Abnormal Response to Granulocyte Colony-Stimulating Factor (G-CSF) in Canine Cyclic Hematopoiesis Is Not Caused by Altered G-CSF Receptor Expression

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A decrease in responsiveness to granulocyte colony-stimulating factor (G-CSF) has been implicated in the pathophysiology of cyclic hematopoiesis. Using the canine model of cyclic neutropenia, we examined the response of neutrophil precursors to G-CSF in vitro and G-CSF receptor expression in neutrophils from grey collie dogs to determine whether the abnormal response observed to G-CSF in vivo in this disorder is present at the level of the progenitor cell and is caused by defective G-CSF receptor expression. Bone marrow mononuclear cells from grey collie dogs required sevenfold higher G-CSF concentrations than normal dog cells to achieve half-maximal colony growth (56 pmol/L vs 8 pmol/L). Receptor binding assays with 125I-labeled G-CSF and Scatchard analyses of the equilibrium binding data were consistent with expression of a single class of high-affinity receptors for G-CSF on neutrophils from both normal dogs and grey collies with similar receptor numbers (56 to 446 sites/cell vs 78 to 189 sites/cell) and binding affinities (28 to 206 pmol/L vs 84 to 195 pmol/L). Chemical cross-linking studies identified a G-CSF binding protein of approximately 120 kD on neutrophils from grey collies, similar in size to that on normal dog neutrophils. No abnormal G-CSF receptor mRNA transcripts were detected in neutrophils from grey collie dogs by Northern blot analysis. Treatment of both normal and grey collie neutrophils with G-CSF rapidly induced tyrosine phosphorylation of an 80-kD protein that behaved like canine c-ras. These results demonstrate that the abnormal responsiveness to G-CSF in canine cyclic hematopoiesis is present in neutrophil precursors and is not associated with demonstrable alterations in the number, binding affinity, or overall size of the G-CSF receptor in neutrophils, or with defective tyrosine phosphorylation of p80. These data suggest that cyclic hematopoiesis is caused by a defect in the G-CSF signal transduction pathway at a point distal to G-CSF receptor binding that does not involve the early biochemical events leading to p80 tyrosine phosphorylation.

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Cyclical hematopoiesis is a rare disorder in humans and grey collie dogs characterized by regular cyclical oscillations in blood counts and periodic severe neutropenia frequently complicated by bacterial infections.1-9 Although the mechanisms underlying this disorder are unknown, recent clinical trials have shown that supraphysiologic doses of granulocyte colony-stimulating factor (G-CSF) ameliorate cyclic neutropenia in humans and eliminate cyclical hematopoiesis in dogs with this disorder.10-12

Abnormal responsiveness to G-CSF may be an important pathophysiological mechanism underlying congenital cyclic hematopoiesis. We previously reported that abnormally increased concentrations in vitro of G-CSF and granulocyte-macrophage–CSF (GM-CSF) but not interleukin-3 (IL-3) were required to stimulate maximal granulocytic colony formation by bone marrow (BM) cell populations from patients with congenital cyclic neutropenia.13 The requirement for increased concentrations of growth factor could be demonstrated at the level of CD34+ BM progenitor cells and was most striking with G-CSF. These studies suggest that the abnormality causing cyclic hematopoiesis in humans with cyclic neutropenia lies in growth factor receptor binding or in the postreceptor signal transduction system that drives granulocytopenia. To investigate this hypothesis in the grey collie model, we examined BM mononuclear cells and neutrophils from grey collie dogs to determine whether G-CSF responsiveness and G-CSF receptor expression were abnormal.

Materials and Methods

Dogs and blood sampling. Grey collie dogs (ages 1 to 4 years) and normal mongrel dogs were housed in American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited animal care facilities as previously described.14-16 Daily blood counts were performed between 7 AM and 9 AM, including a white blood cell count with differential, hematocrit, reticulocyte, and platelet determinations. Blood samples for neutrophil isolations required between 50 and 120 mL and were timed in grey collies to maximize the neutrophil yield.

