Effect of Recombinant Human Granulocyte Colony-Stimulating Factor on Hematopoiesis of Normal Dogs and on Hematopoietic Recovery After Otherwise Lethal Total Body Irradiation

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This study was designed to test whether recombinant human G-CSF (rh G-CSF) affects hematopoiesis in normal dogs and, if so, to test the effects of G-CSF in dogs given otherwise lethal total body irradiation (TBI). Rh G-CSF given subcutaneously at 10 or 100 μg/kg/d for 14 days to two normal dogs increased peripheral blood neutrophils eight to tenfold and monocytes four to sixfold above controls. Lymphocyte counts remained unchanged at the lower dose and increased threefold at the higher dose of rh G-CSF. No significant changes were observed in eosinophil, platelet, reticulocyte, or hematocrit levels. After 2 weeks of treatment with rh G-CSF, bone marrow displayed myeloid hyperplasia and left-shifted granulocytopenia. After discontinuation of rh G-CSF, peripheral leukocyte counts returned to control levels within three days. Five dogs administered 400 cGy TBI at 10 cGy/min from two opposing 60Co sources and no marrow infusion or growth factor, all developed profound pancytopenia and died between 17 and 23 days after TBI with infections secondary to marrow aplasia. Four of five dogs treated within two hours after 400 cGy TBI with 100 μg rh G-CSF/kg/d subcutaneously twice a day for 21 days showed complete and sustained endogenous hematopoietic recovery. In contrast, five dogs irradiated with 400 cGy TBI and treated with 100 μg rh G-CSF/kg/d starting on day 7 after TBI all died between days 17 and 20 after TBI with infections secondary to marrow aplasia. Rh G-CSF, if administered shortly after irradiation, can reverse the otherwise lethal myelosuppressive effect of radiation exposure.

MATERIALS AND METHODS

Experimental animals. Seventeen dogs of both sexes and various breeds (Beagles, Fox Hounds, mongrels) 6 to 13 months of age were used in the study. Animals 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. All dogs 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 of this study was approved by the Institutional Animal Care and Use Committee of the FHCRC.

Recombinant human G-CSF. The rh G-CSF used in this study was prepared and provided by Amgen Corporation (Thousand Oaks, CA). The gene encoding human G-CSF was cloned from the 5637 cell line, expressed in Escherichia coli and purified to homogeneity by reverse-phase high-performance liquid chromatography. The purified rh G-CSF contained <0.5 ng endotoxin/mg of protein as measured by the Limulus amebocyte lysate assay. The specific activity of rh G-CSF was 1 x 10^5 units/mg protein when assayed by serial dilution in a colony forming unit-granulocyte/macrophage (CFU-GM) assay. Rh G-CSF was administered subcutaneously twice a day in 1 mL 0.9% NaCl containing 0.1% normal heat-inactivated dog serum.

Total-body irradiation and postirradiation care. Dogs were given 400 cGy TBI at 10 cGy per minute from two opposing 60Co sources. The day of TBI was designated day 0. After TBI, parenteral...
fluids, electrolytes, platelet transfusions, and antibiotics were administered as described. All blood products used for transfusions were irradiated in vitro (1500 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. Autopsies with histological examinations were performed on all dogs that died.

**Rh G-CSF treatment after TBI.** Dogs were treated with 100 μg rh G-CSF/kg/d subcutaneously twice a day starting either within two hours after 400 cGy TBI or on day 7 after TBI. Treatment lasted for 21 days or until death, whichever occurred first.

**Marrow biopsy.** For marrow biopsy dogs were anesthetized by intravenous (IV) injection of Fentanyl (Innovar-Vet, Pitman-Moore, Inc, Washington Crossing, NJ) at 0.03 mg/kg. The collection site was aseptically cleansed with a povidone-iodine scrub and alcohol rinse. Marrow biopsies were obtained from normal dogs before and after 14 days of rh G-CSF injections from the humoral condyle using Jamshidi bone marrow needles (American Pharmaceutical Co, Valencia, CA).

**RESULTS**

**Effects of rh G-CSF in normal dogs.** Two dogs were treated with either 10 or 100 μg rh G-CSF/kg/d subcutaneously twice a day for 14 days. Rh G-CSF was well tolerated and no systemic toxicity was observed. Both dogs showed increased peripheral blood neutrophil counts within 24 hours of starting the injections of the growth factor (Fig 1A). By day 14 neutrophils reached levels which were eight to ten times higher than the preinfusion counts and those of two control dogs receiving either 60 μg heat-inactivated rh IL-3/kg/d for 14 days (range indicated by two horizontal lines) or 10 μg human serum albumin/kg/d (data not shown). After discontinuation of rh G-CSF, the neutrophil counts returned to control levels within three days. Peripheral blood monocytes were increased four to sixfold (Fig 1B). Lymphocyte counts remained unchanged at 10 μg rh G-CSF/kg/d and increased threefold at 100 μg rh G-CSF/kg/d (Fig 1C). No significant changes were observed in platelet counts (Fig 1D), eosinophil, reticulocyte, or hematocrit levels (data not shown). Marrow biopsies were taken before and after 14 days of rh G-CSF injections, and slides were evaluated without knowledge of treatment modality. Compared with the normocellular marrow histology before rh-G-CSF treatment, the marrows of the rh G-CSF--treated dogs were hypercellular with myeloid hyperplasia and left-shifted granulopoiesis.

