Effect of Recombinant Canine Granulocyte-Macrophage Colony-Stimulating Factor on Hematopoietic Recovery After Otherwise Lethal Total Body Irradiation


Recombinant canine granulocyte-macrophage colony-stimulating factor (rcGM-CSF) was studied in normal dogs and in dogs receiving otherwise lethal total body irradiation (TBI) without marrow transplant. Five normal dogs receiving 25 µg/kg of rcGM-CSF by subcutaneous (SC) injection twice daily (BID) for 14 days showed increases in peripheral blood neutrophil counts of three to five times the baseline. Platelet counts decreased during administration of rcGM-CSF to a mean nadir of 52,800. Ten dogs received 400 cGy TBI at 10 cGy/min from two opposing 60Co sources and no marrow graft. Within 2 hours of TBI, rcGM-CSF was begun at a dose of 50 µg/kg SC BID for 5 doses and then continued at 25 µg/kg SC BID for 21 days. Only 1 of the 10 dogs receiving rcGM-CSF survived with complete and sustained recovery of hematopoiesis. One of 13 historical control dogs survived after 400 cGy with no hematopoietic growth factor or marrow infusion. Results with rcGM-CSF were compared with previous and concurrent data with G-CSF studied in the same model. Of 10 dogs receiving G-CSF, 8 survived with complete and sustained hematopoietic recovery, a significantly better survival than that seen with rcGM-CSF (p = .006). Neutrophil counts were sustained at higher levels after TBI for the first 18 days in the G-CSF group (p < .016) and the neutrophil nadirs were higher. No differences in neutrophil nadirs were noted between the rcGM-CSF and control groups. Dogs treated with rcGM-CSF experienced a more rapid decline of platelet counts than G-CSF-treated or control dogs over the first 18 days (p < .001). The nadir of the platelet count was higher in the control group than in either the G-CSF or rcGM-CSF group and no significant difference was observed between the G-CSF and rcGM-CSF groups. After otherwise lethal TBI (400 cGy) in dogs, rcGM-CSF was not effective in promoting hematopoietic recovery or improving survival.

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MATERIALS AND METHODS

Experimental animals. Fifteen beagles of either sex, at a median age of 9 (range, 7 to 32) months received rcGM-CSF in this study. Five beagles in this group received rcGM-CSF and no TBI. Ten other beagles in this group received no hematopoietic growth factor after 400 cGy TBI and constitute the "no growth factor" group (historical). Ten dogs received G-CSF, 5 dogs received 100 pg/kg/d recombinant human G-CSF (rhG-CSF) (historical), and 5 dogs received 10 pg/kg/d rcG-CSF (concurrent) after 400 cGy TBI. The doses of the human and canine G-CSF selected for the study, when administered to normal dogs not receiving TBI, gave similar increases in peripheral blood neutrophils and were, therefore, considered equivalent. Seven of these dogs receiving G-CSF after 400 cGy TBI have been previously reported and 3 more dogs receiving rcG-CSF were added to the group for this study. All dogs were either raised at the Fred Hutchinson Cancer Research Center (FHCRC) or purchased from commercial US Department of Agriculture licensed dealers. Dogs were quarantined on arrival, screened for evidence of disease, and observed for a minimum of 2 months before being released for use. They were dewormed and vaccinated for rabies, distemper, leptospirosis, hepatitis, and parvovirus. They were housed in an American Association for Accreditation of Laboratory Animal Care accredited facility in standard indoor runs, and provided commercial dog chow and chlorinated tap water ad libitum. Animal holding areas were maintained at 70 ± 2°F with 50% ± 10% relative humidity, using at least 15 air changes per hour of 100% conditioned fresh air. The dogs were on a 12-hour light/dark full-spectrum lighting cycle with no twilight. The protocol for this study was approved by the Institutional Animal Care and Use Committee of the FHCRC.

