -THF to THF (19), making more CH_2 -THF available for DNA synthesis. Overall reduction of cellular folate pools results in diminished methionine synthesis and, as a consequence, the availability of methionine becomes limited.

In this study, prolonged methionine deficiency not accompanied by coexisting folate deficiency resulted in severely depressed UdR incorporation. However, the UdR incorporation recovered quickly and markedly upon the addition of methionine, but the improvement was much less striking with the addition of folate. This suggests that methionine deficiency itself brings about serious metabolic derangement leading to decreased DNA synthesis which cannot be replaced by folates. These findings are in agreement with reports that described the deleterious effects of amino acid deprivation on DNA and RNA syntheses of cultured cells (20–22).

In summary, our results confirm the close relationship between folate and methionine, and indicate that folate deficiency may become manifest through concomitant methionine deprivation. Since in human megaloblastic anemia folate metabolism is presumed to be disturbed by cobalamin deficiency and such cells are in a functional folate deficiency, our present observations may be relevant to the improving effect of methionine on DNA synthesis in human megaloblastic cells.

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