**Effects of rh G-CSF in dogs after lethal TBI.** Five dogs were given 400 cGy TBI at 10 cGy/min from two opposing 60Co sources and no marrow infusion or additional growth

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**Fig 1.** Effect of twice daily (14 days) subcutaneous injections of recombinant human G-CSF (rh G-CSF) on peripheral blood neutrophils (A), monocytes (B), lymphocytes (C), and platelets (D) of two normal dogs. Rh G-CSF was administered at 10 or 100 μg/kg/d. The two horizontal lines indicate the range of the peripheral blood counts of a control dog given continuous IV infusion of 80 μg heat-inactivated (100°C, 30') rh IL-3/kg/d for 14 days.
All dogs developed profound pancytopenia following the irradiation and died between 17 and 23 days after TBI with infections secondary to marrow aplasia (Fig 2). In four of the dogs, marrow histology at autopsy showed an empty marrow with no hematopoietic cells. One dog had 5% of normal marrow cellularity with few myeloid and erythroid precursor cells, but no megakaryocytes (Table 1).

Five other dogs were irradiated with 400 cGy TBI at 10 cGy/min as before and, in addition, were treated with 100 µg rh G-CSF/kg/d subcutaneously twice a day for 21 days starting within two hours after TBI. Four dogs showed complete and sustained hematopoietic recovery and are now surviving 120 to 180 days after TBI with normal peripheral blood counts (Fig 3). One dog, C726, died on day 19 after TBI because of acute bacterial pneumonia secondary to marrow aplasia. On the day of his death peripheral blood neutrophil count was zero and marrow histology at autopsy showed 5% of normal marrow cellularity with few myeloid precursor cells but no erythroid or megakaryocytic precursors (Table 1).

A third group of five dogs was irradiated with 400 cGy TBI at 10 cGy/min as before and then treated with 100 µg rh G-CSF/kg/d subcutaneously twice a day from day 7 until day 20 after TBI or until death. All five dogs died between days 17 and 20 after TBI with infections secondary to marrow aplasia (Fig 4). In three dogs marrow histology at autopsy showed no signs of hematopoiesis and in two dogs 5% of normal marrow cellularity was seen containing myeloid precursor cells but no erythroid or megakaryocytic precursors (Table 1).

**DISCUSSION**

This study demonstrates that rh G-CSF stimulates hematopoiesis in normal dogs and allows sustained endogenous hematopoietic recovery in dogs after otherwise lethal TBI. The results seen in normal dogs treated with rh G-CSF

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**Table 1. Survival of Dogs After 4 Gy of TBI Without or With rh G-CSF Treatment Starting Either Immediately or Seven Days After TBI**

<table>
<thead>
<tr>
<th>Dog No.</th>
<th>G-CSF After TBI</th>
<th>Duration of Treatment (Days Past TBI)</th>
<th>Survival (d)</th>
<th>Marrow Cellularity at Autopsy</th>
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</thead>
<tbody>
<tr>
<td>C506</td>
<td>No</td>
<td>—</td>
<td>19</td>
<td>0%</td>
</tr>
<tr>
<td>C518</td>
<td>No</td>
<td>—</td>
<td>22</td>
<td>5%; myeloid + erythroid precursors</td>
</tr>
<tr>
<td>C520</td>
<td>No</td>
<td>—</td>
<td>23</td>
<td>0%</td>
</tr>
<tr>
<td>C530</td>
<td>No</td>
<td>—</td>
<td>20</td>
<td>0%</td>
</tr>
<tr>
<td>C581</td>
<td>No</td>
<td>—</td>
<td>17</td>
<td>0%</td>
</tr>
<tr>
<td>C726</td>
<td>Yes</td>
<td>0-19</td>
<td>19</td>
<td>5%; myeloid precursors</td>
</tr>
<tr>
<td>C784</td>
<td>Yes</td>
<td>0-20</td>
<td>&gt;150</td>
<td>—</td>
</tr>
<tr>
<td>C820</td>
<td>Yes</td>
<td>0-20</td>
<td>&gt;120</td>
<td>—</td>
</tr>
<tr>
<td>C834</td>
<td>Yes</td>
<td>0-20</td>
<td>&gt;180</td>
<td>—</td>
</tr>
<tr>
<td>C861</td>
<td>Yes</td>
<td>0-20</td>
<td>&gt;120</td>
<td>—</td>
</tr>
<tr>
<td>C639</td>
<td>Yes</td>
<td>7-18</td>
<td>18</td>
<td>5%; myeloid precursors</td>
</tr>
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<td>18</td>
<td>0%</td>
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confirm, in part, observations described by Lothrop et al. In accordance with that report, we saw a rapid and persistent leukocytosis that was mainly due to an increase of neutrophils and, to a smaller extent, to an increase of monocytes. In contrast to the observations by Lothrop et al., we did not see an elevation of lymphocyte counts at the dose of 10 μg rh G-CSF/kg/d but only when we increased the dose to 100 μg/kg/d. At both dosages of rh G-CSF we did not find any significant changes in eosinophil, platelet, reticulocyte, or hematocrit levels, similar to the report by Lothrop et al. The stimulatory activity of rh G-CSF in dogs is similar to results in mice, hamsters, and monkeys. In all four species, rh G-CSF injections induce a rapid increase of peripheral blood neutrophils. Neutrophil counts decrease within two to three days after discontinuing rh G-CSF application indicating that the effect of rh G-CSF is only transient. In dogs and mice, monocytes were mildly elevated while they remained unchanged in monkeys and hamsters. Lymphocytes were increased in dogs and monkeys, but only at dosages >10 μg/kg/d rh G-CSF and were unchanged in hamsters and mice at the dosages studied. In all four species, no stimulation of eosinophil, platelet, and reticulocyte counts or hemoglobin concentration was noted.

