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Development of a model of melphalan-induced gastrointestinal toxicity in mice*

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Summary. The tolerated dose of melphalan is limited by bone marrow suppression; when this complication is ameliorated by bone marrow transplantation, the dose-limiting toxicity becomes gastrointestinal mucositis. No intervention to date has been successful in modulating this life-threatening complication of melphalan. We conducted studies to develop a murine model of melphalan-induced gastrointestinal toxicity to facilitate the preclinical identification of effective strategies for reducing this toxicity. Melphalan given at the 90% lethal dosage produced severe gastrointestinal mucositis and mortality (13 of 23 treated mice). Syngeneic bone marrow transplantation, effective in preventing the myeloablation produced by total-body irradiation, was ineffective in preventing melphalan-induced mortality (16 of 23 treated mice), indicating that gastrointestinal mucositis was the dose-limiting toxicity. On the basis of the results of previous studies, which revealed that depletion of glutathione enhances the antineoplastic activity of melphalan and that glutathione is required for murine intestinal function, we attempted to modulate melphalan-induced gastrointestinal toxicity by the administration of glutathione (8–10 mmol/kg given in 1 ml sterile water by gavage at 12-h intervals for 4–8 doses). Glutathione therapy failed to produce a significant increase in mucosal glutathione content in animals treated with melphalan plus glutathione gavage as compared with those receiving melphalan alone ($P > 0.05$), and histologic mucosal injury secondary to melphalan was not reduced. The administration of glutathione in the presence or absence of concomitant bone marrow transplantation did not decrease melphalan-induced mortality (melphalan alone, 16/26 deaths; melphalan plus glutathione, 14/25 deaths; melphalan plus glutathione plus bone marrow transplantation, 20/26 deaths). Studies using a reduced melphalan dose (50% lethal dosage) produced similar results, with no survival benefit being seen following glutathione administration. Our studies suggest that melphalan-induced mucositis can be studied in a mouse model in which this complication is dose-limiting. Although glutathione administration at the dose and schedules initially studied is not effective in reducing this damage, other therapeutic strategies such as the use of alternative glutathione regimens or other thiols can be effectively studied in this system.

Introduction

The dose-limiting toxicity of melphalan in conventional oncologic practice is bone marrow suppression [4], an observation correctly predicted in early toxicology studies in mice, dogs, and primates [13]. Dose escalation made possible by autologous or allogeneic bone marrow transplantation, however, results in the appearance of a new dose-limiting nonhematopoietic toxicity, gastrointestinal mucositis [7, 8]. Unfortunately, no intervention has been developed to date that diminishes this serious and potentially life-threatening complication of melphalan. Progress in this area has been limited by the absence of a convenient animal model suitable for study of the gastrointestinal toxicity.

We report the development of a model of melphalan-induced toxicity in mice in which gastrointestinal mucositis is the lethal complication of melphalan given at the 90% lethal dose. This model provides the opportunity to define in preclinical studies a therapy that will be effective in ameliorating melphalan-induced mucositis.

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Materials and methods

Animals

All studies used male or female athymic BALB/c mice (nu/nu genotype; age, 6 weeks or older). Animals were maintained as previously described [3].

Drugs

Melphalan (L-phenylalanine mustard), provided courtesy of Burroughs Wellcome Co. (Research Triangle Park, N. C.), was given at a dose of 95.7 or 85.5 mg/m^2, which represent the 90% and 50% lethal doses, respectively, as a single intraperitoneal injection in 17% dimethylsulfoxide. Glutathione (Sigma Chemical Co., St. Louis, Mo.) was given in 1 ml sterile water (pH adjusted to 5.5–6 with NaOH) by gavage at 12-h intervals for 4–8 doses.

Total-body irradiation

Mice were irradiated in a Mark 1-68 R irradiator (J. L. Shepard and Associates, San Fernando, Calif.) at a rate of 17.4 cGy/s.

Bone marrow transplantation

Bone marrow harvest and preparation was carried out under sterile conditions. Donor animals were killed by cervical dislocation. The femurs were surgically removed, the ends were cut off, and the marrow was flushed out with 3–4 ml Dulbecco's phosphate-buffered saline. The suspension of marrow cells, maintained at 4°C, was centrifuged at 7000 rpm for 7 min. After the pellets had been resuspended in Dulbecco's phosphate-buffered saline and pooled, the WBC was determined using a hemocytometer. The sample volume was than adjusted such that each irradiated or melphalan-treated (90% lethal dose) animal received 10^7 cells in 0.2 ml via the tail vein when injected at 24 h following irradiation or melphalan treatment.

