Melphalan Reduces the Severity of Experimental Colitis in Mice by Blocking Tumor Necrosis Factor-α Signaling Pathway

GALINA SHMARINA
ALEXANDER PUKHALSKY
VLADIMIR ALIOSHKIN
Alex Sabelnikov
Melphalan Reduces the Severity of Experimental Colitis in Mice by Blocking Tumor Necrosis Factor-α Signaling Pathway

GALINA SHMARINA, ALEXANDER PUKHALSKY, VLADIMIR ALIOSHKIN, AND ALEX SABELNIKOV

aResearch Centre for Medical Genetics, Moscow 115478, Russia
bG.N. Gabrichevsky Institute of Epidemiology and Microbiology, Moscow 115478, Russia
cEast Carolina University, Greenville, North Carolina 27858, USA

ABSTRACT: Melphalan is an alkylating agent, which is commonly used as an antineoplastic drug. Its cytostatic effect can be realized in humans in the dose range of 0.6–1.4 mg/kg body weight. However, previously it was shown that in the case of gradual dose decrease, the number of targets for alkylation was also reduced and the drug lost its cytostatic properties switching to cell growth modifier. It has been postulated that application of alkylating agents in such ultra-low concentrations (50- to 100-fold lower than cytostatic ones) may result in a beneficial effect in the therapy of diseases associated with mucosa inflammation. The aim of the article was to investigate the effect of ultra-low doses of melphalan in the murine experimental colitis induced by the replacement of drinking water with 5% solution of dextran sulphate sodium (DSS). Daily administration of melphalan (25 μg/kg body weight) markedly reduced the severity of DSS-colitis as determined by clinical and quantitative histological criteria. Both systemic and local anti-inflammatory effects of melphalan have been observed. The possible mechanisms of the beneficial effect of the drug have been discussed.

KEYWORDS: alkylating agents; melphalan; experimental colitis; inflammation; NF-κB; TNF-α

INTRODUCTION

Alkylating agents (cyclophosphamide, chlorambucil, melphalan) are commonly used as antineoplastic and immunosuppressive drugs. Their cytostatic

Address of correspondence: Galina V. Shmarina, Research Centre for Medical Genetics, 1 Moskvorechsch Street, Moscow 115478, Russia. Voice: +7-095-111-85-68; fax: +7-095-324-07-02.

doi: 10.1196/annals.1397.075
effect is mainly associated with cross-linking of DNA double strands\(^1\) and, at higher concentrations, with induction of DNA strand breaks.\(^2\) Obviously, DNA is not a single target for alkylation in the cell, but the others, such as RNA, secondary messengers, or membrane receptors, do not play any role in the cytostatic effect if the drug is used at a DNA-altering dose. In the case of a gradual dose decrease, the number of targets for alkylation is also reduced and the drug switches from cytostatic to cell growth modifier.\(^3\) Thus, alkylating agents in the concentration 10-fold lower than cytostatic ones (30 \(\mu\)g/mL versus 300 \(\mu\)g/mL) affect interleukin-2 (IL-2) production by activated lymphocytes and result in reduced proliferation of the cells.\(^4,5\) Ultra-low doses (100-fold lower than cytostatic concentrations) seem to increase lymphocyte proliferation due to selective deterioration of IL-2 receptor (IL-2R) \(\beta\) chain on the surface of CD8\(^+\) cells.\(^6\) IL-2R is not a unique receptor, which may be blocked with alkylating agents. Ultra-low concentrations of mafosfamide protected fibroblastoid cell line against tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\))-induced cytotoxicity.\(^3\) The aforesaid data suggest that alkylating drugs applied in ultra-low doses should result in a beneficial effect in the therapy of diseases associated with mucosa inflammation.

The aim of the article was to investigate the effect of ultra-low doses of alkylating drug melphalan in the murine experimental colitis.

**MATERIALS AND METHODS**

**Animals**

Male BALB/cJLac mice weighing 18–20 g were obtained from the Laboratory Animals Centre of the Research Centre of Oncology (Russian Academy of Medical Science, Moscow, Russia). All the animals were kept on standard diet and had free access to water.

