Sulfobetaine (Dimethylsulfoniopropionate) and Glycine Betaine Show Incompatible Involvement in Crucial Ehrlich Ascites Carcinoma in Mice

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Abstract: *Background/Aim: The role of methylation reactions in cancer was examined using the methylating agents, sulfobetaine [dimethylsulfonioproponate (DMSP)], and glycine betaine (GB), in murine crucial Ehrlich ascites carcinoma (EAC) for up to 10 days. Results: DMSP administration in EAC-bearing mice mitigated EAC, while GB administration clearly promoted EAC. However, the immune cell profiles did not differ largely between animals receiving DMSP and those receiving GB. Moreover, DMSP and GB had merely any effects on proliferation of EAC cells in vitro. Injection of DMSP into normal mice interestingly led to macrophage accumulation in the peritoneal cavity in a dose-dependent manner at early rearing. Conclusion: These results indicate that GB promoted EAC by the methylation of cancer promotor gene, whereas DMSP ameliorated EAC by the accumulation of activated macrophages with a rapid response and long life span during cancer progression.*

Glycine betaine (GB) is found in a variety of natural and processed foods (1, 2). GB effectively acts as a methyl donor (3-5) and as an osmolyte (3, 6). Sulfobetaine [dimethylsulfoniopropionate (DMSP)] occurs in ocean-dwelling bacteria, micro- and macro-algae, and halophytic plants (7-10) and functions as a methyl donor in fish and animals (11) and osmolytes in aquatic plants (10). DMSP and GB have very similar metabolic functions and form zwitterions at neutral pH. We previously investigated the effects of DMSP on a variety of inflammatory and degenerative disorders in the viscera and brains of animals and found it to have favorable effects in ameliorating diseases (12).

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Among diseases affected by DMSP, cancer continues to increase in humans, with high risk especially in aged people (13). Therefore, while a number of chemopreventative drugs have been used singly and combinatorially for cancer therapy (14, 15), drugs eradicating cancer have not been found.

Ehrlich ascites carcinoma (EAC) cells elicit critical free-cell cancers in the peritoneal cavity when intraperitoneally (*i.p.*) injected into mice (16), with death occurring ~2 weeks after injection (16, 17). Therefore, this mouse model is likely to be very useful for investigating the therapeutic efficacy of potential anticancer drugs in humans.

We, therefore, attempted to investigate the role of methylation in the promotion of EAC using DMSP and GB.

Materials and Methods

Reagents, cells, and animals. DMSP was synthesized by refluxing dimethylsulfide and 3-bromopropionic acid, washing with ethyl ether and crystallizing the product in methanol to a purification level of 99.8% by elemental analysis (18). Betaine (glycine betaine) (GB-HCl) was obtained from Wako Pure Chemicals Co. Ltd., Osaka, Japan, and washed several times with ethyl ether. 3-Bromopropionate was purchased from Tokyo Kasei Kogyo Co. Ltd., Japan. Harm's medium and fluid thioglycollate (TG) medium (DAIGO) were obtained from Wako, Pure, Chemical, Co. Ltd. Unless otherwise stated, other chemicals were purchased from Wako Pure Chemicals, Co. Ltd.

EAC cells were kindly donated by the Cancer Cell Repository, Research Institute for Tuberculosis and Cancer, Tohoku University, Sendai, Japan. The cells were maintained *in vivo* in mice by weekly *i.p.* transplantation. Three-week-old ICR/Jcl male mice were purchased from CLEA Japan Inc., Osaka, Japan. The mice had free access to distilled water and solid diets (MF; CLEA Japan Inc.) and were maintained in a room with a 12-h dark/light cycle at around 60% relative humidity and at 24±2˚C in a pathogen-free environment. The experiments with mice were performed under sterile conditions to the greatest extent possible.

Animals treatments. Care and treatment of experimental animals were performed in accordance with the National Institutes of Health Guide (19) and also with the Koshien University Guide for the Care and Use of Laboratory Animals.

