Effect of Nitrous Oxide on Human Bone Marrow Cells and Its Synergistic Effect with Methionine and Methotrexate on Functional Folate Deficiency

Yasuhiko Kano, Shinobu Sakamoto, Koeri Sakuraya, Tatsuya Kubota, Keita Hida, Keiichi Suda, and Fumimaro Takaku


ABSTRACT

5-Methyltetrahydrofolate homocysteine methyltransferase activity in human bone marrow cells obtained from patients undergoing surgical operation became low after about 4 hr of nitrous oxide (N₂O) anesthesia. The deoxyuridine suppression test performed on these bone marrow cells also became abnormal after about 6 hr of N₂O anesthesia. The incorporation of [³H]thymidine into DNA in the bone marrow cells preincubated with methionine or methotrexate was much higher after N₂O anesthesia than before anesthesia. Since N₂O and methionine or methotrexate have a synergistic effect on depletion of functional folate, N₂O alone or in combination with methionine or methotrexate might be of value for cancer treatment and deserve clinical trials.

INTRODUCTION

Since the 1950s, nitrous oxide (N₂O), an anesthetic agent, has been known to suppress cell proliferation and cause megaloblastic changes in hematopoietic cells (12). In 1959 and 1963, N₂O inhalation was used to treat patients with chronic and acute myelogenous leukemia (8, 13). As a result, N₂O was proven to have an effect on the reduction of leukemia cells. The effect of N₂O, however, was transient, and leukemia cells resumed rapid proliferation after withdrawal of N₂O inhalation. It has been reported that N₂O oxidizes vitamin B₁₂ from the cob(II)alamin to the cob(III)alamin form (2). In addition, it has been shown recently that N₂O induces a functional vitamin B₁₂ deficiency and inhibits 5-methyltetrahydrofolate homocysteine methyltransferase in rat liver (7). This means that N₂O impairs not only vitamin B₁₂ metabolism but also folate metabolism. However, metabolism of vitamin B₁₂ and folate in humans is not always the same as that found in experimental animals, and the effect of N₂O on human bone marrow cells has not been examined in detail (1, 5).

In this report, we measured 5-methyltetrahydrofolate homocysteine methyltransferase activity in human bone marrow cells from patients undergoing surgical operation under N₂O anesthesia. In addition, utilizing the dUrd³ suppression test, a good marker for defining biochemical megaloblastosis due to deficiency of folate and vitamin B₁₂ (6), we studied (a) the effect of N₂O and (b) methionine and MTX-caused alterations in folate metabolism in human bone marrow cells exposed to N₂O. Our data show that N₂O inhibits 5-methyltetrahydrofolate homocysteine methyltransferase in human bone marrow cells within a few hr of N₂O anesthesia and suggest that N₂O impairs cell growth and the combined use of N₂O with methionine or MTX is more effective against cancer than is single administration of each agent.

MATERIALS AND METHODS

Materials. [³H]dTThd and 5-¹⁴C]methyltetrahydrofolate were purchased from the Radiochemical Centre, Amersham, England. Other chemicals were obtained from the following sources: methionine (Wako, Tokyo, Japan); S-adenosylmethionine, dithiothreitol, and nonradioabeled 5-methyltetrahydrofolate (Sigma Chemical Co., St. Louis, Mo.); ω-homocysteine (Nakarai, Kyoto, Japan); MTX (Lederle, Tokyo, Japan); and Hanks’ medium (Nissui, Tokyo, Japan).

Patients. Twenty-two patients were assigned in a random fashion to either one of the following 2 groups. Group A (n = 7) was anesthetized with halothane:air:oxygen (1:81:18) and Group B (n = 15) was anesthetized with halothane:N₂O:oxygen (1:81:18). The duration of anesthesia ranged from 2 to 11 hr. No patients with hematological, cardiovascular, and respiratory diseases were included in this study. Serum vitamin B₁₂ and folate levels of the patients were all within the normal range.

