Prolonged Nitrous Oxide Exposure Inhibits Settlement of Transplanted Hemopoietic Stem Cells in Murine Spleen

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In order to clarify the mechanism of hemopoietic depression induced by nitrous oxide inhalation, effects of prolonged nitrous oxide exposure on the settlement of transplanted bone marrow cells were investigated. Mice were continuously exposed to mixed gas containig 50% nitrous oxide, 21% oxygen and 29% nitrogen for 7 days and then they were irradiated with 850 rads. By the irradiation, endogenous pluripotent hemopoietic stem cells (CFU-S) almost disappeared in the mice. Normal syngenic murine bone marrow cells were injected intravenously and the numbers of CFU-S, which settled in the bone marrow and spleen 2 hr after injection, were measured. There was no difference of the numbers of CFU-S settled in the bone marrow between nitrous oxide and control gas exposed mice. In contrast, the numbers of CFU-S in the spleen of nitrous oxide exposed mice were approximately 60% of the control. These results and our previous data suggest that hemopoietic inhibitory effects of nitrous oxide in mice are due to a damage of splenic hemopoietic microenvironment, that supports the settlement of hemopoietic stem cells. (Key words: nitrous oxide, hemopoietic stem cells (CFU-S), hemopoietic supportive microenvironment, bone marrow transplantation)

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Nitrous oxide has been reported to be toxic to the hemopoietic tissues of man in 1956[1–3]. Since then there have been several clinical reports on leukocytopenia and/or acute bone marrow depression, resulting from prolonged administration of nitrous oxide in man[3–5]. Several experiments using rodents showed that long-term exposure to nitrous oxide induced significant leukocytopenia[7,8] and histocytological hypoplastic changes in the bone marrow[9].

However, there has been no report on the effects of nitrous oxide on the hemopoietic stem cells *in vivo*. We investigated recently the effects of nitrous oxide on the numbers of pluripotent hemopoietic stem cells (CFU-S) and granulocyte-macrophage progenitor cells (GM-CFC) in murine bone marrow and spleen[10]. Prolonged exposure to nitrous oxide elicited decreases in the numbers of these cells in both the bone marrow and the spleen. The decrease was especially remarkable, in the spleen, compared to a slight decrease in the bone marrow. More recently, we showed that recovery of hemopoietic stem cells was delayed in mice which were given

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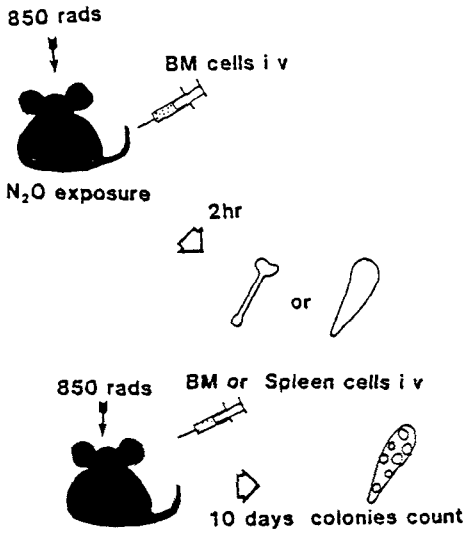

Fig. 1. Determination of CFU-S, settled in the bone marrow and spleen.
Detailed explanations were described in Materials and Methods.

low dose-irradiation followed by continuous exposure to nitrous oxide[11]. The delay of recovery was also distinct in the spleen, but only slight in the bone marrow. These results suggest that nitrous oxide may not directly affect the growth and differentiation of the hemopoietic stem cells but causes damage to the hemopoietic microenvironment, which supports settlement, proliferation and differentiation of hemopoietic stem cells, and that the damage is localized in the spleen rather than the bone marrow. However, there is no direct evidence for this hypothesis.

Present study was carried out to estimate a possible direct effect of nitrous oxide on the function of hemopoietic microenvironment. Settlement of the transplanted hemopoietic stem cells in the bone marrow and spleen of prolonged nitrous oxide exposed mice were investigated.