Recombinant hematopoietic growth factors. The cloning of the cDNA of rcGM-CSF has been previously reported. The recombinant protein for in vivo studies was produced by Amgen (Thousand Oaks, CA). It was expressed in Escherichia coli and purified by precipitation and cation-exchange chromatography. Endotoxin levels were less than 2.5 endotoxin U/mL as determined by the limulus amebocyte lysate assay (Associates of Cape Cod, Wood Hole, MA). Purified material was stored at 4°C. For administration, rcGM-CSF was diluted in normal saline (Abbott Laboratories, Chicago, IL) containing 0.5% normal heat-inactivated dog serum. The rcGM-CSF was administered to dogs after 400 cGy TBI by subcutaneous (SC) injection twice daily (BID) at 50 μg/kg for the first 5 doses and then continued as 25 μg/kg (50 μg/kg/d) for 21 days or until death, whichever occurred first. The rcG-CSF (produced by Amgen) in this study was produced as previously reported. The rcG-CSF was administered at 10 μg/kg/d by SC BID for 21 days.

TBI and supportive care. All dogs received 400 cGy TBI at 10 cGy/min from two opposing 60Co sources. The day of TBI was designated day 0. No marrow grafts were infused in any dog. After TBI, parenteral fluids, electrolytes, platelet transfusions, and antibiotics were administered as described. All blood products used for transfusions were irradiated in vitro (1,500 cGy) to inactivate pluripotent hematopoietic stem cells and immunologically competent cells. Hematocrit, reticulocyte, leukocyte, platelet, and differential counts were obtained before and daily after TBI. Necropsies with histologic examinations were performed routinely on all dogs that died.

Marrow biopsy. For marrow biopsy, dogs were sedated with ketamine hydrochloride (Aveco Co, Inc, Fort Dodge, IA). The collection site was aseptically cleansed with a povidone-iodine scrub and alcohol rinse. Marrow biopsies were obtained from dogs from...
Effects of GM-CSF in dogs after sublethal TBI

The decrease in platelet counts in five dogs that received rcGM-CSF at 50 μg/kg/d SC for 14 days. Day 0 was the first day of GM-CSF treatment. Symbols are the same as in Fig 1.

Statistical methods. Log-rank tests were used to compare survival time between groups of dogs that received rcGM-CSF, G-CSF, or no treatment after 400 cGy TBI. Bonferroni adjustments for performing multiple tests were applied when data from a group of dogs were used in more than one pairwise testing procedure. Daily peripheral blood cell counts were plotted on a logarithmic scale versus time. For dogs that received radiation, the means of the log blood counts for each day were calculated for the rcGM-CSF and G-CSF groups and for control dogs. Graphically, these results were displayed with cubic spline curves connecting the daily means for each group to show mean log blood count as a relatively smooth function of time. Blood count profiles after TBI in the rcGM-CSF and G-CSF groups were compared by modeling mean log blood counts as a 5th degree polynomial in time with a constant difference between group means. We then tested whether this constant was significantly different from zero using the generalized estimating equation (GEE) technique with independent working covariance matrix. Blood count nadirs were calculated for dogs that survived at least 18 days. Testing for differences among the three groups in nadirs of platelet and neutrophil counts was performed using the Kruskal-Wallis test. If the overall test indicated differences among the three groups in nadirs of platelet and neutrophil counts as a 5th degree polynomial in time with a constant difference between group means.

Table 1. Survival of Dogs Receiving 400 cGy TBI and Subsequent Treatment With rcGM-CSF

<table>
<thead>
<tr>
<th>Dog Identification</th>
<th>Survival (d)</th>
<th>Cause of Death</th>
<th>Marrow Cellularity at Necropsy*</th>
</tr>
</thead>
<tbody>
<tr>
<td>BC9004</td>
<td>14</td>
<td>Pneumonia</td>
<td>20%; P, L</td>
</tr>
<tr>
<td>D176</td>
<td>18</td>
<td>Pneumonia</td>
<td>15%; P, L</td>
</tr>
<tr>
<td>D189</td>
<td>19</td>
<td>Pneumonia</td>
<td>15%; P, L</td>
</tr>
<tr>
<td>D197</td>
<td>&gt;50</td>
<td>Euthanized</td>
<td>No necropsy</td>
</tr>
<tr>
<td>D207</td>
<td>12</td>
<td>Pneumonia</td>
<td>10%; P, L</td>
</tr>
<tr>
<td>D208</td>
<td>19</td>
<td>Pneumonia</td>
<td>0%</td>
</tr>
<tr>
<td>D260</td>
<td>13</td>
<td>Pneumonia</td>
<td>45%; P, L, Mo</td>
</tr>
<tr>
<td>D423</td>
<td>21</td>
<td>Pneumonia</td>
<td>15%; P, L, Mo, Meg</td>
</tr>
<tr>
<td>D434</td>
<td>21</td>
<td>Sepsis</td>
<td>130%; P, L, Mo, Meg</td>
</tr>
<tr>
<td>D436</td>
<td>11</td>
<td>Sepsis</td>
<td>15%; P, L, Mo</td>
</tr>
</tbody>
</table>

*Percentage is the marrow cellularity estimated from a biopsy. Lineage of cells constituting the marrow at necropsy are designated: P, plasma cells; L, lymphocytes; Mo, monocytes; Meg, megakaryocytes. No significant numbers of myeloid or erythroid precursors were observed.