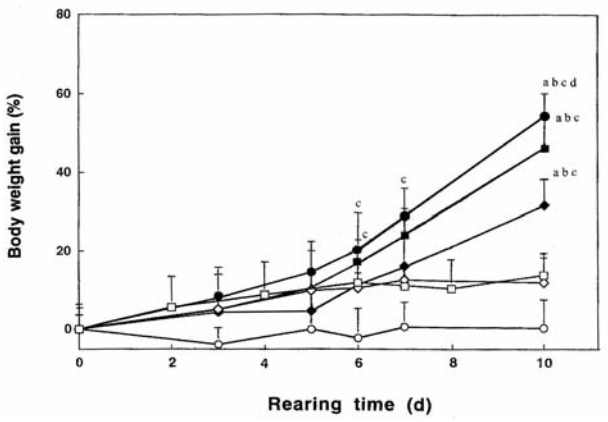

Figure 1. *Effects of dimethylsulfoniopropionate (DMSP) and glycine-betaine (GB) on body weights of Ehrlich ascites carcinoma (EAC)-bearing mice at the indicated times for up to 10 days. Data are means±SD (n=8).* □, ◊, ○, ■, ●, ♦: *control, DMSP, GB, EAC-control, EAC-GB, and EAC-DMSP groups. p<0.05 (vs. [a]control, [b]DMSP, [c]GB, and [d]EAC-DMSP groups at the indicated times, by Tukey-Kramer test).*

Preparation of test mice. After preliminary acclimatization for one week, the mice (35.4±0.21g) were divided into six groups with eight mice per group. Subsequently, 0.5 ml aliquots of saline, DMSP, and GB solutions [5 mM each (0.55 and 0.29 mg/mouse, respectively] were initially administered *i.p.* to the six groups of mice. An appropriate volume of ascitic fluid containing EAC cells was withdrawn from the peritoneal cavity of EAC-bearing mice, centrifuged, and then suspended in Harm's 12F medium before further centrifugation. EAC cells obtained were suspended in Ham's medium containing 8% fetal bovine serum. The EAC cell suspension (0.5 ml, 2×10^6 cells) was then administered *i.p.* to three groups mice treated with saline, DMSP, or GB solutions. Thus, the test group comprised of six sub-groups: Control, DMSP, GB, EAC-control, EAC-DMSP, and EAC-GB.

Determination of body weights and ascitic fluid. The mice were housed for 10 days and body weights were measured in all the groups at the specified times. Ascitic fluids in the peritoneal cavity of the test mice were collected carefully into plastic tubes first from a hole in the abdomen by a syringe and then the cut end of the abdominal cavity with scissors under deep ether anesthesia and the volume of ascitic fluid was measured at 10 days.

Preparation and determination of white blood cells (WBCs) and WBC differential. The blood was drawn from the portal vein of test mice immediately after the removal of ascitic fluid. The blood was agitated with Turk solution in a Melangeur to count WBCs. WBCs were counted in a Neubauer hematocytometer under magnification (×100-400). To prepare a blood smear, an appropriate amount of blood was spread evenly in a layer on a glass slide and dried in air. The slide was fixed in methanol. Wright solution was then added for 2 min. After removal of residual solution, phosphate buffer was added to the slide, and then the slide was washed with distilled water and dried. Giemsa solution was added to the slide for 10-20 min, followed by washing with distilled water and drying. Then the glass slide was immersed in xylene solution and mounted. The WBCs and lymphocytes and neutrophils were carefully examined using microscopic observation (20).

Isolation of macrophages. Two milliliters of 20 mM DMSP solution (final) were injected *i.p.* into five normal 4-week-old mice and their abdomens were softly massaged to equally spread the test solution into the peritoneal cavity. Three days later, the ascitic fluid in the peritoneal cavity was collected in a plastic Petri dish from the cut end of the abdomen of test mice and residual ascitic fluid was further collected by washing the peritoneal cavity with cold phosphate-buffered saline (PBS). The cells were then allowed to adhere for 2 h at 37˚C, and non-adherent cells were removed by aspiration and washing. The adherent cells (macrophages) were then washed twice and carefully collected using a rubber scraper after dislodging with chilled PBS (20).

Incubation of DMSP and GB solutions with EAC cells and macrophages. EAC cells and macrophages freshly prepared as described above were incubated with 5 and 20 mM DMSP and 20 mM GB solutions (final) in Harm's medium (8 ml) with fetal bovine serum (5×10^4 cells/ml, each) in a controlled atmosphere at 37˚C for up to 5 h. One milliliter of the medium was removed from the incubation mixture at different times. The number of EAC cells and macrophages in the medium was counted using a Neubauer hemocytometer according to the trypan blue dye exclusion method.

Effects of DMSP and TG medium on the accumulation of macrophages. Two milliliters of 5, 10, and 20 mM DMSP solutions and medium with TG, an inflammatory agent (21), was administered *i.p.* to five normal 4-week-old male mice. After three days, accumulated macrophages were isolated and counted as described above.

Statistical analyses. Statistical analyses were performed using ANOVA, Tukey-Kramer, and Fisher's protected least significant difference (PLSD) tests (StatView, version 5; SAS Institute, Inc., Cary, North Carolina, USA).