Materials and Methods

Exposure to Nitrous oxide

Seven-week-old male mice of C3H/HeSlc strain (Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu) were used at the start of these studies. Nitrous oxide exposure was carried out in chambers with 21% O_2, 29% N_2 and 50% N_2O at 21–24°C and about 70% humidity. The gas-mixture was circulated through the chambers by an anesthetic machine. Carbon dioxide was absorbed with soda lime placed on the floor of chambers. Mice were housed at 4 to 5 mice per cage, and fed with the standard diet and water *ad lib.* The chambers were opened every 48 hours for cleaning and the exchange of food and water. These mice were continuously exposed to nitrous oxide for 7 days. The control mice were housed in the same manner expect for exposure to the gas-mixture containing 21% O_2 and 79% N_2. As previously reported[11], mice exposed to nitrous oxide appeared alert and behaved normally.

Assay of the number of transplanted pluripotent hemopoietic stem cells (CFU-S), which settled in the bone marrow and spleen

In order to determine the capacity of hemopoietic supportive microenvironment in the bone marrow and spleen, which settle transplanted pluripotent hemopoietic stem cells (CFU-S), following double transfer experiments[12] were carried out (fig. 1).

1) Preparation of bone marrow cells for transplantation

Normal syngenic murine bone marrow cells were flushed into α – minimal essential medium (α–MEM) from tibiae and femora, using an injection syringe. The cells from 3 mice were pooled and counted. After measuring the number of bone marrow cells, the cell suspension was diluted to 5×10^7 cells/ml.

2) Transplantation of bone marrow cells

Recipient mice to which nitrous oxide or control gas were given whole-body X-irradiation with 850 rads of cobalt 60 (at approximately 80 rads per minute). Soon after irradiation, 0.2 ml of normal bone marrow cell suspension (1×10^7 bone marrow cells) was injected intravenously to recipient mice.

3) Determination of numbers of CFU-S that settled in the bone marrow and the spleen after the bone marrow transplantation

Recipient mice, which had received trans-

Table 1. Effects of N_2O on the settlement of CFU-S in the spleen after bone marrow transplantation

	N_2O	Control
No. of mice	10	10
No. of transplanted CFU-S	$2.10 \pm 0.52 \times 10^3$	
No. of total cells in spleen 2 hours after transplantation	5.70×10^7	9.80×10^7
No. of CFU-S settled in spleen 2 hours after transplantation	292 ± 76	480 ± 122
Spleen weight of mice for CFU-S assay* (mg)	38.2 ± 7.6	53.7 ± 13.0
Seeding efficiency (f)**	0.14	0.23

Mice were continuously exposed to N_2O or control gas for 7 days, then were given X-irradiation. To these mice, normal bone marrow cells (0.9×10^7) were injected i.v. After 2 hours numbers of total cells and CFU-S in the spleen were assayed. For the assay of CFU-S, 1/20 of total spleen cells were injected per mice. Mice for the assay of CFU-S, were sacrificed after 10 days, and weights* and numbers of colonies in the spleen were determined. Seeding efficiency (f)** = No. of CFU-S settled in the spleen after 2 hours of transplantation/No. of transplanted CFU-S.

Table 2. Effects of N_2O on the settlement of CFU-S in the bone marrow after bone marrow transplantation

	N_2O	Control
No. of mice	9	9
No. of transplanted CFU-S	$2.24 \pm 0.38 \times 10^3$	
No. of total cells in tibia 2 hours after transplantation	5.38×10^6	4.72×10^6
No. of CFU-S settled in tibia 2 hours after transplantation	31 ± 10	33 ± 8
Spleen weight of mice for CFU-S assay (mg)	23.1 ± 2.0	22.9 ± 1.1
Seeding efficiency (f)	0.014	0.015

Data were determined as described in Methods and table 1. In assays of CFU-S, 1/5 of total tibial cells were injected i.v. per mouse.

Seeding efficiency (f)* = No. of CFU-S settled in tibia after 2 hours of transplantation/No. of transplanted CFU-S.

planted bone marrow cells, were housed under N_2O in control gas conditions for 2 hours and then mice sacrificed for assays of CFU-S in the spleen and bone marrow, as previously described. Briefly, appropriate numbers of spleen cells and bone marrow cells were injected intravenously into lethally irradiated (850 rad of cobalt 60) recipient mice, and macroscopic colonies on the surface of the spleen were counted as CFU-S 10 days later[13].