Enzyme and Cell Preparations. Bone marrow cells were aspirated in the operating room just before the beginning and termination of anesthesia (hereafter referred to as before and after anesthesia) and separated by centrifugation on Ficoll-Hypaque density gradients. For the assay of 5-methyltetrahydrofolate homocysteine methyltransferase, the cells were washed 3 times in phosphate-buffered saline and then resuspended with 50 mM potassium phosphate buffer, pH 7.2, with 1.5 mM reduced glutathione. The cell suspension was subjected to 4 cycles of rapid freezing and thawing. The suspension was centrifuged at 100,000 x g for 60 min, and the supernatant was assayed for 5-methyltetrahydrofolate homocysteine methyltransferase activity. For the dUrd suppression test, the cells were washed 3 times in Hanks’ medium and then resuspended in Hanks’ medium at the concentration of 3 x 10⁶ cells/ml. All the procedures for cell separation were carried out at 4°.

Assay of 5-Methyltetrahydrofolate Homocysteine Methyltransferase (Apoenzyme). We measured this enzyme activity in 4 cases of Group A and 8 cases of Group B. The enzyme assay was carried out according to the method described previously without vitamin B₁₂ in the reaction mixture (11).

The reaction mixture contained in a total volume of 200 μl: 100 mM sodium phosphate buffer, pH 7.4; 20 mM dithiothreitol; 10 mM ascorbic acid; 60 μM S-adenosylmethionine; 600 μM ω-homocysteine; 600 μM [⁵-¹⁴C]methyltetrahydrofolate (1.8 x 10⁵ dpm/nmol); and 100 μl of the enzyme solution. After incubation for 60 min at 37° in the dark, the reaction was stopped by chilling in ice and adding 1.6 ml of 75% isopropyl alcohol. The supernatant was added to a screw-topped centrifuge tube containing 0.25 ml of 50 mM cytosine bromide in 0.1 N acetic acid. The tube was tightly capped and incubated at 100° for

Received February 27, 1981; accepted July 30, 1981.
5 min, followed by 5 min of incubation at 0°. Two ml toluene were added, and the mixture was shaken vigorously on a Thermomixer. After centrifugation for 10 min at 1000 x g, a 1-ml aliquot of the toluene extract was removed for determination of radioactivity by a liquid scintillation spectrophotometer. The enzyme activity was represented as nmol of [14C]methionine formed per 1 x 10⁸ cells per 60 min.

dUrd Suppression Tests. The test was performed according to the method described by Metz et al. (14) with a modification. One-tenth ml of bone marrow cell suspension and 0.1 ml of each patient's serum were added to the following cell culture tubes containing: (a) no dUrd (control); (b) dUrd (1 x 10⁻⁴ M); (c) dUrd (1 x 10⁻⁴ M) + 1 mg of methionine; (d) dUrd (1 x 10⁻⁴ M) + MTX (1 x 10⁻⁸ M); (e) dUrd (1 x 10⁻⁴ M) + MTX (1 x 10⁻⁷ M); (f) dUrd (1 x 10⁻⁴ M) + MTX (1 x 10⁻⁶ M); and (g) dUrd (1 x 10⁻⁴ M) + MTX (1 x 10⁻⁵ M). Fifty µg of ascorbic acid were also added to all tubes, and the final volume of each tube was adjusted to 1.0 ml with Hanks' medium. The tests were set up in duplicate. The tubes were incubated at 37° for 90 min. After the addition of 1 µCi of [³H]dThd (specific activity, 20 Ci/mmol), the tubes were again incubated at 37° for 90 min. The cells were then washed once with cold phosphate-buffered saline, lysed to remove hemoglobin, and delivered onto Whatman GF/C filter discs. The discs were immersed in 10% trichloroacetic acid at 4° overnight, and then washed 2 times with 5% trichloroacetic acid. After the discs were dried, [³H]dThd incorporation into DMA was counted in a Packard scintillation counter. Less than 10% of the mean control value was regarded as normal in the present study.

