RAPID COMMUNICATION

Absence of Abnormalities of c-kit or Its Ligand in Two Patients With Diamond-Blackfan Anemia

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As Diamond-Blackfan anemia shares clinical features with W and Steel defects in mice, we investigated the possibility that this human disorder might result from an abnormality of the c-kit receptor or its ligand, stem cell factor (SCF). For these studies, full nucleotide sequences for coding regions of c-kit and SCF were generated for two Diamond-Blackfan anemia patients and were normal. Similarly, the kds of SCF receptors on their marrow (31 pmol/L, 43 pmol/L) were comparable with those found in three normal controls (50 pmol/L, 55 pmol/L, 27 pmol/L). Serum SCF concentrations were 6.9 ng/mL in patient A, 14.6 ng/mL in patient B, who has been in hematologic remission since adolescence, and 2.7 ng/mL in the 3-year-old daughter of patient B, who also has Diamond-Blackfan anemia but is transfusion-dependent. It is possible that the SCF level in patient B increased with puberty, leading to her remission. These data provide evidence that Diamond-Blackfan anemia does not result from structural abnormalities of c-kit or SCF.

DIAMOND-BLACKFAN anemia is a congenital macrocytic anemia. The numbers of erythroid precursors in the marrow are decreased and few reticulocytes are seen in the blood, but the production and morphology of granulocytes and platelets are normal. As these findings are similar to the hematologic abnormalities in mice with defects of either the W (c-kit) or Steel (stem cell factor [SCF], kit-ligand) locus, several investigators have hypothesized that Diamond-Blackfan anemia may have an analogous pathogenesis. For this reason, we characterized c-kit and SCF cDNA and protein in two patients with Diamond-Blackfan anemia.

MATERIALS AND METHODS

Patient description. Studies were performed on patients A and B. The clinical presentations, hematologic data, and marrow culture studies for these individuals have been described previously (ref 4; patients 1 and 4, respectively).

The anemia in patient A (now age 22) was first detected at age 7 years and was unresponsive to steroids, cyclophasphamide, and splenectomy. He remains transfusion-dependent. Currently his pretransfusion hematocrit is 13, with a reticulocyte count of 0, a white blood cell (WBC) count of 9.4 with a normal differential, and a platelet count of 327,000. His marrow aspirate has erythroid hypoplasia (the ratio of erythroid to granulocytic cells [E/G] = 0.1). Pronormoblasts are increased in number and have prominent nucleoli. The subsequent erythroid maturation is megaloblastic. Fetal hemoglobin (HgbF) is elevated, and the serum erythropoietin level is 8,800 mU/mL (normal 5 to 20 mU/mL). There is no evidence of human parvovirus B19 in his serum or marrow by polymerase chain reaction (PCR) and Southern analysis (personal communication, Neal Young, Bethesda, MD, August 1991). Marrow cytogenetics are normal.

Patient B (now age 26) presented with Diamond-Blackfan anemia at age 6 months and responded to high-dose prednisone. In the 3-year-old daughter of patient B, who also has Diamond-Blackfan anemia but is transfusion-dependent. It is possible that the SCF level in patient B increased with puberty, leading to her remission. These data provide evidence that Diamond-Blackfan anemia does not result from structural abnormalities of c-kit or SCF.

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incubated with the 125I-SCF for 4 hours in a shaking water bath at 15°C. Our initial experiments demonstrated plateau binding with these conditions and minimal (< 20%) internalization of 125I-SCF. At the conclusion of the incubation, cell-associated 125I-SCF was separated from free 125I-SCF by sedimentation through phthalate oil. Both cell-bound and free 125I-SCF were quantitated, and equations for one or two classes of receptors were fitted to the data using the Ligand program.