After demonstrating stimulation of hematopoiesis in nor-
eral dogs, the effect of rh G-CSF in dogs after lethal TBI was studied. The "LD<sub>50</sub>" in dogs given single-dose TBI, supportive care, and no marrow infusion is at 400 cGy in our laboratory based on previous\(^1\) and concurrent data (Fig 2). When dogs were treated immediately after 400 cGy TBI with rh G-CSF for 21 days, four of five dogs had complete and sustained endogenous hematopoietic recovery. In contrast, when rh G-CSF injections were started seven days after TBI, all dogs died between days 17 and 20 after TBI because of infections secondary to marrow aplasia, similar to the control dogs not receiving rh G-CSF. These results indicate first that a number of pluripotent hematopoietic stem cells sufficient to allow for sustained hematopoietic recovery survive an otherwise lethal dose of 400 cGy TBI. This finding confirms previous observations in our laboratory where dogs given DLA-identical marrow transplants after 450 cGy single-dose TBI showed transient allogetic engraftment followed by graft rejection and autologous marrow recovery.\(^10\)

Second, our experiments demonstrate that treatment with rh G-CSF immediately after TBI shortens the period of neutropenia such that lethal infections are prevented. With current supportive care including platelet transfusions, prophylactic systemic broad spectrum antibiotics and selective gut decontamination, we are able to keep dogs with TBI-induced pancytopenia alive for about 21 days. If the marrow does not start to produce mature neutrophils by this time, the dogs usually die of sepsis or pneumonia, as shown in the control dogs not receiving rh G-CSF after 400 cGy TBI. The neutrophil counts of the dogs that were treated with rh G-CSF from day 0 on, started to increase again around day 15 except for one dog that showed only a temporary recovery of neutrophils and then died due to infection. As we cannot currently keep pancytopenic animals alive for more than approximately 3 weeks, we do not know how long it would have taken for the dogs without rh G-CSF treatment to show hematopoietic recovery. Based on the experiments where we could protect dogs after 450 cGy from lethal infections by a transient DLA-identical marrow graft, we can assume that it would probably take about the same time, 40 days, for the control dogs to reach 1,000 neutrophils/mm\(^3\) after 400 cGy TBI. As dogs treated with rh G-CSF from day 0 on reached 1,000 neutrophils/mm\(^3\) around day 20, we can hypothesize that treatment with rh G-CSF probably shortened the period of neutropenia after 400 cGy TBI by 50%.

Accelerated recovery from chemotherapy-induced neutropenia by rh G-CSF treatment has been described in mice,\(^4,7\) hamsters,\(^6\) monkeys,\(^8\) and humans,\(^11,12\) but only after sublethal dosages of cytotoxic drugs. There has been no controlled report so far describing survival and sustained endogenous hematopoietic recovery after otherwise lethal TBI or chemotherapy by treatment with hematopoietic growth factors after the lethal conditioning.

A third conclusion from our experiments is that the time interval between TBI and start of rh G-CSF treatment is important. Only the animals that were started on rh G-CSF treatment directly after TBI survived, while the dogs given rh G-CSF from day 7 on, died with infections due to marrow aplasia. We do not know the reasons for this different outcome. As rh G-CSF, based on in vitro data, stimulates only neutrophil precursor cells, it is possible that only a temporary increase of neutrophil production would be seen when rh G-CSF is given after TBI, resulting in a rapid exhaustion of the number of neutrophil precursor cells surviving TBI. The accelerated and sustained increase of peripheral neutrophils by rh G-CSF, however, suggests that this growth factor acts also on early hematopoietic precursor cells either directly or through accessory cells. If this is the case, it would be understandable that the earlier the growth factor was given, the more profound the effect over time. Giving it on day 0 allowed the animals to reach a sustained number of peripheral neutrophils by the end of the third week such that lethal infections were prevented.

Some of the effect of rh G-CSF may also have been to improve neutrophil function\(^13,14\) in addition to increasing cell number. This effect was not measured in this study.

In summary, we have described that rh G-CSF stimulates hematopoiesis in normal dogs and allows sustained endogenous hematopoietic recovery after otherwise lethal TBI when administered immediately after irradiation.

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