† End of study.

RESULTS

Effects of rcGM-CSF in normal dogs. The effects of rcGM-CSF in normal dogs administered SC BID for 14 days at 1 μg/kg/d (1 dog), 10 μg/kg/d (4 dogs), 30 μg/kg/d (1 dog), and 50 μg/kg/d (1 dog) have been reported. The mean increase in peripheral blood neutrophil counts in the 4 dogs receiving 10 μg/kg/d was only two times the prestudy baseline. In an effort to define a dose of rcGM-CSF that increased
neutrophils to that seen with rcG-CSF, the effects of rcGM-CSF at 50 μg/kg/d for 14 days was explored in 5 more dogs. The mean increase in peripheral blood neutrophil counts was 6.0 times the prestudy baseline (range, 3.0 to 9.3) (Fig 1). An increase was also noted in the peripheral blood monocyte counts. There was no increase in eosinophil counts. The platelet counts started to increase within 24 hours of the start of rcGM-CSF (Fig 2). The platelet counts remained decreased until the termination of rcGM-CSF, after which platelet counts started to increase. The mean nadir of the platelet count during rcGM-CSF administration was 52,800 platelets/μL (median, 57,000 platelets/μL). At 50 μg/kg/d of rcGM-CSF, the 5 normal dogs were eating, active, and maintained their weights.

**Effects of rcGM-CSF on survival after otherwise lethal TBI.** Ten dogs received 400 cGy TBI without marrow infusion, and then received rcGM-CSF at 50 μg/kg for the first 5 doses and 25 μg/kg rcGM-CSF SC BID for 21 days or until the death of the dog. One of the 10 dogs showed complete and sustained hematopoietic recovery (Table 1). Another dog showed evidence of hematopoietic recovery, having 3 days of increasing peripheral blood neutrophil counts, but died secondary to a septic event. Eight dogs died between days 11 and 21 after TBI from sepsis secondary to marrow aplasia. Seven dogs had evidence of pneumonia, 5 of which were considered to be hemorrhagic at necropsy or on studies of lung histopathology. One dog, which died on day 11, had a laceration on the back of the neck that may have led to the terminal septic event. Bacteriological cultures from blood or lung tissue were positive for Klebsiella, Proteus, or enterococcus in 5 of the 9 dogs that died of sepsis. Two other dogs had bacteria identified on the histopathologic samples obtained at necropsy. Of the 2 remaining dogs in which there were no positive cultures, 1 dog died of a sepsis syndrome and the other had a hemorrhagic pneumonia. In addition to the hemorrhage related to pneumonia, 5 dogs had hemorrhagic events of the skin, lymph node, kidney, or pericardium. There was no difference in the number or severity of hemorrhagic events noted after TBI at necropsy between the rcGM-CSF group and the group not receiving hematopoietic growth factor. Ten dogs received 400 cGy TBI without marrow infusion or hematopoietic growth factors. The overall survival of 13 dogs in the untreated group was not different from that observed in the rcGM-CSF group. Of the 12 dogs that did not survive, 10 were evaluated by necropsy. Eight dogs had evidence of pneumonia, 5 of which were hemorrhagic at necropsy or studies of lung histopathology. In addition to the hemorrhage related to pneumonia, 5 dogs had hemorrhagic events of the skin, lymph node, kidney, or pericardium. There was no difference in the number or severity of hemorrhagic events noted after TBI at necropsy between the rcGM-CSF group and the group not receiving hematopoietic growth factor. Ten dogs received 400 cGy TBI without marrow infusion, followed by the administration of G-CSF SC BID. The probability of survival in the G-CSF group was significantly higher than in the rcGM-CSF group (P = .006). Eight of the 10 dogs showed complete and sustained hematopoietic recovery, including 4 of the 5 dogs receiving rcG-CSF concurrently with the study of rcGM-CSF. Both dogs that died in the G-CSF group had evidence of pneumonia at necropsy, 1 of which was hemorrhagic. The other dog had evidence of a small bowel hemorrhage.

