Interferon-α restores β1-integrin-dependent, collagen-mediated platelet aggregation in a patient with chronic myelogenous leukemia

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Although interferon-α (IFN-α) induces hematologic remissions in 70% to 80% of patients with chronic myelogenous leukemia (CML) and complete or near-complete cytogenetic remissions in 10% to 20% of patients, the exact mechanisms underlying these clinical results remain unclear. We have hypothesized that IFN-α acts at least in part through restoration of β1-integrin function on malignant hematopoietic progenitors that can promote adhesion of malignant progenitors to the marrow microenvironment. This may then restore microenvironmental inhibition of progenitor proliferation and induce tumor dormancy. We demonstrate that IFN-α administration to a patient suffering from a clinically severe bleeding diathesis reversed the defective collagen-mediated aggregation of platelets expressing normal numbers of functionally inactive collagen receptors. This is the first in vivo demonstration that IFN-α can up-regulate the function of adhesion receptors in CML and supports the premise that IFN-α induces remissions by restoring normal integrin-mediated interactions between progenitors and microenvironmental components. (J Lab Clin Med 1998;131:163-9)

Abbreviations: BSA = bovine serum albumin; CML = chronic myelogenous leukemia; GP = glycoprotein; HBSS = Hanks' buffered salt solution; IFN-α = α-interferon; Ph = Philadelphia chromosome; Ph+, Ph- = Philadelphia chromosome-positive, Philadelphia chromosome-negative; RBC = red blood cell; WBC = white blood cell

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Although IFN-α has been shown to induce hematologic remissions in 70% to 80% of patients with CML and to induce complete or near-complete cytogenetic remissions in 10% to 20% of patients, the exact mechanisms underlying these clinical results remain unclear. We have hypothesized that IFN-α acts at least in part through restoration of β1-integrin function on malignant hematopoietic progenitors that can promote adhesion of malignant progenitors to the marrow microenvironment. This may then restore microenvironmental inhibition of progenitor proliferation and induce tumor dormancy.

CASE REPORT

P.C. is a 59-year old white woman who was diagnosed with Ph+, chronic-phase CML in 1987 (Fig. 1). She was treated with hydroxyurea with good control of peripheral

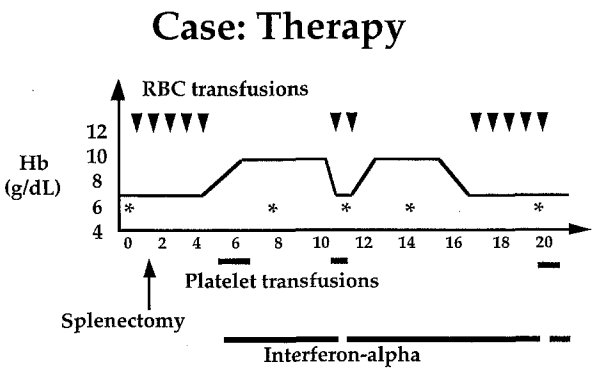

Fig. 1. Clinical course. Indicated are treatment with IFN-α, need for red cell and platelet transfusions, and times (*) at which platelet aggregation studies were done. The *x*-axis units of time are months.

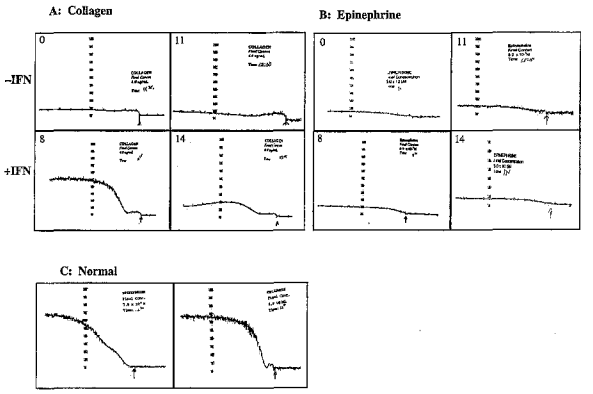

Fig. 2. Platelet aggregation studies. Platelet aggregation with 4μg collagen (**A**) and 5×10^{-5} epinephrine (**B**) were done twice while the patient was receiving IFN-α (8 and 14 months after referral to the University of Minnesota) and twice while the patient was untreated (before starting IFN-α treatment and 11 months after referral). Platelet aggregation with collagen and epinephrine was done in a normal control subject (**C**).