Protection by bone marrow transplantation. The efficacy of bone marrow transplantation was assessed by survival analysis of mice receiving bone marrow 24 h after undergoing total-body irradiation with 850–1000 cGy or treatment with melphalan (90% lethal dose).

Histology studies. Groups of mice were killed at 5 days following melphalan treatment (90% lethal dose) by cervical dislocation, and the entire intestine was removed and fixed by immersion in 10% formalin. Four cross sections of small intestine were taken at equivalent intervals and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin and examined by light microscopy. The severity of intestinal epithelial injury was graded using the following criteria: grade 0, no lesion; grade 1, occasional (<10%) crypts contain individual necrotic cells; grade 2, many (>10%) crypts contain necrotic cells but the crypt architecture is intact [often accompanied by irregularities (shape and polarity) of remaining crypt-cell nuclei]; grade 3, many (>10%) crypts contain necrotic cells showing focal loss of crypt architecture (<20%), some villi are shortened, and variable hypertrophy/hyperbasophilia is apparent in the remaining crypt cells; and grade 4, same as grade 3 except that the loss of crypt architecture and villus shortening are more extensive.

Experimental design

Measurement of the glutathione content of gastrointestinal mucosa. Groups of 3–6 mice were treated in experiments 1–5 with melphalan (90% lethal dose) alone or in combination with glutathione gavage (8 mmol/kg) at 12-h intervals for 8 doses ending 6 h prior to treatment with melphalan or normal saline. All animals were killed by cervical dislocation at 5–78 h following melphalan administration, and a section extending from the duodenum to the cecum was immediately excised. The lumen was perfused with 10 ml cold saline and opened to expose the mucosa. The mucosal surface was blotted dry, and the mucosal layer was gently scraped off the muscularis with a spatula, weighed, and homogenized in 1.0 ml 10% 5′-sulfosalicylic acid. Homogenates were centrifuged for 5 min at 4000 g (4°C), and the supernatant was assayed for glutathione content as previously described [14].

Survival studies. In experiments 6 and 7, groups of 6–10 mice were gavaged with glutathione (8 mmol/kg) or normal saline at 12-h intervals for 8 doses and melphalan (90% lethal dose) was given 6 h later. In experiment 8, groups of 10 mice were gavaged with glutathione (8 mmol/kg) or normal saline at 8 h, 4 h, and 15 min prior to treatment with melphalan (90% lethal dose). In experiment 9, groups of 10 mice were gavaged with glutathione (8 mmol/kg) or normal saline at 8 h, 4 h, and 15 min prior to treatment with reduced-dose melphalan (50% lethal dose). In experiments 6–8, animals receiving glutathione were randomized to receive or not receive donor bone marrow at 24 h after melphalan administration, with all animals subsequently being monitored daily for survival. In experiment 9, all animals were given donor bone marrow 24 h after melphalan.

Histology studies. In animals that died during experiment 8 after undergoing treatment with melphalan and bone marrow transplantation, the right femur was removed, fixed in Zenker's solution, and decalcified, and sections were cut out and stained with hematoxylin and eosin. Groups of 3–6 mice in experiments 10–12 were subjected to glutathione gavage prior to treatment with melphalan and were killed 5 days later; the entire intestine was removed, fixed, and examined, and the histologic damage was graded on a scale ranging from 0 to 4 as described above.

Table 1. Modulation of murine mortality secondary to total-body irradiation or melphalan by bone marrow transplantation

Treatment	Bone marrow transplantation	Mortality
Total-body irradiation (850 cGy)	No	6/6
	Yes	0/6*
Total-body irradiation (950 cGy)	No	4/5
	Yes	1/6*
Total-body irradiation (1000 cGy)	No	5/6
	Yes	0/5*
Melphalan[a]	No	13/23
	Yes	17/23[ND]

[a] Melphalan was given i.p. in 17% dimethylsulfoxide at 95.7 mg/m^2, the 90% lethal dose

* Difference between transplanted and nontransplanted mouse survival statistically significant ($P \leq 0.01$)

ND, Difference between transplanted and nontransplanted mouse survival not statistically significant ($P > 0.05$)

Results

Efficacy of bone marrow transplantation

Mice receiving 850 cGy total-body irradiation developed petechiae and purpura 9–10 days later and died between day 13 and day 16 postirradiation. When the dose was increased to 950 or 1000 cGy, deaths occurred between