Results

Determination of body weight and volume of ascitic fluid. Body weights were measured in all groups at specified times for up to 10 days. The results are given in Figure 1. The body weights of the EAC-GB and EAC-control groups in this order rapidly increased over the10-day period. The body weights in the EAC-DMSP group decreased more remarkably than those in the EAC-GB group and clearly tended to decrease more than those of the EAC-control group for up to 10 days. The body weights of the Control and DMSP groups increased slightly and similarly up to 10 days, whereas those of the GB group remained unchangeable, showing almost no growth.

The volumes of ascitic fluid in all groups were examined on the 10th day. The results are given in Table I. No fluid was found in the Control, DMSP and GB groups. However, the volumes of the fluids in the EAC-control and EAC-GB groups were larger than that of the EAC-DMSP group ($p<0.05$).

Table I. *Effects of dimethylsulfoniopropionate (DMSP) and Glycinebetaine (GB) on the hematological parameters of Ehrlich ascitic carcinoma (EAC)-bearing mice on the 10th day after the start of the experiment*

Group	Ascitic fluid (ml)	WBC ($\times 10^6$ cells/μl)	Lymphocyte (%)	Neutrophil (%)
Control	–	3.9±2.10	69.5±6.93	19.5±0.78
DMSP	–	3.7±0.35	73.1±2.16	19.7±0.54
GB	–	3.8±80.41	72.1±2.11	19.5±0.53
EAC-control	3.9±0.80	31.6±3.81[a]	34.2±5.26 [ac]	64.3±4.30
EAC-DMSP	2.2±0.19[b]	8.8±1.13[ab]	75.5±3.10[b]	21.5±2.73[b]
EAC-GB	4.6±1.20[c]	8.9±0.65[ab]	70.5±2.03[b]	27.3±2.46[abc]

Data are means±SD (n=8). –: Negligible effect. $p<0.05$ (*vs*. [a]control, [b]EAC-control and [c]EAC-DMSP groups, by Tukey-Kramer test or PLSD test.)

Table II. *Effects of dimethylsulfoniopropionate (DMSP) and glycinebetaine (GB) on proliferation of Ehrlich ascitic carcinoma (EAC) cells (A) and macrophages (B) grown on Harm's medium containing fetal bovine serum*

A Group	Incubation times (h)		
	1	3	5
	EAC cells ($\times 10^5$)		
Control	13.8±1.28	15.3±1.23	14.7±1.71
DMSP (5 mM)	14.1±0.87	15.6±1.23	15.9±1.08
DMSP (20mM)	14.7±1.89	15.9±1.50	16.5±1.89
GB (5 mM)	13.8±0.93	14.4±1.08	15.3±1.20
GB (20 mM)	15.3±2.16	14.3±2.34	9.7±2.31[abcd]
B Group			
	Machrophages ($\times 10^5$)		
Control	5.4±0.78	6.9±0.74	6.6±0.75
DMSP (5 mM)	5.6±0.71	7.0±0.64	6.8±0.76
DMSP (20 mM)	6.1±0.74	7.2±0.76	6.8±0.48
GB (5 mM)	5.9±0.68	6.5±0.86	6.4±0.70
GB (20 mM)	6.1±0.95	4.3±0.82[abcd]	2.2±0.86[abcd]

Data are means±SD (n=5). $p<0.05$ (*vs*. [a]control, [b]5mM DMSP, [c]20 mM DMSP, and [d]5 mM GB groups at the indicated times, by Tukey-Kramer test).

Determination of WBCs and differential. The effect of 5 mM DMSP and GB solutions on the amounts and types of WBCs in EAC-bearing mice were estimated at 10 days. The results are given in Table I. The number of WBCs in the EAC-control group corresponded to about an 8-fold increase compared to the control group and to a ~3.6-fold increase compared to the EAC-DMSP and EAC-GB groups. In contrast, the number of WBCs in the EAC-DMSP and EAC-GB groups corresponded to a ~2-fold increase compared to that of the control group. The number of lymphocytes in the EAC-control group was the highest among all groups, at ~3-fold that of the control group. Additionally, the number of neutrophils in the EAC-control group corresponded to ~29-fold that of the control group, whereas the number of lymphocytes increased by ~2.9- and 2.7-fold and the number of neutrophils by ~5.3- and 7.7-fold in the EAC-DMSP and EAC-GB groups, respectively, compared to those in the control group.