counts and symptoms. Six years after the initial diagnosis, she experienced bruising, repeated episodes of epistaxis, and gastrointestinal bleeding necessitating transfusion of 20 units of RBCs over the next year. Aside from some mild gastritis, which was treated with H2-blockers and later omeprazole, results of esophagogastroduodenoscopies, colonoscopies, and an abdominal arteriography did not reveal a localized source for her gastrointestinal bleeding. During that time she developed frank splenomegaly, thrombocytopenia ranging between 50 and 120 × 10^9/L, and significant leukocytosis ranging between 30 and 120 × 10^9/L. She was referred to the University of Minnesota for consideration of autologous stem cell transplantation. Her medical history is also significant for granulosa cell carcinoma of the ovary 15 years earlier, which was treated with radical hysterectomy and cobalt radiation therapy from the umbilicus to the symphysis pubis for an estimated dose of 3200 cGy. She also previously underwent partial thyroidectomy for a benign thyroid cyst and cholecystectomy.

When she was first seen at the University of Minnesota, we noted several large bruises diffusely over her extremities and trunk and small petechiae but no telangiectasiae in the oral mucosa. The spleen was palpable 15 cm below the left costal margin. During the month of February, she continued to have frank melanotic stools requiring transfusion of 6 units of RBCs to keep her hemoglobin above 8 gm/dl. Reticulocytosis was between 8% and 10%. The WBC count ranged between 100 and 145 × 10^9/L and the platelet count between 80 and 120 × 10^9/L. RBCs were morphologically hypochromic and microcytic, and there was significant polychromasia, anisopoikilocytosis, and rare tear drop cells. The WBC differential was 70% neutrophils, 8% monocytes, 5% metamyelocytes, 7% myelocytes, 2% promyelocytes, 7% basophils, and <1% blasts. There was marked platelet anisocytosis, with the largest platelets seen being approximately 16 μm across. Twenty percent of the platelets were agranular and 80% were hypogranular. A bone marrow aspirate and biopsy were performed that confirmed the diagnosis of CML in apparent chronic phase. Decreased iron stores were noted. Cytogenetic analysis demonstrated 20/20 metaphases with a single Ph chromosome. Serum iron level was <10μgm/dl (normal range 35 to 180 μg/dl) and serum transferrin level was 485 μgm/dl (normal range 210 to 360 μg/dl). Plasma coagulation studies were essentially normal, with a prothrombin time of 10.6 seconds (international normalized ratio of 0.95), a partial thromboplastic time of 29.7 seconds, a thrombin time of 14.8 seconds, and a Clauss fibrinogen value of 0.54 gm/dl. von Willebrand antigen as determined by enzyme-linked immunosorbent assay as well as the ristocetin cofactor activity were elevated. Distribution of von Willebrand multimers as determined by crossed immunoelectrophoresis was normal. Bleeding time was prolonged (>20 minutes; normal <8 minutes), and platelet aggregation studies demonstrated normal aggregation with serotonin, adenosine diphosphate and ristocetin. A weak primary aggregation but no secondary aggregation with epinephrine was observed, a feature that is seen in 10% to 15% of normal individuals. More importantly, a complete absence of aggregation was noted with 2 and 4 μg/ml collagen (Fig. 2). Further, these aggregation studies also indicate that no shape change occurred in the platelets after the addition of collagen. Repeat esophagogastroduodenoscopy, colonoscopy, arteriography, and tagged red cell scan again failed to demonstrate a lesion responsible for the gastrointestinal bleeding. All patient blood samples obtained for experimental purposes were obtained according to guidelines from the Committee on the Use of Human Subjects in Research at the University of Minnesota.