RESULTS

5-Methyltetrahydrofolate Homocysteine Methyltransferase Activity. As shown in Chart 1, 5-methyltetrahydrofolate homocysteine methyltransferase activity in Group A was 1.04 ± 0.26 (S.D.) before anesthesia and 0.97 ± 0.22 after anesthesia. No difference was detected between them. In Group B, 5-methyltetrahydrofolate homocysteine methyltransferase activity was 1.10 ± 0.33 before anesthesia and 0.80 ± 0.51 after anesthesia. The enzyme activity tended to decrease after 4 hr of N₂O anesthesia.

Effect of dUrd. As shown in Charts 2 and 3 and in Table 1, the dUrd suppression test of Group A was normal in all cases before and after anesthesia.

In Group B, it was also normal in all cases before anesthesia. [³H]dThd uptake increased in 14 patients from Group B after anesthesia. However, this increase usually occurred within the normal range, and it was much higher in the dUrd and 1 x 10⁻⁶ M MTX after anesthesia than in the dUrd and 1 x 10⁻⁵ M MTX before anesthesia. However, the increase was much more apparent after anesthesia at any concentration of MTX than it was before anesthesia. The degree of [³H]dThd uptake between dUrd plus 1 x 10⁻⁴ M MTX and dUrd alone. Higher concentrations of MTX markedly increased [³H]dThd uptake before as well as after anesthesia. However, the increase was much more apparent after anesthesia at any concentration of MTX than it was before anesthesia. The degree of [³H]dThd uptake was a little lower in the dUrd and 1 x 10⁻⁷ M MTX after anesthesia group than in the dUrd and 1 x 10⁻⁸ M MTX before anesthesia group. However, it was much higher in the dUrd and 1 x 10⁻⁸ M MTX after anesthesia group than in the dUrd and 1 x 10⁻⁸ M MTX before anesthesia group.

DISCUSSION

5-Methyltetrahydrofolate homocysteine methyltransferase
This may be related to the difference of vitamin B12 and folate in the effects of MTX in vitro on [3H]dUrd uptake, depletion of functional folate follows the administration of these agents. Although the sites of action of N2O and MTX are different, depletion of functional folate follows the administration of these agents.

<table>
<thead>
<tr>
<th>Group A (n = 7)</th>
<th>dUrd Before</th>
<th>dUrd + methionine Before</th>
<th>dUrd + MTX (1 x 10^-8 M) Before</th>
<th>dUrd + MTX (1 x 10^-7 M) Before</th>
<th>dUrd + MTX (1 x 10^-6 M) Before</th>
<th>dUrd + MTX (1 x 10^-5 M) Before</th>
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<tbody>
<tr>
<td>dUrd Before</td>
<td>5.1 ± 2.4^a</td>
<td>7.1 ± 3.2</td>
<td>4.0 ± 2.2</td>
<td>19.2 ± 5.0</td>
<td>41.1 ± 14.3</td>
<td>49.0 ± 22.4</td>
</tr>
<tr>
<td>dUrd + MTX</td>
<td>6.7 ± 6.3</td>
<td>9.1 ± 3.2</td>
<td>3.9 ± 1.4</td>
<td>15.1 ± 8.0</td>
<td>30.0 ± 13.7</td>
<td>50.0 ± 21.9</td>
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<tr>
<td>p</td>
<td>0.05</td>
<td>1.03</td>
<td>0.31</td>
<td>1.28</td>
<td>1.49</td>
<td>0.07</td>
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<tr>
<td>p</td>
<td>NS</td>
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<tr>
<th>Group B (n = 15)</th>
<th>dUrd Before</th>
<th>dUrd + methionine Before</th>
<th>dUrd + MTX (1 x 10^-8 M) Before</th>
<th>dUrd + MTX (1 x 10^-7 M) Before</th>
<th>dUrd + MTX (1 x 10^-6 M) Before</th>
<th>dUrd + MTX (1 x 10^-5 M) Before</th>
</tr>
</thead>
<tbody>
<tr>
<td>dUrd Before</td>
<td>3.9 ± 1.6</td>
<td>7.4 ± 2.5</td>
<td>4.2 ± 2.0</td>
<td>26.3 ± 10.3</td>
<td>53.1 ± 21.2</td>
<td>56.4 ± 27.4</td>
</tr>
<tr>
<td>dUrd + MTX</td>
<td>9.9 ± 6.1</td>
<td>23.2 ± 10.1</td>
<td>10.4 ± 6.1</td>
<td>48.2 ± 17.2</td>
<td>85.2 ± 23.2</td>
<td>88.0 ± 28.7</td>
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<tr>
<td>p</td>
<td>4.48</td>
<td>6.35</td>
<td>4.58</td>
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<tr>
<td>p</td>
<td>&lt;0.001</td>
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^a Mean ± S.D.
^b Statistical comparisons of paired data between before and after anesthesia with various compounds.
^c NS, not significant (>0.1).