In vitro colony formation assays. In vitro BM colony formation was used to assess the potential responsiveness of canine marrow cells to G-CSF. Assays were performed as previously described, using normal and grey collie BM. BM was aspirated into heparin, diluted, and subjected to Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradient centrifugation. To reduce background growth factor production by cells in the marrow, two cycles of adherence to the surface of plastic flasks (T-75; Falcon, Lincoln Park, NJ) for 1 hour at 37°C were performed. The mononuclear cells were plated at 1 x 105 cells/mL in the presence of 10% fetal calf serum, 5% normal dog serum, and varying concentrations of exogenous G-CSF. Recombinant human and canine G-CSF (rhG-CSF and rcG-CSF) were kindly provided by Dr Lawrence Souza (Amgen, Thousand Oaks, CA) and Dr Brian Druker (Dana-Farber Cancer Institute, Boston, MA).

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CA). Colonies (greater than 50 cells) were counted at 8 to 10 days ("total colonies"). As a positive control, phytohemagglutinin-stimulated canine leukocyte conditioned medium (PHA-LCM) was routinely assayed as an estimate of maximal colony-forming capacity for each marrow specimen. Data are presented as percent of maximal colony formation. 

**Isolation of neutrophils.** Neutrophils were isolated from heparinized peripheral blood by ficoll-hypaque gradient centrifugation followed by sedimentation in 3% dextran sulfate as previously described. Cells (95% to 98% neutrophils) were washed once with phosphate-buffered saline (PBS) and then resuspended in RPM1 1640 (GIBCO) containing 0.5% BSA (fraction V; Sigma) at 5 X 10^6 cells/mL. Neutrophils were pretreated with 1 mmol/L DFP for 30 minutes at 4°C to inhibit proteases before G-CSF stimulation. Cells were then warmed to 37°C and treated with varying concentrations of G-CSF from 0.01 to 10 ng/mL for 15 minutes. After stimulation, cells were washed in PBS and lysed in lysis buffer containing 25 mmol/L HEPES and 4 mg/mL bovine serum albumin (BSA), with the pH adjusted to 7.4.

**Iodination of rhG-CSF.** All receptor binding studies were performed with rhG-CSF. Five micrograms of the rhG-CSF analogue Met-Lys-rhG-CSF (kindly provided by L. Souza and T. Boone, Amgen) was radioactively labeled using lactoperoxidase, glucose oxidase, and 1 mCi NaI (Amersham, Arlington Heights, IL) as previously described. The 125I-labeled G-CSF was purified by G-50 sephadex gel filtration. This procedure resulted in specific radioactivities for 125I-G-CSF ranging from 1.7 to 4.6 X 10^6 cpm/ng as determined by self-displacement analyses.

**G-CSF binding assay.** Neutrophils (2.0 to 5.0 X 10^6) were incubated for 3 hours at 4°C in binding buffer containing varying concentrations of 125I-G-CSF in the range of 0.01 to 10 mmol/L in the presence or absence of a 100-fold excess of unlabeled G-CSF in a final volume of 400 mL. After incubation, the cells were resuspended, transferred onto a 0.75-ML ice-cold mixture of 75% calf serum in binding buffer and then centrifuged. The supernatants were aspirated and the cell pellets were counted in a gamma counter. All binding experiments were performed using duplicate incubations. Specific binding was defined as the amount of binding competed by a 100-fold excess of unlabeled G-CSF. Equilibrium binding data were analyzed according to the method of Scatchard and by weighted nonlinear least-squares curve fitting as described by Munson and Rodbard. Objective statistical criteria (F test, extra-sum squares modeling between models.)