The patient was taken to the operating room for splenectomy, because the massive splenomegaly was thought to contribute to her RBC transfusion requirements and thrombocytopenia. An intra-operative ileoscopy revealed the presence of a bleeding lesion mid ileum and a second

lesion in the jejunum, which were resected. Pathologic examination of the spleen was compatible with CML. The ileal and jejunal lesions revealed acute mucosal/submucosal hemorrhage and granulation tissue. After surgery she was continued on hydroxyurea (1500 mg/day) with better control of her platelet count, which ranged between 400 and 500 $\times$ 10^9/L, and her WBC count, which ranged between 13 and 25 $\times$ 10^9/L. However, she continued to have episodes of melanotic diarrhea for which she was rehospitalized on three different occasions during the next 3 months. Repeated upper and lower endoscopies, arteriographies, and tagged red cell scans did not reveal a localized lesion. She required transfusion with 19 units of RBCs. She was given a trial of epsilon amino caproic acid that was discontinued because of intolerance. Subsequently she was started on 4.5 MU/day IFN-α, and hydroxyurea was decreased to 500 mg/day. During the first month of IFN-α therapy, she also received twice-weekly 6-packs of random donor platelets. During that period her bruises disappeared and she had no significant gastrointestinal blood loss. Her peripheral counts stabilized with a hemoglobin value between 8.5 and 10 gm/dl, a WBC count between 10 and 20 $\times$ 10^9/L, and a platelet count between 200 and 300 $\times$ 10^9/L. One month after IFN-α therapy was started, platelet transfusions were stopped and she was maintained on hydroxyurea and 4.5 MU/day IFN-α without significant change in her peripheral blood parameters. Platelet aggregation studies were repeated, which now demonstrated normal aggregation with collagen. Both the initial shape change seen after the addition of collagen and the ensuing aggregation were restored. However, abnormal aggregation with epinephrine persisted (Fig. 2). Furthermore, platelet morphology continued to be abnormal. Four months later, IFN-α therapy was stopped in preparation for autologous transplantation. However, 10 days later, she again developed bruising and melanotic diarrhea. Seven days after IFN-α treatment was stopped, her hemoglobin level was 9.6 gm/dl but dropped to 6.1 gm/dl after a further 7 days. She required transfusion of 5 units of RBCs over the next 2 weeks. Platelet aggregation studies performed 14 days after IFN-α treatment was stopped demonstrated again a complete absence of aggregation with collagen (Fig. 2). Immunophenotypic analysis of her platelets demonstrated the presence of normal numbers of the CD36, CD49b, and CD61 adhesion receptors. Fourteen days after IFN-α was restarted, no new bruises appeared and her melanotic diarrhea ceased. The bleeding time repeated 2 months after re-initiation of IFN-α was 5 and 6 minutes, platelet aggregation studies demonstrated aggregation with collagen (Fig. 2), and immuno-phenotypic analysis of the platelets demonstrated again the expression of normal levels of CD36, CD49b, CD29, and CD61 receptors.

Five months later, accelerated-phase CML developed in the patient, with thrombocytosis, leukocytosis, increasing basophilia, and anemia not as a result of bleeding, which required increasing amounts of hydroxyurea (up to 4 gm/dl). The combination of IFN-α and hydroxyurea