Incubation of EAC cells and macrophages in vitro with 5 and 20 mM DMSP and GB solutions. Incubation of EAC cells with 5 and 20 mM DMSP or GB solutions was performed in Ham's medium with fetal bovine serum for up to 5 h. The results are given in Table II. The proliferation of EAC cells in the control, 5 and 20 mM DMSP-treated, and 5 mM GB-treated groups remained unchanged for the duration of the experiment, whereas proliferation of EAC cells in the 20 mM GB-treated group was restricted only at 5 h ($p<0.05$). Similarly, macrophages in the control, 5 and 20 mM DMSP-treated, and 5 mM GB-treated groups also remained unchanged for the duration of the experiment, whereas macrophages in the 20 mM GB-treated group were restricted in a time-dependent manner ($p<0.05$).

Effect of DMSP concentration and TG medium on macrophage accumulation. Two milliliters of 5, 10, and 20 mM DMSP solutions or TG medium were administered to normal 4-week-old male mice, and the number of accumulated macrophages was examined three days after injection. The results are given in Table III. DMSP administration increased the number of macrophages in a dose-dependent manner (R^2=0.980) in the peritoneal cavity, without apparent side-effects. Administration of TG medium also increased macrophage accumulation similarly to 20 mM DMSP solution.

Discussion

The hematological profile comparisons revealed that the number of WBCs, lymphocytes, and neutrophils in EAC bearing-mice administered 5 mM DMSP increased by ~1.4-, 0.8-, and 3.4-fold, respectively, compared to administration of 10-mM DMSP under the same experimental conditions (20). While the number of lymphocytes was largest, the number of neutrophils was incredibly large in EAC-bearing mice. This fact indicates that WBCs, lymphocytes and neutrophils, particularly neutrophils, actively function in the more promoted-states of EAC upon the administration of 5 mM DMSP compared to the promoted-state of EAC in the administration of 10 mM DMSP. The administration of 5 or 10 mM DMSP solution to EAC-bearing mice reduced the volume of ascitic fluid by ~44% or 62% (20), respectively, compared to those in EAC-bearing mice. Furthermore, the profiles of immune cells in the EAC-DMSP and EAC-GB groups was almost similar, while increases in the body weights due to ascitic fluid were very different. These findings clearly indicate that the

Table III. *Effects of dimethylsulfoniopropionate (DMSP) and thioglycollate (TG) medium on accumulation of macrophages*

Group	Macrophages ($\times 10^5$ cells/ml)	Stimulation (%)
Control	8.1±3.61	100
DMSP (5 mM)	28.6±2.43[a]	353
DMSP (10 mM)	39.4±2.62[ab]	484
DMSP (20 mM)	65.7±3.10[abc]	811
TG medium	59.4±6.10[abc]	733

Data are means±SD (n=5). $p<0.05$ (*vs* .[a]control, [b]5 mM DMSP, and [c]10 mM DMSP groups, by Tukey-Kramer test)

administration of DMSP and GB to EAC-bearing mice leads to different outcomes.

Incubation of EAC cells with 5 mM DMSP and GB solutions scarcely inhibited EAC cell proliferation up to 5 h ($p<0.05$). Therefore, DMSP and GB are likely not to function as osmolytes for EAC cells. However, incubation of EAC cells with 20 mM GB, but not 20 mM DMSP, inhibited EAC cell proliferation at 5 h. Higher concentrations of GB are likely cytotoxic not only to EAC cells but also to normal cells, as shown in Figure 1.

In EAC-bearing mice, GB does not act as an osmolyte or an activator of immune systems, but GB likely acts as an effective methyl donor. GB reacts with homocysteine to form methionine and dimethylglycine by Betaine-Homocysteine S-methyltransferase (BHMT) (4-6). Methionine is further converted to S-adenosylmethionine by methionine S-adenosyltransferase. Thus, GB plays a central role as a methyl donor in metabolism in animals and humans (3-5). However, it remains unclear whether methylation mediated by GB (22) or folate and vitamin B12 (23) ameliorates cancer (24). Recently, many reports showed that the hypermethylation of tumor-promotor genes leads to tumor progressions (25-27). Our results indicate that ascitic fluid and body weight significantly increased upon administration of GB, or methylmethionine (20), a more effective methyl donor than GB (11), compared to those of EAC-bearing mice. Accordingly, these results strongly support that EAC, which originated from breast cancer in mice (28), occurs and progresses more rapidly following GB-mediated methylation in EAC-bearing mice. This DNA methylation appears to go beyond the inhibition of BHMT by dimethylglycine (29) or S-adenosylmethionine (4, 30). This is supported by the fact that the trans-sulfuration reaction that forms cysteine *via* cystathionine from homocysteine does not actively occur after GB administration (4, 5). Furthermore, this is supported by the fact that the administration of GB, DMSP, and folic acid to rats with hyperhomocysteinemia rapidly reduces homocysteine, while plasma levels of cysteine and cysteinylglycine do not increase, but rather decrease at early stages of the disease (unpublished data).