Results

No effect of N_2O on radiosensitivity of murine hemopoietic stem cells

To determine whether or not endogenous hemopoietic stem cells in N_2O-exposed mice survived after 850 rads of X-irradiation, the following examination was carried out. Mice were exposed to N_2O or control gas after irradiation, and colonies in the spleen, derived from survived endogenous CFU-S, were counted 10 days later. The numbers of endogenous CFU-S in control gas and N_2O-exposed mice were 0.2 ± 0.6 ($n = 9$) and 0.2 ± 0.4 ($n = 5$), respectively. There was no difference between them. These results suggests that the inhalation of N_2O does not affect the radiosensitivity of hemopoietic stem cells.

Inhibitory effects of N_2O on settlements of CFU-S in the spleen

As shown in table 1, the number of CFU-S settled in the spleen was 480, two hr after injection of approximate 2,100 of CFU-S in control mice. Calculating from these data, the seeding efficiency (f) of CFU-S in the spleen was 0.23. The value almost corresponded well to those of previous investigators[12–14]. In contrast, in N_2O-exposed mice, cellularity and the number of CFU-S in the spleen were significantly less than those in control mice (58% and 61% of control, respectively). The seeding efficiency of CFU-S in the spleen of N_2O-exposed mice was 0.14.

No effect of N_2O on the settlements of CFU-S in bone marrow

Settlements of CFU-S in the tibia of N_2O-exposed mice were examined (table 2). Cellularity and the numbers of CFU-S were not different between control-gas and N_2O-exposed mice. Seeding efficiencies (f) were essentially the same. The f value of CFU-S in tibia (0.14–0.15) also corresponded to those of previous reports[15,16].

Discussion

Recently, we have examined the inhibitory effects of N_2O on hemopoiesis using techniques for clonal assay of pluripotent hemopoietic stem cells (CFU-S)[13] and granulocyte-macrophage precursor cells (GM-CFC)[17]. Inhalation of N_2O was found to cause a marked inhibition of hemopoiesis in murine spleen, but only slightly in bone marrow[10]. Furthermore, N_2O exposure inhibited the recovery of CFU-S and GM-CFC after irradiation[11]. Such inhibition was also remarkable in the spleen, but only slight in the bone marrow. In the present study, it was found that the inhalation of N_2O inhibited settlements of transplanted CFU-S in the spleen, but not in the bone marrow.

Hemopoietic depressions are thought to be induced by two mechanisms[18]. One is a direct damage to hemopoietic stem cells, such as by X-irradiation and by anti-tumor drugs. The other is an indirect growth inhibition of the stem cells via hemopoietic supportive microenvironment, which can support proliferation and differentiation of hemopoietic stem cells. The existence of hemopoietic depressions by these mechanisms was demonstrated by skillful analysis of genetic anemic mice using CFU-S assay, showing that the anemia of genetically anemic W/W^v and Sl/Sl^d mice are due to the defect of the stem cells and hemopoietic supportive microenvironments, respectively[19,20]. The principal mechanism of N_2O-induced hemopoietic depression may be the latter for the following reasons: 1) N_2O did not directly affect the colony formation GM-CFC *in vitro*, as reported by Nunn and his colleagues[21]. 2) Extent of the decreases in the numbers of CFU-S and GM-CFC, induced by *in vivo* inhalation of N_2O, showed the difference between the spleen and the bone marrow[10]. If N_2O had directly inhibited the proliferation and differentiation of hemopoietic stem cells, above difference would not have been observed. 3) The similar difference was observed in the recovery of hemopoietic stem cells after irradiation in mice exposed to N_2O[11]. 4) Furthermore, the present data directly demonstrated the inhibitory effects of N_2O on one of the functions of hemopoietic supportive microenvironment.

It is well known that the spleen is one of the hemopoietic tissues in adult mice and a primary organ for acute hemopoietic response to exogenous stimuli, such as bacterial infection and X-irradiation[22–24]. The differences between splenic and medullary hemopoiesis are attributable to the difference in the hemopoietic supportive microenvironments. Present results suggest that N_2O inhalation causes damage of splenic hemopoietic microenvironments, which can support the settlement of transplanted CFU-S. Although the seeding efficiency of CFU-S is one aspect of the function of hemopoietic supportive microenvironments, N_2O may inhibit murine hemopoiesis by way of hemopoietic supportive microenvironments.

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