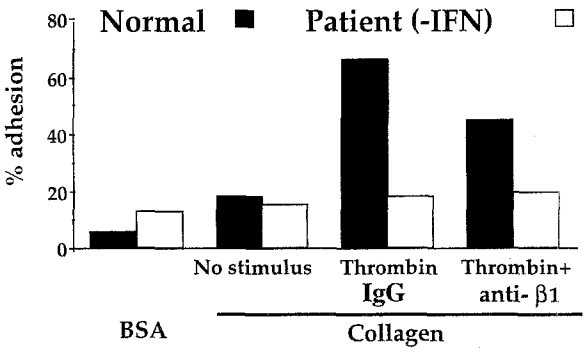

Fig. 3. Platelet adhesion to collagen. Platelets were collected from 50 ml peripheral blood from a normal volunteer donor and from the patient after informed consent was obtained according to guidelines approved by the Committee on the Use of Human Subjects for Research at the University of Minnesota. Platelets were labeled with 50 μCi ^{51}Cr (specific activity 200 to 500 mCi/mg ^{51}Cr) (DuPont, Wilmington, Del.) for 30 minutes; washed twice with Ca/Mg-free HBSS (Gibco, Grand Island, N.Y.), 0.5% BSA (Sigma Chemical Co., St. Louis, Mo., 10% citrate phosphate dextrose, and 1 U/ml heparin (pH 6.5)(Sigma); washed once with Ca/Mg-free HBSS and 0.5% BSA (pH 7.4); and resuspended. Platelets were then exposed to either mouse immunoglobulin G (1 μg/ml, Sigma) or anti-β1-integrin antibodies (1:400 dilution of the murine anti-β1-antibody P4C10, purchased from Sigma) for 15 minutes and allowed to adhere to wells coated with collagen type IV (50 μg/ml, Gibco) in a 48-well plate for 1 hour in the presence or absence of 1 U/ml thrombin (Sigma). Nonadherent platelets were removed by three washes with Ca/Mg-free HBSS, 0.5% BSA, BSA at pH 7.4, and adherent platelets collected after lysis with 10 mol/L NaOH. Percent adhesion was determined by the following equation: (Mean cpm in adherent fraction) – (Spontaneous release of mean cpm) $\times$ 100% = (Percent adhesion in mean cpm in [adherent + nonadherent fraction]) – (Spontaneous release in mean cpm).

abruptly induced thrombocytopenia ($<20 \times 10^9$/L) and neutropenia (absolute neutrophil count $< 1 \times 10^9$/L), necessitating interruption of treatment with both IFN-α and hydroxyurea. Interestingly, before interruption of the IFN-α therapy she did not have clinical signs of bleeding, despite the finding that her platelet count was only 18 $\times$ 10^9/L. Five days after therapy with IFN-α was stopped, her platelet count had rebounded to 55 $\times$ 10^9/L, at which time she again developed bruises and melena. Repeat platelet aggregation studies demonstrated complete absence of aggregation with collagen (Fig. 2), even though the expression of CD29, CD49b, CD36, and CD61 adhesion receptors was unchanged. To further demonstrate the defect in adhesion to collagen, we assessed the adhesion of platelets to collagen with platelets obtained from a normal donor (after informed consent) and with platelets obtained from the patient while she was not being treated with IFN-α. In contrast to platelets from a normal individual, which adhered to collagen through a β1-integrin-dependent mechanism, no adhesion to collagen was seen for platelets from our patient, either spontaneously or after stimulation with thrombin (Fig. 3). Once her platelet

count had recovered to >100 × 10^9/L, IFN-α was restarted at 2 MU/day. Ten days later, platelet aggregation with collagen normalized and the bleeding subsided.

DISCUSSION

CML is an invariably lethal malignant disease of the hematopoietic stem cell[1] that is characterized by the Philadelphia chromosome[2] and the BCR/ABL gene rearrangement.[3] The resulting oncoprotein, $P210^{BCR/ABL}$, is required and sufficient[4,5] for the malignant transformation of hematopoietic cells. Over the last 10 to 15 years, IFN-α has been widely used to treat patients with CML. Seventy percent to 80% of patients treated with IFN-α obtain a hematologic remission, while IFN-α induces a complete or near-complete cytogenetic remission in 10% to 20% of patients.[6-8] The exact mechanism through which IFN-α induces remissions in CML is not understood. IFN-α does not directly affect CML progenitor proliferation.[9] In isolated chromatin nucleoprotein complexes, IFN-α decreases DNA-polymerase activity, an observation that has been correlated with clinical responses to IFN-α.[10] Decreases in DNA-polymerase levels or $p210^{BCR/ABL}$ may eliminate the growth/survival advantage of malignant Ph+ CML progenitors and lead to remission. IFN-α may also inhibit expression of the oncoprotein $p210^{BCR/ABL}$,[11] which is thought to provide an anti-apoptotic signal.[12,13] However, careful examination of marrow from patients with CML treated to complete cytogenetic remission with IFN-α indicates that some Ph+ progenitors persist, because colonies plucked from methylcellulose progenitor cultures of marrow from patients in remission after IFN-α treatment demonstrate the continued presence of the Ph.[14] This suggests persistence of the malignant clone, albeit in a dormant state, rather than elimination of the Ph+ population.