In acute EAC-bearing mice, we previously found that 5 or 10 mM DMSP activated the delayed-type hypersensitive immune reaction in mice (31) and rats (32). Furthermore, a single administration of 10 mM DMSP to EAC-bearing mice reduced ascitic fluid to nearly one third to that of EAC-bearing mice (20). Free ingestion of 10 and 20 mM DMSP solutions (average of 104 and 208 mg/day/rat) for 33 weeks resulted in complete healing of chronic 3'-methyl-4-dimethylaminoazobenzen-induced-liver cancer in rats without side-effects (32). In particular, *i.p.* administration of 10 and 20 mM DMSP prior to injection of EAC cells prolonged the lives of the majority of EAC bearing-mice for more than 300 days (31). These facts suggest that immune system activation remains effective for a long time without subsequent DMSP treatment in EAC-bearing mice.

Of note, single *i.p.* administration of a large amount of DMSP (3.2 M, 1 ml) to rats, and oral and sequential administration of high-dose DMSP (208 mg/day/rat) for up to 33 weeks to young rats (32) or a single *i.p.* injection of DMSP (180 mM, 1 ml) to juvenile mice caused no toxicity. These findings indicate that it is possible to administer DMSP orally and intraperitoneally at high and more frequent doses to animals suffering from acute and chronic cancer without side-effects *in vivo*. In fact, this was clearly verified, as *i.p.* administration (0.5 ml) of 70 mM DMSP completely eradicated EAC without any side-effects and with no toxicity in a recent study (33).

Intraperitoneal administration of 5-20 mM DMSP to normal 4-week-old male mice elicited activated macrophage accumulation in a dose-dependent manner at early rearing times. The activated macrophages appear to be noticeably involved in the healing of EAC-bearing mice without toxicity, because activated macrophages rapidly and significantly respond to cancer cells (34, 35), and have a long life-span (36). This insight is strongly supported by the fact that incubation of isolated macrophages with EAC cells significantly reduced the number of EAC cells in vitro (33).

Furthermore, *i.p.* administration of DMSP reduced the volume of ascitic fluid in a dose-dependent fashion ($R^2=0.964$) (33) and increased activated macrophages in a dose-dependent manner ($R^2=0.980$). Therefore, the decrease in ascitic fluid and subsequent increase in survival of EAC-bearing mice are directly attributable to the increase in activated macrophages following administration of DMSP in EAC-bearing mice.

There is no report of macrophage accumulation following administration of high GB concentrations. Rather, incubation of macrophages (5×10^4 cells/ml) with GB (20 mM) on Harm's medium with fetal bovine serum inhibited macrophage proliferation by about 58.9% and 66.7% at 3 and 5 h, respectively, compared to that of the corresponding control groups.

These results and other associated findings suggest that GB acts as a methyl donor for the methylation of tumor-promotor

genes, thereby promoting cancer, whereas DMSP functions as a strong activator of T-lymphocytes (31, 32), neutrophils, and, in particular, activated macrophages in the innate immune system without a cytokine storm in EAC-bearing mice with no side-effects. Furthermore, resident activated macrophages in normal mice increased by a 2-fold by administration of TG medium (37), while recruited activated macrophages increased by a ~7- and ~ 8-fold by administration of TG medium and DMSP (Table III). Therefore, DMSP accelerates monocyte-/macrophage differentiation of monocytes from myeloid progenitor cells (34-36) and regenerates inflamed and/or damaged cells and tissues to lineage cellular population via multipotent stem cells formed from hematopoietic stem cells in the bone marrow without a cytokine storm and no toxicity in a complex network (34, 35, 38) in acute and chronic cancer in mice (20, 31, 33) and rats (32). Accordingly, DMSP not only destroys the capacity of tumor cell populations to evolve leading to effective killing of EAC cells (39) but also overcomes resistance: the double-edged sword of anticancer drugs (40). Therefore, DMSP most likely plays a vital role for cancer therapy.

In conclusion, our results demonstrate that GB clearly promoted, while DMSP ameliorated, the progress of EAC in mice. These findings are likely due to GB-induced methylation of the tumor-promotor gene, whereas DMSP increased activated-macrophage recruitment and regenerated damaged cells in the microenvironment by recruiting tissue-specific stem cells.

Conflicts of Interest

The Authors declare that they have no competing interests.

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