The dUrd suppression test was abnormal in bone marrow cells from 7 of the 15 patients anesthetized with N2O. It tended to remain within the normal range up to 6 hr of anesthesia with N2O but became abnormal in 5 of the 6 patients with longer than 6-hr anesthesia. These abnormalities in dUrd suppression coincided with the lowering of the activity of 5-methyltetrahydrofolic acid homocysteine methyltransferase after 4 hr of N2O anesthesia. On the other hand, Amess et al. (1) reported that the dUrd suppression test was abnormal in only 3 out of the 9 patients 24 hr after anesthesia by 5 to 12 hr of inhalation of 50% N2O. This discrepancy may be attributed to the differences in the time of bone marrow aspiration performed after anesthesia and the concentration of N2O used.

The effects of methionine and MTX on bone marrow cells treated with N2O are interesting. By preincubation with dUrd and methionine, bone marrow cells from patients anesthetized with N2O increased the incorporation of [3H]dThd much more than bone marrow cells from a patient without N2O anesthesia. As an end product inhibitor (4), methionine inhibits 5-methyltetrahydrofolate homocysteine methyltransferase. N2O and methionine inhibit the enzyme and manifest a synergistic effect to deplete functional folate.

A similar tendency was obtained by the use of N2O and MTX. By preincubation with dUrd plus MTX, bone marrow cells from patients with N2O anesthesia increased the incorporation of [3H]dThd much more than did bone marrow cells from the same patients before anesthesia. Especially marked was the increase in [3H]dThd incorporation by preincubated bone marrow cells exposed to N2O with dUrd plus 1 × 10^-8 M MTX. N2O inhibits the proliferation of malignant cells more potent depletion of functional folate than does a single administration of each agent.

Our results are based on the investigation using bone marrow cells from patients undergoing surgery but suggest that the combination therapy with N2O and methionine or MTX may have a stronger anticancer action.

N2O inhibits 5-methyltetrahydrofolate homocysteine methyltransferase activity through inactivation of vitamin B12. 5-Methyltetrahydrofolate homocysteine methyltransferase activity in hematopoietic cells has been reported to be very low (15). Among them, leukemic cells from the patients with acute myelogenous leukemia and chronic myelogenous leukemia usually have lower enzyme activity than normal bone marrow cells (9). It has been reported that N2O effectively suppressed the proliferation of leukemic cells of acute myelogenous leukemia and chronic myelogenous leukemia (8, 13). These data may suggest that N2O inhibits the proliferation of malignant cells with low levels of 5-methyltetrahydrofolate homocysteine methyltransferase activity like leukemic cells from patients with acute and chronic myelogenous leukemia.

Recently, high-dose MTX therapy has come into wide use against osteosarcoma, lymphoma, and various kinds of carcinoma. The combined use of N2O and MTX can reduce the dose of MTX and may have a stronger effect upon malignant cells. N2O also has a strong anesthetic action and is effective for alleviation of pain of patients with cancer. N2O therapy alone against osteosarcoma, lymphoma, and various kinds of carcinoma. The combined use of N2O and MTX can reduce the dose of MTX and may have a stronger effect upon malignant cells. N2O also has a strong anesthetic action and is effective for alleviation of pain of patients with cancer. N2O therapy alone or in combination with methionine or MTX is encouraging as a form of cancer treatment and worth clinical trials.

REFERENCES
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