An alternative explanation for the hematopoietic remissions seen with IFN-α treatment is that IFN-α reverses the abnormal circulation and unregulated proliferation of malignant progenitors by restoring defective adhesion mechanisms. Several studies demonstrate that Ph+ hematopoietic progenitors from CML marrow adhere significantly less well to marrow stromal feeders and to the extracellular matrix component fibronectin than do progenitors obtained from normal marrow.[15-18] This lack of adhesion contributes to the uncontrolled proliferation of the Ph+ clone.[17] However, CML Ph+ progenitors express normal numbers of the α4β1 and α5β1 integrins,[15,16] adhesion receptors responsible for the interaction of normal progenitors with stroma and fibronectin.[19] Interestingly, preincubation of Ph+ progenitors for at least 12 hours with >100 U IFN-α restores progenitor adhesion to stromal feeders or fibronectin and restores microenvironmental regulation of their proliferation.[16,17] Because antibodies against the α4, α5, or β1 integrins[16] can inhibit the IFN-α-induced adhesion, these studies indicate that β1-integrins, although present, are functionally abnormal in CML.

Presence of adhesion receptors on the cell surface does not necessarily indicate that the receptor has functional significance.[20-22] Certain integrins are indeed constitutively expressed on the cell surface in a non-functional or low-affinity state, but they can be switched to a functional, high-affinity state by the adhesive ligand itself ("activating antibodies") or by signaling through other adhesion receptors or cytokines. We believe that IFN-α-induced restoration of β1-integrin-mediated adhesive mechanisms may at least in part explain the abrogation of the abnormal trafficking and abnormal growth regulation of CML progenitors by IFN-α.

In our patient, we demonstrate that the bleeding diathesis is caused at least in part by defective platelet aggregation with collagen. The interaction of platelets with collagen is considered to be of primary importance in the arrest of bleeding. After damage to the vessel wall, platelets coming in contact with exposed collagen fibrils in the subendothelium spread along it, which results in activation and secretion of intracellular effectors leading to aggregation.[23,24] The principal receptors responsible for aggregation with collagen are the GPIa/IIa integrin (α2β1 or CD49b/CD29)[25] in association with the GPIV or CD36 receptor[26] and the GPIIb/IIIa integrin (GPIIb, β3 or CD61).[27] A selective platelet aggregation defect with collagen has been described in rare patients in whom the number of α2β1 integrins expressed on platelets was significantly decreased.[28-30] In contrast to these reported cases, expression levels of the collagen receptor α2β1 as well as the CD36 and CD61 receptors on platelets in our patient were normal. Lack of aggregation with collagen must therefore be the result of either a functional defect in the collagen receptor or a downstream component of platelet aggregation. Platelet aggregation studies demonstrated also a loss of the secondary phase of platelet aggregation with epinephrine. Various in vitro abnormalities of platelet function have been described in patients with CML and other myeloproliferative syndromes, including hypoaggregation in response to epinephrine, adensosine diphosphate, thrombin, and platelet-activating factor.[31-33] Of these, impaired responsiveness to epinephrine—which has been attributed to de-

creased numbers of platelet α-adrenergic receptors—has in most studies been the most commonly encountered abnormality.[34] Associated impairment, but not complete loss, of collagen aggregation has been described that has been attributed to an acquired nucleotide storage defect.[32] Because aggregation with collagen, but not epinephrine, was normalized on three separate occasions after IFN-α treatment, we believe that a defect at the collagen receptor level itself is more likely than a defect in a downstream component of platelet aggregation. The initial interaction of platelets with an agonist, including collagen, results in a change in shape from discs to spheres, which translates to an initial increase in light transmission when examined in an aggregometer. The shape change after the addition of collagen was seen only for platelets obtained while the patient was treated with IFN-α but not when obtained at the time the patient was not treated with IFN-α, further indicating that the collagen aggregation defect in our patient is at the receptor level.