Autoradiography. Because the frequency of erythroid precursor cells in marrow from Diamond-Blackfan patients differs substantially from that of normal marrow, we used autoradiography to assess binding of 125I-SCF to individual marrow blasts. Marrow mononuclear cells from patient A and from a normal adult were incubated with 125I-SCF (0.5 nmol/L) in binding buffer for 1 hour at 37°C. At the conclusion of incubation, the cells were sedimented through Percoll (Sigma; density 1.028 g/mL) to separate cell-bound from free 125I-SCF. Cytocpreparations were made in a cytospin (Shandon Southern Co, Pittsburgh, PA) and were processed for autoradiography. After a 3-week exposure, the slides were developed and stained with Wright-Giemsa. Grains overlaying the cells and in an area immediately adjacent to each cell were counted; the grain count in the adjacent area was then subtracted.

Quantification of SCF protein in serum. An enzyme immunoassay was used to measure SCF protein in serum samples (Bennett L, Langley KL, Wypych J, Zsebo KM: manuscript in preparation). In brief, serum samples were added to microtiter wells (Dynatech Laboratories, Inc, Alexandria, VA) that had previously been coated with affinity purified rabbit antihuman SCF polyclonal antibodies (AMGEN). Purified recombinant human SCF (1-248) expressed in Chinese hamster ovary (CHO) cells was used to generate a standard curve. Bound SCF was identified using the 7H6 antihuman SCF monoclonal antibody (AMGEN) covalently coupled to horseradish peroxidase. Absorbance at 450 nmol/L was read using a Vmax Kinetic Microplate Reader (Molecular Devices Corp, Menlo Park, CA).

RESULTS

Sequencing of cDNAs for c-kit and SCF. PCR amplification of regions of the SCF and c-kit coding regions in cDNA of both patients produced single major bands of the sizes generated from the published sequences. Direct sequencing of partially overlapping PCR-produced cDNA fragments which in aggregate spanned the entire coding regions of SCF and of c-kit showed no differences from the published data and no evident double sequences (sites of heterozygosity).

SCF receptor quantitation. Equilibrium binding experiments with 125I-SCF were performed with marrow mononuclear cells from patients A and B, and from three normal individuals. Marrow cells from patient A displayed a single class of high-affinity SCF receptors with a kd of 31 pmol/L (Fig 1), and approximately 72 receptors per cell. A second full equilibrium binding experiment with marrow mononuclear cells from patient A showed the kd of the SCF receptor to be 37 pmol/L, with 98 receptors per cell (data not shown). Cells from patient B were found to exhibit high-affinity SCF receptors (kd 43 pmol/L) with 190 receptors per cell (Fig 2). The binding affinity of the SCF receptor on marrow cells from three different normal adults were 50 pmol/L (Fig 3), 55 pmol/L, and 27 pmol/L. The number of receptors in the three normal volunteers was 410 ± 165 receptors per cell (mean ± SD).
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variation in the concentration of SCF with or without unlabeled SCF as factor and colony stimulating factor-1. W mutations result in defective progenitor cells. Steel mutations result from the reciprocal translocation.

We and other investigators have hypothesized that Diamond-Blackfan anemia might result from defects in the ligand for the SCF receptor. SCF, which is produced by marrow stromal fibroblasts, is structurally similar to that in patients with Diamond-Blackfan anemia, and progenitor cells are particularly rich in SCF receptors. In further studies we demonstrated that marrow cells from the two patients with Diamond-Blackfan anemia retain the ability to bind SCF with high affinity, indicating that the ligand binding domain of the SCF receptor is expressed on these cells and is intact. The binding affinity of their SCF receptors was very similar to that found on normal marrow cells. However, the number of SCF receptors in the entire population of marrow mononuclear cells appeared to be lower in the Diamond-Blackfan patients (particularly in the severely anemic patient) than in the normal adults. Because normal human erythroid precursor and progenitor cells are particularly rich in SCF receptors, the relative depletion of erythroid cells in the marrows of Diamond-Blackfan patients could explain these results. Direct comparison of [125I]-SCF binding to normal and Diamond-Blackfan hematopoietic precursor cells showed a similar number of grains associated with individual blasts.