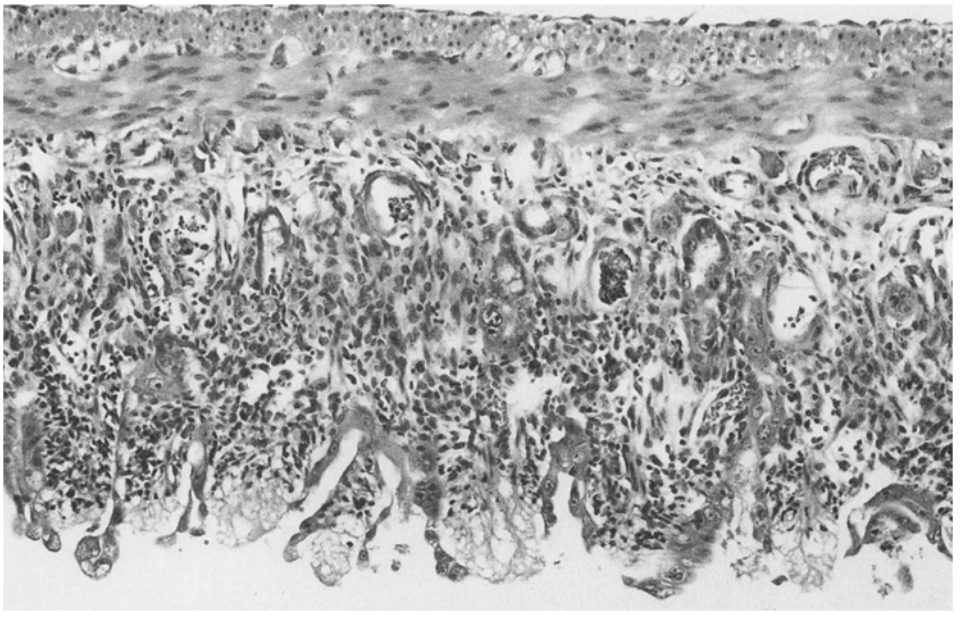

Fig. 1. Melphalan-induced injury in the small intestine, manifesting as necrosis and loss of the crypt epithelium and irregularities in the size and shape of the remaining crypt cells. Note that villi are shortened. (Hematoxylin & eosin-stained section, × 152)

day 8 and day 11 postirradiation. Bone marrow transplantation substantially reduced the mortality of mice receiving total-body irradiation but did not alter that produced by melphalan (Table 1), with deaths occurring between day 5 and day 7 after melphalan treatment and between day 5 and day 7 following therapy with melphalan plus bone marrow transplantation.

Histologic analysis

Treatment of mice with melphalan produced moderate to severe intestinal damage, with a mean grade of 3.3 ($n = 3$), 2.4 ($n = 5$), and 2.0 ($n = 3$), respectively, being recorded in three separate experiments (Fig. 1).

Modulation of melphalan-induced toxicity by glutathione gavage

Mucosal glutathione levels. The mean mucosal glutathione level determined in control mice was 2.93 ± 0.56 nmol/mg wet weight of tissue. The modulation of mucosal glutathione levels following treatment with melphalan or glutathione gavage alone or in combination is shown in Table 2. Treatment with melphalan did not produce a reproducible decrease in mucosal glutathione content as measured at 5–78 h after drug administration. Although glutathione gavage did occasionally result in a significant increase ($P < 0.01$) in glutathione content as compared with control values (experiment 3), it did not produce a significant increase ($P > 0.05$) in animals treated with melphalan plus glutathione gavage as compared with those receiving melphalan alone.

Survival studies. In experiments 6 and 7, the mortality of mice treated with melphalan (5/6 and 5/10 mice in experiments 6 and 7, respectively) was not decreased by glutathione gavage (3/5 and 3/10 mice in experiments 6 and 7, respectively). Furthermore, glutathione gavage in combination with bone marrow transplantation did not reduce melphalan-induced mortality (6/6 and 5/10 mice in experiments 6 and 7, respectively). In experiment 8, the mortality following melphalan administration (6/10 mice) was also not decreased by glutathione gavage (8/10) or glutathione gavage plus bone marrow transplantation (9/10). In experiment 9, the mortality following melphalan administration plus bone marrow transplantation (8/10) was not reduced by glutathione gavage plus bone marrow transplantation (5/10).