**Comparison of hematologic recovery of dogs receiving rcGM-CSF with that of untreated dogs and dogs receiving G-CSF after otherwise lethal TBI.** Over the first 18 days after TBI, neutrophil counts in the rcGM-CSF group were significantly lower than those in the G-CSF group (P = .048) (Fig 4). No difference could be shown between the rcGM-CSF group and the group that did not receive growth factor. In a separate analysis of neutrophil counts, the nadirs that...
EFFECTS OF GM-CSF IN DOGS AFTER SUBLETHAL TBI

Fig 4. The average log neutrophil counts after 400 cGy TBI and no marrow infusion in dogs receiving GM-CSF, G-CSF, or no hematopoietic growth factor. Significant differences were found for the GM-CSF versus G-CSF (P = .048) and G-CSF versus control (P = .017) comparisons using the GEE technique with a polynomial model for mean log neutrophil count. No significant difference was found between the GM-CSF and control groups. Actual data points are plotted for daily neutrophil counts.

were reached after TBI in each of the three groups were compared by a Kruskal-Wallis test. There was a significant difference detected among the neutrophil nadirs (P < .05), and multiple comparison procedures for pairwise differences between groups showed that the G-CSF group had higher nadirs than both the rcGM-CSF group and the group receiving no hematopoietic growth factors. The rcGM-CSF group and untreated group did not differ with respect to neutrophil nadirs.

Over the first 18 days after TBI, platelet counts in the rcGM-CSF group were significantly lower than those in the G-CSF group (P < .001) (Fig 5). The Kruskal-Wallis test for differences among the platelet nadirs for the three groups was significant (P < .05). Pairwise comparisons indicated that the group not receiving hematopoietic growth factors had platelet count nadirs that were higher than for both the rcGM-CSF and G-CSF groups. A difference in platelet count nadirs could not be demonstrated between the rcGM-CSF and G-CSF groups.

DISCUSSION

In this model of "just lethal" TBI (400 cGy), administration of rcGM-CSF was not associated with significant hematopoietic recovery or improved survival at a dose that was sufficient to increase neutrophil counts sixfold in dogs not receiving TBI. Survival in this model has previously been shown to depend on recovery of neutrophil counts because the most frequent cause of death is sepsis and pneumonia. Over the first 18 days, the neutrophil counts in the rcGM-CSF group were lower than those in the G-CSF group. There was a marked early increase in neutrophil counts in the G-CSF group, which may or may not have had an effect on survival. The difference in the nadirs of the neutrophil counts was likely more critical to the survival endpoint in this study. The nadirs were significantly lower in the rcGM-CSF group than in the G-CSF group. All the dogs that died in the rcGM-CSF group were neutropenic and had pneumonia or sepsis. Only 1 dog had recovering neutrophil counts and increased marrow cellularity. The dog was still neutropenic and marrow showed predominantly monocytoid cells, plasma cells, and lymphocytes at the time of death from sepsis.

At the doses used in this study, rcGM-CSF induced a significant thrombocytopenia in dogs not receiving TBI. Previous studies in dogs receiving GM-CSF showed a shortened survival of platelets using 51Cr-labeling. It was not unexpected, therefore, that the rcGM-CSF group had a more rapid
Fig 5. The average log platelet counts after 400 cGy TBI and no marrow infusion in dogs receiving GM-CSF, G-CSF, or no hematopoietic growth factor. Significant differences were found for the GM-CSF versus G-CSF (P < .001) and the GM-CSF versus control (P < .001) comparisons using the GEE technique with a polynomial model for mean log platelet count over the first 18 days. No significant differences were found between the G-CSF and control groups. Actual data points are plotted for daily platelet counts.