**Affinity labeling and immunoprecipitation of 125I-G-CSF ligand receptor complexes from dog neutrophils.** A total of 5 X 10^6 neutrophils in 1.0 mL binding buffer was incubated for 3 hours at 4°C with 500 pmol/L 125I-G-CSF in the presence or absence of a 100-fold excess of unlabeled G-CSF. The cells were washed twice with ice-cold PBS and resuspended in 1.0 mL cold PBS, pH 8.3, to which disuccinimidyl suberate (DSS) was added at a final concentration of 1 mmol/L. The reaction was quenched after 30 minutes by the addition of 100 mmol/L Tris-HCl, pH 7.4, and the samples were washed twice with cold PBS. To reduce background nonspecific binding and uncross-linked 125I-G-CSF, the pellets were resuspended in 70 mmol/L sodium acetate and 50 mmol/L NaCl, pH 4.0, for 10 minutes at 0°C. The samples were washed twice with cold PBS and gently rocked for 20 minutes at 4°C in 1.0 mL lysis buffer consisting of PBS with 1% Triton, 2 mmol/L phenylmethylsulfonyl fluoride (PMSF; Sigma, St Louis, MO), 10 mmol/L pepstatin (Sigma), 1 mg/mL aprotinin (Sigma), 2 mmol/L EDTA, 10 mmol/L leupeptin (Sigma), and 1 mmol/L diisopropyl-fluorophosphate (DFP; Sigma). The supernatants from spun samples were incubated overnight at 4°C with 30 mL of rabbit antihuman G-CSF antisera that was generated in our laboratory, then 12 hours later incubated with 10% washed staphylococcal A protein (Pansorbin; Calbiochem, La Jolla, CA) for 4 hours at 4°C. The spun samples were washed three times in lysis buffer and then resuspended in sodium dodecyl sulfate (SDS) sample buffer containing 5% 2-mercaptoethanol. Aliquots of the supernatants were applied to a 7.5% SDS-polyacrylamide gel. The dried gel was autoradiographed for 2 weeks at -70°C with an intensifying screen.

**Isolation of RNA and Northern analysis.** Total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction. Ten-microgram total cellular RNA samples were fractionated by electrophoresis through a 1% agarose formaldehyde gel, transferred to nylon membranes, and hybridized to a minimum of 10^6 cpm/mL 32P-labeled probe for the human G-CSF receptor. A probe for the human G-CSF receptor was prepared by Xba I digestion of the 2.5-kb Bgl II bound insert of the human G-CSF receptor cDNA clone D7 (kindly provided by Alf Larsen, Immunex, Seattle, WA) ligated into the Bam site of pBluescript II SK and labeled with [α-32P]dATP by random oligonucleotide priming. The filters were hybridized at 42°C for 16 hours, washed to a final stringency of 0.25X SSC, and exposed to Kodak X-OMat films (Eastman Kodak, Rochester, NY).

**Immunoblotting of cell lysates.** Neutrophils were isolated as described above after which the cell pellet was washed twice with Hank's Balanced Salt Solution (GIBCO, Grand Island, NY). Red blood cells were lysed by incubation for 30 minutes at 4°C in ammonium chloride (8.29 g/L, pH 7.27). The neutrophil pellet was resuspended in RPM1 1640 (GIBCO) containing 0.5% BSA (fraction V; Sigma) at 5 X 10^6 cells/mL. Neutrophils were pretreated with 1 mmol/L DFP for 30 minutes at 4°C to inhibit proteases before G-CSF stimulation. Cells were then warmed to 37°C and treated with varying concentrations of G-CSF from 0.01 to 10 ng/mL for 15 minutes. After stimulation, cells were washed in PBS and lysed in lysis buffer (20 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 10% glycerol, 1% NP-40), containing 1 mmol/L PMSF, 0.15 U/mL aprotinin, 10 μg/mL leupeptin, and 1 mmol/L sodium orthovanadate (Sigma) at 4°C for 20 minutes at a concentration of 10^6 cells/mL. Insoluble material was removed by centrifugation at 4°C for 10 minutes at 10,000g. Whole cell lysates (5 X 10^6 cells) were mixed 1:1 with 2X SDS sample buffer containing 2-mercaptoethanol and heated at 95°C for 3 minutes before one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 7.5% polyacrylamide. After electrophoresis, proteins were electrophoretically transferred to 0.2 μ nitrocellulose (Schleicher & Schuell, Keene, NH) in a buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol at 0.4 Amp for 4 hours at 4°C. Residual binding sites were blocked by incubation in TBS (10 mmol/L Tris, pH 8.0, 150 mmol/L NaCl) containing 2% gelatin for 60 minutes at 25°C. The blots were incubated overnight at room temperature with the antiphosphorysine antibody 4G10 (1.5 μg/mL) in TBST (TBS with 0.05% Tween 20). The 4G10 antibody was generated using phosphorylamine as the immunogen and antibody reactions were detected as described.

**Prestained molecular weight markers (BRL, Gaithersburg, MD) were also loaded onto the gel.**

**RESULTS**

**In vitro colony growth.** BM mononuclear cells from grey collie dogs required sevenfold higher concentrations of G-CSF to achieve half-maximal colony growth than cells from normal dogs (56 pmol/L vs 8 pmol/L), as shown in Fig 1. Similar dose-response curves were obtained using either rG-CSF or rhG-CSF and were therefore pooled for presentation.