Histology studies. Bone marrow sections obtained from mice in experiment 8 revealed evidence of marked pancytopenia (<5% cellularity) in 6 mice and 25%–50% cellularity in 3 mice. Glutathione gavage did not significantly reduce ($P > 0.05$) the intestinal damage produced by melphalan (Table 3).

Discussion

The initial preclinical evaluation of melphalan revealed that myelosuppression was the toxic, dose-limiting factor of this alkylating agent [13]. Clinical trials confirmed these observations, with granulocytopenia and thrombocytopenia limiting the dose escalation to 20–45 mg/m^2 [4]. Amelioration of lethal bone marrow suppression by autologous or allogeneic transplantations, however, has led to the use of much higher melphalan doses and to the appearance of a new dose-limiting toxicity gastrointestinal mucositis [7, 8]. This potentially lethal complication, which is further exacerbated by the use of other chemotherapeutic agents such as etoposide or cyclophosphamide, has no effective treatment other than supportive care.

The present studies were carried out to develop a murine model of melphalan-induced gastrointestinal mu-

Table 2. Murine levels of jejunoileal mucosal glutathione following treatment with melphalan and/or glutathione gavage

Treatment groups[a]	Interval between melphalan administration and glutathione measurement (h)	Number of mice studied	Glutathione[b] (μmol/g wet weight)
Experiment 1	24		
Control		3	2.81±0.03
Melphalan		3	3.01±0.30
Experiment 1	48		
Control		3	3.36±0.39
Melphalan		3	2.56±0.35
Experiment 1	78		
Control		3	2.43±0.08
Melphalan		3	2.19±0.25
Experiment 2	48		
Control		5	2.23±0.30
Melphalan		6	2.56±0.48
Melphalan + glutathione gavage		6	3.53±0.84
Experiment 3	24		
Control		4	3.49±0.60
Melphalan		4	4.28±0.45
Glutathione gavage		4	5.32±0.86
Melphalan + glutathione gavage		4	5.04±0.30
Experiment 4	48		
Control		3	3.40±0.58
Melphalan		3	3.55±0.19
Glutathione gavage		3	3.81±0.76
Melphalan + glutathione gavage		3	3.95±0.41
Experiment 5	5		
Control		4	2.98±0.58
Melphalan		4	3.45±0.18
Glutathione gavage		4	3.82±0.85
Melphalan + glutathione gavage		4	3.95±1.02

[a] In all experiments, melphalan was given i.p. in 17% dimethylsulfoxide at 95.7 mg/m^2, the 90% lethal dose. Glutathione, (8 mmol/kg) was given in 1 ml sterile water (pH adjusted to 5.5–6) by gavage at 12-h intervals for 8 doses

[b] Mean values ±SD

Table 3. Modulation of melphalan-induced gastrointestinal damage by glutathione gavage in mice

Experiment	Number of mice	Intestinal damage[d, e]
10[a]		
Melphalan	3	3.3
Water gavage – melphalan – water gavage	7	3.1
Glutathione gavage – melphalan – -glutathione gavage	10	3.3
11[b]		
Melphalan	5	2.4
Glutathione gavage – melphalan	6	1.8
12[c]		
Controls	3	0
Glutathione gavage	3	0
Melphalan	3	2.0
Glutathione gavage – melphalan	5	1.8

[a] Treatment protocol: melphalan was given i.p. in 17% dimethylsulfoxide at 95.7 mg/m^2, the 90% lethal dose; water gavage 1 ml sterile water) was done at 12-h intervals for 4 doses, followed by melphalan, then water gavage at 12-h intervals for 8 doses; glutathione gavage was carried out at 10 mmol/kg glutathione in 1 ml sterile water (pH adjusted to 5.5–6) at 12-h intervals for 4 doses, followed by melphalan, then glutathione gavage at 12-h intervals for 8 doses; mice were killed and intestinal sections were taken at 5 days after melphalan administration

[b] Treatment protocol: melphalan was given as described for experiment 10; glutathione was given at 8 mmol/kg in 1 ml sterile water (pH adjusted to 5.5–6) at 12-h intervals for 8 doses, followed by melphalan; mice were killed and intestinal sections were taken at 5 days after melphalan administration

[c] Treatment protocol: melphalan was given as described for experiment 10; glutathione was given at 8 mmol/kg in 1 ml sterile water (pH adjusted to 5.5–6) at 12-h intervals for 8 doses, followed by melphalan; mice were killed and intestinal sections were taken at 5 days after melphalan administration