**Chemical Reagent and the Drug**

Dextran sulphate sodium (DSS), M.W. 36,000–50,000 (ICN Biomedicals, Inc., Aurora, OH), was used for the experimental colitis induction. DSS-colitis treatment was performed with an alkylating drug melphalan (Alkeran\(^6\), The Wellcome Foundation Ltd., London, UK).

**Experimental Procedure**

For the induction of colitis, normal drinking water was replaced with a 5% (w/v) solution of DSS for 10 days. Melphalan was administered daily
intraperitoneally (i.p.) at a dose of 25 μg/kg body weight in 200 μL of phosphate-buffered saline (PBS) beginning on the day of DSS exposure. Time-matched controls consisted of mice-administered DSS or melphalan only, and those injected with PBS. All experimental procedures were approved by the Committee of Animal Research at the Research Centre for Medical Genetics.

**General Assessment of Colitis**

Mean DSS/water and pure water consumption was noted per cage and day. Mice were weighed daily and visually inspected. The following parameters were measured: pose, hair condition, rectal bleeding, diarrhea, and blood in the stool. Each parameter was evaluated from 0 to 4. The total clinical score was the sum of the scores of each parameter. The maximum possible score is 20. On day 6, five animals from each group were used for colitis assessment. The blood was collected from the orbital venous plexus with a glass capillary pipette; then hematocrit and white blood cell number were evaluated. Animals were killed by cervical dislocation. The entire bowel (from stomach to the anus) and the spleen were excised. Bowel length and spleen weight were measured.

**Histology**

Samples of proximal colon (2–3 cm from the ileocecal junction) were fixed in 10% neutral buffered formalin, dehydrated and paraffin-embedded. Seven-μM sections were collected on coded slides, stained with hematoxylin and eosin, and scored in a blind fashion by two investigators as described.³ Three independent parameters were measured: severity of inflammation (0, none; 1, slight; 2, moderate; 3, severe), crypt damage (0, none; 1, basal one-third portion damaged; 2, the basal two-thirds portion damaged; 3, the entire crypt damaged but the surface epithelium intact; 4, the entire crypt and epithelium lost), and depth of injury (0, none; 1, mucosal; 2, mucosal and submucosal; 3, transmural). The total histological score was the sum of the scores of each parameter. The maximum possible score is 10.

**Analysis of NF-κB Activation**

To determine whether melphalan treatment affects the activation of transcriptional factor NF-κB, nuclear translocation of NF-κB was examined with TransAM NF-κB kit (Active Motif, Inc., Carlsbad, CA). A pool of L929 cells was preincubated for 1 h with actinomycin D (10 μg/mL). Subsequently, the cells were treated for another 1 h with melphalan (0.3 μg/mL) and then exposed to TNF-α (64 pg/mL) for 15 min. Nuclear extracts were prepared and tested for NF-κB p65 according to the manufacturer’s recommendation.
**L929 Cell Viability Assessment**

L929 cell were seeded in 96-well plates in complete medium and incubated until monolayer formation. Then the cells were treated with actinomycin D (10 μg/mL) and melphalan (0.1–1 μg/mL) as described above, and exposed to TNF-α (16–64 pg/mL) for 18 h. Subsequently, the cells were stained with 0.2% crystal violet, and then the plates were washed, dried, and read at 540 nm.

**Statistical Analysis**

The results are expressed as means ± SEM. The significance of differences between the groups was evaluated using Student’s *t*-test. Significance was defined at *P* = 0.05.