**Receptor expression studies.** Neutrophils from three grey collie and three normal dogs were examined in a total of eight separate experiments for G-CSF receptor expression. Representative binding curves and Scatchard analyses are shown in Fig 2. Neutrophils from normal dogs express a single class of high-affinity receptors with 56 to 446 binding sites per cell and a kd of 28 to 206 pmol/L (n = 4, Fig
G-CSF RECEPTOR IN CANINE CYCLIC HEMATOPOIESIS

Fig 1. In vitro colony assays. Nonadherent BM mononuclear cells from normal dogs (C) and grey collies (B) were plated at 1 x 10^6 cells/mL in NCTC 109 medium containing 10% FCS, 5% normal dog serum, and varying concentrations of rG-CSF or rhG-CSF. Colonies (>50 cells) were counted at 10 days. The percentage of maximal colony growth in each individual experiment is plotted as a function of G-CSF concentration. Data presented are derived from the mean of triplicate samples in each experiment, with a total of five experiments from normal dogs and four experiments in grey collies. Error bars represent ±1 SD.

2A). Similar receptor numbers for G-CSF with a comparable dissociation constant were observed on neutrophils from grey collies with 78 to 199 receptors per cell and a KD of 84 to 195 pmol/L (n = 4, Fig 2B). G-CSF receptor expression was also analyzed in one grey collie dog before and during treatment with rhG-CSF. Before initiation of G-CSF therapy, the number of G-CSF receptors on neutrophils from this dog was 85 per cell and, on a repeat determination 18 months later while on G-CSF, 86 per cell (data not shown).

Cross-linking studies. Experiments were performed to determine the size of the G-CSF binding proteins on neutrophils from normal and grey collie dogs. As shown in Fig 3, when neutrophils from normal dogs and grey collies were incubated with 125I-labeled G-CSF, then covalently cross-linked with DSS, immunoprecipitated with polyclonal anti-G-CSF antibody, and analyzed by SDS-PAGE under reducing conditions, an identical major labeled band with a molecular weight of 139 kD was observed (see arrow). This band was completely abolished on both normal and grey collie neutrophils when a 100-fold excess of unlabeled G-CSF was present during the incubation. Because the molecular weight of G-CSF is 19 kD, the calculated molecular weight of the G-CSF binding protein on normal and grey collie neutrophils is approximately 120 kD. Similar results were obtained and confirmed by densitometry using BM mononuclear cells from normal and grey collie dogs (data not shown).

Northern analysis. G-CSF receptor expression in normal and grey collie cells was also examined by Northern blot analysis. As shown in Fig 4, G-CSF receptor mRNA transcripts of identical size were detected in normal and grey collie neutrophils. The canine neutrophil G-CSF receptor transcripts migrated with a slightly slower mobility than the human neutrophil transcript.

Antiphosphotyrosine immunoblots. Neutrophils from both normal and grey collie dogs were stimulated with G-CSF at 0.01, 0.1, 1.0, and 10 ng/mL and evaluated by immunoblotting with an antiphosphotyrosine antibody to determine whether the pattern of tyrosine phosphorylation induced by G-CSF in grey collie cells differed from that observed in normal dog cells. Rapid and prominent tyrosine

Fig 2. Saturation binding curve for 125I-labeled G-CSF and Scatchard analyses. Binding of 125I-labeled recombinant G-CSF to neutrophils from a normal dog (A) and grey collie (B) is shown with Scatchard transformations of the data in the inset. Neutrophils (5 x 10^6 cells) were incubated for 3 hours at 4°C with varying concentrations of 125I-G-CSF in the absence or presence of a 100-fold excess of unlabeled G-CSF. Specific binding shown on the vertical axis is the amount of binding competed by excess unlabeled G-CSF. Binding parameters were determined by Scatchard analysis of the data points from the saturation binding curve using the LIGAND program. In this representative experiment, the calculated number of binding sites on normal dog cells was 65 per cell with a KD of 84 pmol/L, whereas 103 sites per cells with a KD of 112 pmol/L were detected on grey collie cells.
phosphorylation of a protein of relative molecular weight of 80 kD was seen in both normal and grey collie neutrophils (Fig 5, arrow) and was most apparent at G-CSF concentrations of 1 ng/mL and 10 ng/mL.