The clinical observation that both the bleeding diathesis and the collagen-dependent aggregation defect reoccurred when IFN-α therapy was stopped but disappeared when IFN-α therapy was resumed supports our in vitro observation that IFN-α upregulates the affinity state of integrin adhesion receptors in CML and also supports the hypothesis that IFN-α acts at least in part by restoring normal integrin-mediated interactions between progenitors and bone marrow microenvironmental components. Why integrins are present in a low-affinity state on CML cells is currently unknown. CML is characterized by the BCR/ABL gene rearrangement encoding the novel tyrosine kinase $P210^{BCR/ABL}$, which is necessary and sufficient for the malignant transformation of hematopoietic progenitors.[4,5] Introduction of BCR/ABL cDNA in fibroblasts has been associated with adhesion-independent fibroblast proliferation,[35] strongly suggesting that $P210^{BCR/ABL}$ interferes with integrin-mediated signaling. $P210^{BCR/ABL}$ binds significantly more to F-actin.[36] Compared with p145ABL, $P210^{BCR/ABL}$ has increased tyrosine kinase activity, which results in activation of the Ras pathway[37] and phosphorylation of a number of intracellular adaptor proteins such as FAK,[38] Paxillin,[39] and Crkl,[40] all of which may interfere with normal integrin-dependent signaling. Studies from our group suggest that elimination of $P210^{BCR/ABL}$ in CML clonogenic cells by treating CD34+ cells with anti-BCR/ABL anti-sense oligodeoxynucleotides restores β1-integrin-dependent adhesion to stroma and fibronectin (manuscript submitted). This links the presence of the $P210^{BCR/ABL}$ tyrosine kinase to the defect seen in β1-integrin function in hematopoietic progenitors.

Although our patient had been diagnosed with Ph+ CML in 1987, the bleeding diathesis did not develop until 1993. Furthermore, a similar bleeding diathesis is not a common occurrence in Ph+ CML. This suggests that presence of the $P210^{BCR/ABL}$ in the megakaryocytic lineage may not be sufficient to induce clinically relevant α2β1 integrin dysfunction. Because clinical characteristics compatible with accelerated phase disease (splenomegaly, leukocytosis)[41] coincided with the occurrence of the bleeding diathesis, it is possible that activation of additional oncogenes[42] in combination with the $p210^{BCR/ABL}$ are responsible for the profound defect seen in platelet aggregation in this patient.

The mechanisms underlying the restoration to "normal" phenotype after treatment of CML cells with IFN-α remain to be elucidated. IFN-α increases adhesion receptor expression levels in other biologic systems, including L-selectin,[43] LFA-3,[44] and LAM-1.[45] However, the expression of β1-integrins on clonogenic progenitors from CML marrow[16,17] or platelets from this patient did not increase after IFN-α treatment. Alternatively, IFN-α may act by down-regulating BCR/ABL gene expression.[11] Because anti-BCR/ABL anti-sense oligonucleotides restore β1-integrin-dependent adhesion fibronectin, it is possible that down-regulation of $p210^{BCR/ABL}$ in megakaryocytes by in vivo treatment with IFN-α results in functionally normal collagen receptors on platelet progeny. This would explain the 7- to 14-day delay between initiation of IFN-α therapy and the disappearance of the bleeding diathesis. Finally, IFN-α may act through BCR/ABL-independent mechanisms such as direct activation of cytoskeletal components[46] or activation of phosphatases that may dephosphorylate crucial integrin-associated signal and adaptor molecules.[47]

Although the mechanisms underlying the defect in collagen-mediated aggregation or β1-integrin-dependent adhesion and their restoration after IFN-α treatment remain elusive, this represents the first in vivo evidence that IFN-α may affect β1-integrin-mediated adhesion and signaling. This supports our hypothesis that IFN-α may induce hematologic and cytogenetic remissions in CML by restoring Ph+ progenitor adhesion to and subsequent regulation of their proliferation by the bone marrow microenvironment.

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