As the hematologic phenotype in W and steel mice is similar to that in patients with Diamond-Blackfan anemia, we and other investigators have hypothesized that Diamond-Blackfan anemia might result from defects of c-kit or SCF. In support of this hypothesis, BFU-E from Diamond-Blackfan patients respond dramatically to SCF in vitro, whereas the number of erythroid bursts per 10^5 marrow mononuclear cells and the number of erythroid cells per burst increase with SCF. In some individuals, eg, patient B, the dose-response of BFU-E to recombinant SCF is normal. Plateau frequencies of erythroid bursts were detected with 2.5 ng/mL SCF, raising the possibility that her endogenous SCF was decreased in concentration or abnormal in structure. In other patients, eg, patient A, high concentrations of SCF (> 50 ng/mL) are required in vitro for optimal BFU-E growth. This introduced the question of whether the c-kit receptors on his erythroid progenitors were defective.

To test these hypotheses, cDNA for SCF and c-kit were analyzed. As missense mutations and short deletions are associated with W and SI defects in mice, nucleotide sequencing was required. Full nucleotide sequences for the SCF and c-kit coding regions were generated for each patient after PCR amplification of overlapping portions of cDNA, and were normal.

Heterozygosity, in the form of double sequences caused by single or multiple base missense mutations or short deletions or insertions, or the presence of a PCR product of the wrong size in addition to the correct product was not observed. Although these experiments did not show any mutations, they cannot rule out all possible SCF and c-kit mutations. For example, certain mutations, if present heterozygously, would escape detection with these methods. A mutation which removed an entire end of the cDNA, or which inserted a large enough sequence into the cDNA so that primer pairs spanning it would be too far apart to amplify efficiently, would be missed because only the cDNA of the normal allele would be amplified by PCR and be visualized. In addition, we did not analyze for mutations affecting the control regions of c-kit or SCF. Such abnormalities may be detected with more comprehensive cDNA or genomic DNA cloning methods.

In further studies we demonstrated that marrow cells from the two patients with Diamond-Blackfan anemia retain the ability to bind SCF with high affinity, indicating that the ligand binding domain of the SCF receptor is expressed on these cells and is intact. The binding affinity of their SCF receptors was very similar to that found on normal marrow cells. However, the number of SCF receptors in the entire population of marrow mononuclear cells appeared to be lower in the Diamond-Blackfan patients (particularly in the severely anemic patient) than in the normal adults. Because normal human erythroid precursor and progenitor cells are particularly rich in SCF receptors, the relative depletion of erythroid cells in the marrows of Diamond-Blackfan patients could explain these results. Direct comparison of [125I]-SCF binding to normal and Diamond-Blackfan hematopoietic precursor cells showed a similar number of grains associated with individual blasts.

These studies suggest that structural abnormalities of c-kit or SCF are not likely explanations of Diamond-Blackfan anemia in patients A and B. As Diamond-Blackfan anemia is a clinically heterogenous disorder, such abnormalities could exist in other patients.

An unexpected finding of our study was the extremely high serum level of SCF (14.6 ng/mL) in patient B, who has been in clinical remission since puberty (age 13). This contrasts with the SCF levels in sera from her anemic daughter (2.7 ng/mL), from patient A (6.9 ng/mL), from 30 individuals with acquired pure red blood cell aplasia (mean 3.1 ± 1.7, range 1.8 to 9.8 ng/mL) (J.L.A. L.G.B., K.M.Z.: unpublished data, August 1991) and from 167 normals (mean 3.4 ± 1.0, range 1.5 to 6.6 ng/mL) (L.G.B., K.M.Z.: unpublished data, August 1991). We hypothesize that this high systemic level of SCF led to the improvement in the anemia in patient B. This observation and the impressive response of BFU-E from Diamond-Blackfan patients to SCF in vitro reinforce a potential therapeutic role for SCF in this disorder.
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