days after 400 cGy TBI

The effects of GM-CSF have been studied in other preclinical models of myelosuppression. In two studies of large animals after sublethal doses of TBI, treatment with rhGM-CSF was associated with shorter periods of neutropenia. In one of these studies in which primates had bilateral tibia partially shielded from TBI, the group treated with rhGM-CSF had a shorter time to neutrophil recovery and had increased marrow colony-forming unit–granulocyte-macrophage (CFU-GM) compared with the controls. In the other study, dogs received unshielded sublethal doses of TBI (240 cGy) and GM-CSF. Neutrophil counts recovered more rapidly in the treated group. Besides accelerating recovery of neutrophil counts, GM-CSF augments function of monocytes-macrophages and neutrophils that may have contributed to the improvement of the outcome in other preclinical models of myelosuppression. In mice myelosuppressed by cyclophosphamide, recombinant mouse GM-CSF (rmGM-CSF) administered either before or after a challenge with Pseudomonas aeruginosa or before a challenge with Staphylococcus aureus or Candida albicans resulted in improved survival. However, although GM-CSF does have significant biologic effects, its effectiveness could not be demonstrated in models in which marrow failure was induced by otherwise lethal radiation doses. After an otherwise lethal dose of radiolabeled antibody in mice, rmGM-CSF and interleukin-1 (IL-1) administered for 12 days starting 3 days before the radiation exposure resulted in 100% survival. However, rmGM-CSF alone, before or after the radiation exposure, was minimally effective. In C57BL/6 mice after a lethal dose of TBI (950 cGy) and no marrow infusion, rmGM-CSF administered intravenously (days 0 through 15) significantly prolonged survival compared with the controls receiving no treatment, but only 2 of the 10 mice receiving rmGM-CSF survived more than 40 days. The failure of...
GM-CSF to augment hematopoietic recovery sufficiently for improvement of survival after otherwise lethal doses of TBI in dogs and in the studies of radiolabeled antibody and lethal TBI in mice without marrow infusion may result from its more limited effects on the smaller pool of surviving hematopoietic progenitor cells.

A direct comparison of the effects of rmGM-CSF and rG-CSF on hematopoietic recovery after a sublethal dose of TBI (750 cGy) was performed in mice. The recovery of leukocytes, platelets, and hematocrit was significantly earlier and greater in mice receiving G-CSF than mice receiving GM-CSF at days 14 and 21 after TBI. Mice treated with G-CSF had significant increases in marrow and spleen nucleated cells after TBI. There was not a significant increase of marrow or spleen nucleated cells in the GM-CSF groups when compared with controls. G-CSF significantly enhanced the recovery of CFU-spleen, CFU-C, burst-forming unit-erythrocyte, and CFU-megakaryocyte (CFU-Meg) in marrow on days 7, 14, and 21 after TBI. GM-CSF only enhanced the recovery of CFU-Meg in marrow on day 14. The observations in this sublethal TBI model support a conclusion that GM-CSF after marrow-toxic doses of radiation does not promote hematopoietic recovery to the same degree as that observed with G-CSF. This may occur as a result of the recruitment of dormant hematopoietic stem cells into cell cycle by G-CSF. 27

Although the effects of GM-CSF after otherwise lethal doses of radiation are limited, studies of G-CSF in dogs and mice suggest greater effects. 28-31 G-CSF seems to have its greatest effect when administered early, after exposure to radiation. 29,30 Although G-CSF is capable of reversing the lethality of "just lethal" TBI in most cases, a further increase in TBI 100 to 200 cGy above the "just lethal" dose decreased the effectiveness of G-CSF. 16,30 This likely reflects the need for a critical number of hematopoietic progenitors surviving radiation to permit a rate of hematopoietic recovery compatible with survival.

In summary, rG-CSF increases peripheral blood neutrophils and decreases platelets in normal dogs. The administration of rG-CSF to dogs after otherwise lethal TBI does not improve survival. The primary cause of death was pneumonia and sepsis secondary to neutropenia. This model of otherwise lethal radiation (400 cGy) in the dog has the ability to distinguish between GM-CSF and other hematopoietic growth factors, such as G-CSF and SCF, when the endpoint of clinical interest because in the late 1980s, after two radiation accidents that received international attentions, there were reports and discussions on the use of hematopoietic growth factors to accelerate hematopoietic recovery. 22,23 Current preclinical data would support studies of G-CSF rather than GM-CSF in patients after significant accidental radiation exposure who will be managed without marrow grafting. Further, these studies suggest that, in situations of a severely limited stem cell reserve, G-CSF may be more effective than GM-CSF in eliciting a rapid neutrophil response.

REFERENCES


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Effect of recombinant canine granulocyte-macrophage colony-stimulating factor on hematopoietic recovery after otherwise lethal total body irradiation