[d] Mean values for the following grades: 0, no lesion; 1, occasional (<10%) crypts contain individual necrotic cells; 2, many (>10%) crypts contain necrotic cells but the crypt architecture is intact [often accompanied by irregularities (shape and polarity) of remaining crypt-cell nuclei]; 3, many (>10%) crypts contain necrotic cells showing focal loss of crypt architecture (<20%), some villi are shortened, and variable hypertrophy/hyperbasophilia is apparent in the remaining crypt cells; 4, same as grade 3, but loss of crypt architecture and villus shortening are more extensive

[e] No difference (P >0.05) in intestinal damage observed between mice treated with melphalan and those treated with melphalan plus glutathione gavage

cositis to facilitate the identification of preclinical strategies capable of reducing or eliminating this toxicity. The approach chosen, based on studies conducted by Schmitt et al. [13], was to reduce melphalan-induced myelosuppression by syngeneic bone marrow transplantation, allowing evaluation of the gastrointestinal toxicity of melphalan (as assessed by histologic and survival analysis). Initial experiments using syngeneic bone marrow transplantation in animals previously treated with myeloablative total-body irradiation demonstrated nearly complete protection from lethal bone marrow suppression by the infusion of 10^7 cells/mouse. However, the infusion of similarly harvested cells did not abrogate melphalan lethality, indicating that nonhematopoietic toxicity was responsible for the observed deaths. Histologic evaluation of intestines taken from mice treated with melphalan (and transplanted with syngeneic bone marrow) revealed evidence of severe mucosal damage, consistent with the clinical experience with high-dose melphalan and confirming that this was the organ-limiting toxicity. This model provides the opportunity to evaluate therapeutic interventions designed to ameliorate melphalan-induced mucositis without inducing hematopoietic toxicity.

Previous studies have demonstrated enhancement of the antineoplastic activity of melphalan following buthionine sulfoximine-mediated inhibition of glutathione synthesis [12, 14, 15]. Glutathione is a ubiquitous nonprotein thiol that is protective against a spectrum of cellular toxins, including electrophils such as nitrogen mustard-based alkylating agents. The conjugation (and, presumably,

detoxification) of melphalan by glutathione has also been demonstrated [5], and glutathione has been implicated in the protection of cardiac and skeletal muscle from cyclophosphamide-induced toxicity [6]. Furthermore, the administration of glutathione monoethyl ester diminishes the activity of 4-hydroperoxycyclophosphamide against both tumor cells [9] and chick embryo myocytes [10] in vitro and reduces the hepatic toxicity of cyclophosphamide in vivo [16]. These observations, coupled with the recent demonstration that glutathione is required for intestinal function [11], led us to hypothesize that the administration of exogenous glutathione might ameliorate melphalan-induced gastrointestinal toxicity.

Furthermore, the administration of glutathione by gavage has been shown to reverse BSO-mediated depletion of mucosal glutathione without modulating the levels of this thiol in other organs [11]. This avoids the possibility of increasing the glutathione levels in nongastrointestinal tumors in association with a potential reduction in the antineoplastic activity of melphalan (or other alkylators).

To test our hypothesis, mice were evaluated by histologic and survival analyses for modulation of melphalan-induced toxicity by prolonged gavage with glutathione. Unfortunately, this approach was not successful in reducing histologic mucosal damage or decreasing mortality. Furthermore, although glutathione gavage produced small increases in mucosal glutathione content as compared with that in control animals, no difference was observed between animals receiving melphalan, glutathione gavage, or melphalan plus glutathione gavage. Our failure to protect mice from melphalan-induced gastrointestinal toxicity by glutathione administration may have been due to our inability to increase glutathione levels adequately via the methodology employed. Alternatively, the limiting toxicity may occur at some nonmucosal site that is not affected by glutathione. Indeed, there is no indication from these data that glutathione levels were lowered by melphalan treatment. It should be noted in this regard that although some tumors can be made more sensitive to melphalan toxicity by marked glutathione depletion [14], it cannot be assumed that a higher than normal level of glutathione would protect normal tissue from such toxicity.

The present studies suggest that melphalan-induced mucositis can be studied in a mouse model in which mucositis is dose-limiting by incorporating the use of syngeneic marrow transplantation. Although glutathione gavage at the initial dose and on the schedules studied did not prove to be effective in preventing or reducing mucosal damage, other therapeutic strategies, e.g. the use of alternative glutathione regimens or other thiols such as mesna [1, 2] or glutathione esters [12], can be effectively studied in this system.

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