DISCUSSION

Cyclic hematopoiesis in grey collie dogs is a stem cell disease in which abnormal regulation of hematopoietic cell production in the BM causes cyclic fluctuations of blood cell counts. The canine disease is a close analogue of the human disorder. Clinical trials in humans with this disorder have demonstrated the requirement for supraphysiologic doses of G-CSF to ameliorate neutropenia. In grey collie dogs, neither IL-3 nor GM-CSF altered cyclic fluctuations in blood counts, whereas only high doses of G-CSF obliterated all of the cyclic fluctuations. In humans with congenital cyclic neutropenia, abnormally increased concentrations
of G-CSF are required to stimulate in vitro granulocytic colony formation by BM progenitor cells. On the basis of these observations, a defect in G-CSF signal transduction receptor expression on neutrophils differs from receptor expression on BM mononuclear cells from grey collies required abnormally high concentrations of G-CSF in vitro to achieve half-maximal colony growth compared with cells from normal dogs. This finding in vitro as well as our previous observations in vivo that pharmacologic but not physiologic doses of G-CSF eliminated cyclic oscillations in blood counts in grey collies suggested to us that grey collies might express reduced numbers of G-CSF receptors or receptors with an altered affinity for G-CSF. We therefore examined binding of 125I-labeled G-CSF to neutrophils from grey collies and normal dogs. Scatchard analyses of the equilibrium binding data were consistent with expression of a single class of high-affinity receptors on both normal and grey collie cells with similar receptor numbers and binding affinities. No variation in receptor numbers or affinity was observed in the one dog studied during G-CSF treatment. The number and affinity of G-CSF receptors on canine neutrophils is similar to that previously reported for human neutrophils.

In this study, we also characterized the protein that binds G-CSF on grey collie neutrophils. A binding protein of approximately 120 kDa was identified on neutrophils from both normal and grey collie dogs. No abnormal G-CSF receptor mRNA transcripts were detected in grey collie neutrophils by Northern analysis. These structural data suggested no large defect in the G-CSF receptor itself.

Recently, Druker et al reported rapid tyrosine phosphorylation of p80 in human neutrophils treated with G-CSF. Because this is one of the first biochemical events in the signal transduction pathway activated in neutrophils after G-CSF stimulation, we investigated the patterns of protein phosphorylation in normal and grey collie neutrophils after treatment with G-CSF to determine whether G-CSF receptor function in signal transduction is defective in grey collie cells. A similar dose-response to G-CSF was observed. G-CSF rapidly induced prominent tyrosine phosphorylation of an 80-kD protein, suggesting that this part of the pathway in G-CSF-mediated signal transduction is intact in grey collie cells.

The data presented here complement the previous observations by Kyas et al, who reported normal numbers of G-CSF binding sites on neutrophils from humans with cyclic neutropenia. In their study, no information was provided on the size of the receptor protein or size of the mRNA transcripts of G-CSF receptors. In the present study, using the canine model, we show that the abnormal response to G-CSF in cyclic hematopoiesis is not caused by alterations in the number or binding affinity of the G-CSF receptor expressed on neutrophils. However, it is possible that G-CSF receptor expression on neutrophils differs from receptor expression on precursor cells and that the level of expression or properties of the G-CSF receptor on grey collie precursor cells are abnormal. Also, the size of the G-CSF receptor protein and mRNA transcripts are not demonstrably altered in cyclic hematopoiesis. We cannot exclude the possibility of a small deletion or point mutation in the G-CSF receptor, or expression of different G-CSF receptor molecules arising from alternative splicing mechanisms, which would not be detected by the methods used in our studies. Detection of such a defect would require sequence data on the G-CSF receptor gene in grey collie dogs. However, a sensitive functional assay of the receptor shows that one of the early signal transduction pathways activated by G-CSF is clearly intact in grey collie cells.

Taken together, our results show that the abnormal responsiveness to G-CSF in canine cyclic hematopoiesis is not caused by gross alterations in the number, binding affinity, or size of the neutrophil G-CSF receptor, or by abnormal tyrosine phosphorylation of an 80-kD protein that behaves like canine c-rel. These data suggest that the underlying defect in cyclic hematopoiesis is in the G-CSF signal transduction pathway at a point distal to the G-CSF receptor.

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