**RESULTS**

**Clinical and Macroscopic Assessment**

Treatment with 5% DSS in the drinking water induced fulminant colitis characterized by dramatic appearance of bloody diarrhea. Visible alterations in stool consistency have been observed on day 3. Slight loss of body weight was noted on day 5. There were no significant differences in body weight loss between untreated DSS-mice and DSS-mice treated with melphalan. In the same time, the clinical inflammatory score was significantly, but transiently (days 4, 5, and 6), higher in DSS-mice when compared with DSS-mice treated with melphalan: 6.3 ± 0.4 (DSS) versus 4.8 ± 0.5 (DSS + melphalan) (*P* < 0.03) (day 4); 7.2 ± 0.3 (DSS) versus 5.5 ± 0.3 (DSS + melphalan) (*P* < 0.001) (day 5); and 9.3 ± 0.6 (DSS) versus 7.6 ± 0.5 (DSS + melphalan) (*P* < 0.04) (day 6). The percentage survival of DSS-mice decreased by day 6, dropped by day 7 to 40%, whereas 10 of 15 melphalan-treated mice survived during 7 days of the treatment. These results suggest that onset of severe colitis is delayed in the melphalan-treated mice.

At day 6, five animals from each group were sacrificed and the general signs of colitis had been evaluated. DSS-mice exhibited a significant loss of the bowel length (Table 1). These mice also showed a pronounced elevation in peripheral blood white cells as well as a marked increase in relative spleen weight. Such changes were not so evident in DSS-mice treated with melphalan. Thus, there were no significant differences in white blood count and spleen weight compared with the control animals. Moreover, the white blood count in melphalan-treated DSS-mice was significantly lower than that in mice received DSS only.
TABLE 1. Macroscopic, hematological, and histological changes (day 6 of the treatment)

<table>
<thead>
<tr>
<th>Measure</th>
<th>DSS</th>
<th>DSS + Melphalan</th>
<th>Water controls</th>
<th>Melphalan controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of entire bowel (cm)</td>
<td>51.6 ± 0.8*</td>
<td>57.2 ± 1.2*</td>
<td>64.3 ± 1.9</td>
<td>64.2 ± 1.1</td>
</tr>
<tr>
<td>Spleen weight (% of body weight)</td>
<td>0.89 ± 0.08*</td>
<td>0.78 ± 0.05</td>
<td>0.66 ± 0.03</td>
<td>0.71 ± 0.08</td>
</tr>
<tr>
<td>White blood cells (×10^3/μL)</td>
<td>13.4 ± 1.3*</td>
<td>5.9 ± 0.4**</td>
<td>3.8 ± 1.0</td>
<td>4.1 ± 0.9</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>48.0 ± 5.5</td>
<td>53.0 ± 2.1</td>
<td>51.2 ± 4.4</td>
<td>49.0 ± 2.1</td>
</tr>
<tr>
<td>Histological scores:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severity of inflammation</td>
<td>3.0 ± 0.4</td>
<td>2.2 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crypt damage</td>
<td>3.1 ± 0.2</td>
<td>2.0 ± 0.3**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Depth of injury</td>
<td>4.0 ± 0.1</td>
<td>2.1 ± 0.2**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>10.1 ± 0.4</td>
<td>6.3 ± 0.8**</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.05 vs. relative control; **P < 0.05 vs. DSS-treated group.

Histology

DSS administration induced severe disruption of tissue architecture, including edema, a massive immune cell infiltration, ulceration, and significant area of complete epithelial denudation. The thickness of mucosa seemed to be threefold larger than the muscle layer. The most reproducible histological abnormality was brutal crypt damage, including edema, collapse, or complete destruction. Crypt damage was significantly worse in DSS-mice compared with melphalan-treated animals. Thus, histological scores of DSS-mice were in the range of 9 to 10 (10 is maximum), whereas 4 of 5 animals in the melphalan-treated group had scores of <8. It has been noted that the histology was in accordance with relatively low clinical score in melphalan-treated mice (see TABLE 1).

Ultra-Low Concentrations of Melphalan Impair TNF-α Signaling in L929 Cells

It is known that alkylating agents represent one of the most potent inducers of the cellular stress response, a specific program of gene expression, which includes the induction of JNK/SAPK signaling pathways and transcription activation of c-fos and c-jun, whose gene products are likely to be required in cellular defense against cytotoxic agents. Indeed, earlier we have shown that ultra-low concentrations (0.1 μg/mL) of mafosfamide protect murine fibroblastoid cells (L929) against TNF-α-induced cytotoxicity. As can be seen
in Figure 1 A, the similar results have been observed for melphalan-treated L929 cells. Furthermore, the protection does not depend on de novo protein synthesis, because the cells were preincubated with transcriptional inhibitor actinomycin D before melphalan treatment.

To further investigate the underlying mechanisms of protective effect of alkylating agents, the influence of melphalan on TNF-α-activated NF-κB was studied. No increase in NF-κB activity in nuclear extracts of melphalan-treated fibroblasts was observed. In the same time, 1-h treatment with the drug moderately decreased actinomycin D-induced NF-κB activation and markedly reduced the transcription factor activity in nuclear extracts of actinomycin D-pretreated cells challenged with TNF-α (Fig. 1 B). These data support the suggestion that specific alkylation of components in the cytoplasm or cell membrane by melphalan interferes with TNF-α signaling pathway.

![Graph A](image)

**FIGURE 1.** Ultra-low concentrations of melphalan impair TNF-α-induced cytotoxicity and NF-κB transactivation in L929 cells. (A) L929 cell viability. The data are presented as median values. *P < 0.05, compared to the controls. (B) NF-κB p65 in nuclear extracts of L929 cells. The representative results of one from two experiments are shown.
DISCUSSION

In our study, we used the DSS-murine colitis model system, which was exploited as an established general prototype of the biochemical and molecular events that can lead to intestinal tissue damage. The histological aspects of intestinal damage in DSS-induced colitis share many similarities with those seen in patients affected by inflammatory bowel disease (IBD) and, in particular, ulcerative colitis. Thereafter, this model has been recommended and is a widely used preclinical model for testing the efficacy of treatments for IBD. Here, we have demonstrated that melphalan effectively suppressed the development of DSS-induced colitis in mice. Both systemic and local effects of the drug have been observed.

Permanent toxic effect of DSS upon intestinal epithelium results in the disturbance of its barrier function. Leakage of bacteria and/or their products through the intestinal wall triggers both local and systemic inflammatory response. In addition, the phagocytosis of DSS particles can contribute to intestinal inflammation through stimulation of lamina propria cells and increased production of pro-inflammatory cytokines, including IL-1β, IL-12, IL-18, IFN-γ, and TNF-α. The latter is known to play a pivotal role in DSS-colitis development. In the present study, we have shown that ultra-low concentrations of melphalan prevent cell death of murine fibroblasts treated with TNF-α due to the disruption of the signal transduction by the type I TNF receptor. Therefore, one of the possible mechanisms of melphalan beneficial effect in DSS-induced colitis may be associated with the inhibition of apoptotic cell death pathway in colon epithelium exposed to TNF-α, which has been detected in colon as early as 1 day after the start of DSS treatment, with peak production occurring between day 5 and day 7. Other possible mechanism(s) of melphalan effects seems to be related to facilitation of epithelial repair. This suggestion is indirectly supported by the results of our recent investigation in asthmatic patients treated with inhalation of ultra-low doses of melphalan. In that study, 60% of melphalan-treated patients demonstrated the histological signs of bronchial epithelium regeneration. Moreover, in those patients, a systemic anti-inflammatory effect of the drug was found. In the patients of placebo group, neither the signs of regeneration nor systemic anti-inflammatory effect have been found.

In addition, the anti-inflammatory effect of melphalan observed both in human patients and in DSS-murine model may be also associated with direct action of the drug on activated lymphocytes expressing IL-2R. Such suggestion is based on our previous in vitro experiments indicating that the pretreatment with ultra-low doses of alkylating drugs switches off the β-chain of IL-2R of CD8+ T cells.

In conclusion, the present study suggests that ultra-low doses of melphalan delay the onset of severe colitis in DSS-treated mice by blocking TNF-α signaling pathway. This finding indicates that alkylating drugs may represent a new class of agents with a promising potential in the IBD therapy.
REFERENCES


