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Intracellular growth factors in polycythemia vera and other myeloproliferative disorders

(transforming factors/BALB/c 3T3 cells/platelet-derived growth factor)

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ABSTRACT **In polycythemia vera, idiopathic myelofibrosis, and essential thrombocytosis, hematopoietic cell proliferation is increased in the absence of a recognizable stimulus, suggesting the autonomous production of growth factors in these disorders. Sonicates of peripheral blood mononuclear cells (PBMNC) from patients with polycythemia vera, idiopathic myelofibrosis, and essential thrombocytosis contained soluble factors that stimulated the proliferation of quiescent-confluent 3T3 cells. PBMNC sonicates from normal individuals; from patients with secondary erythrocytosis, chronic myelogenous leukemia, B-cell chronic lymphocytic leukemia, and acute myelogenous leukemia; and from K-562 and HL-60 cells did not stimulate proliferation. Polycythemia vera PBMNC sonicates also induced anchorage-independent colony formation in soft agar by normal rat kidney fibroblasts. Both the mitogenic and transforming activities of the polycythemia vera PBMNC sonicates resided in the T-lymphocyte-depleted mononuclear fraction of the PBMNC and were not secreted. By gel filtration, reversed-phase HPLC and NaDodSO$_4$/PAGE, the mitogenic and transforming activities in the polycythemia vera PBMNC were localized to three proteins with molecular masses of 13-, 17-, and 65-kDa. The 13-kDa protein was only mitogenic, and the 17-kDa protein was only transforming, whereas the 65-kDa protein had both mitogenic and transforming activity. These proteins may be involved in the autonomous hematopoiesis that characterizes polycythemia vera, idiopathic myelofibrosis, and essential thrombocytosis.**

Polycythemia vera (PV), idiopathic myelofibrosis (IMF), and essential thrombocytosis (ET) are clonal disorders involving multipotent hematopoietic stem cells in which the mature progeny are phenotypically normal, but the production of one or more of the formed elements of the blood is increased (1–3). In each instance, the increased production of erythrocytes, granulocytes, or platelets occurs in the absence of a recognizable stimulus. PV is the most common and well studied of the three disorders and can serve as a paradigm for them. In PV, expansion of the erythrocyte mass occurs in the absence of hypoxia or any increase in erythropoietin production (4, 5), even though these signals are normally required before erythropoiesis is stimulated. Furthermore, the erythropoiesis of PV is not suppressed by hyperoxia, suggesting that progenitor cell proliferation is autonomous in this disorder (6). Additional support for this hypothesis comes from *in vitro* studies demonstrating that the erythroid progenitor cells of PV can proliferate in the absence of the hormone erythropoietin (7).

That transformed cells can produce proteins that function in an autostimulatory, autocrine fashion is well established. The first class of such proteins identified were the transforming growth factors, a group of small polypeptides found in medium conditioned by virally (8) or chemically transformed cells (9) and by human tumor cell lines (10). Transforming growth factors were subsequently identified in nontransformed cells as well (11). Transforming growth factors decrease the serum dependence of transformed cells and promote the anchorage-independent growth of normal fibroblasts. A second class of autocrine proteins are those that share structural and functional homology with the platelet-derived growth factor (PDGF). In addition to the extensive homology between the transforming protein of the Simian sarcoma virus and the B chain of PDGF (12–14), PDGF-like growth factors are also produced by human glioma (15), osteosarcoma (16), and fibrosarcoma (17) cell lines.

Since physiologic stimulation of cell proliferation cannot be implicated in the expansion of hematopoiesis occurring in PV, IMF, and ET, we sought to determine whether the production of growth factors by the abnormal clone was a feature of these disorders. Our data indicate that circulating mononuclear cells in PV, IMF, and ET contain intracellular mitogenic and transforming growth factors that are not found in other myeloproliferative disorders or certain leukemic cell lines and that these growth factors are distinct from PDGF and epidermal growth factor.

MATERIALS AND METHODS

Human Subjects. Blood was obtained with informed consent from healthy volunteers and from patients with PV, IMF, ET, secondary erythrocytosis, chronic myelogenous leukemia, and B-cell chronic lymphocytic leukemia. The diagnosis of PV was based on the criteria of the PV study group (18), and none of the patients had received either ^{32}P or cytotoxic drugs. The diagnoses of chronic myelogenous leukemia and B-cell chronic lymphocytic leukemia were based on the presence of the Philadelphia chromosome (9qt;22q−) in the former (19) and the surface marker phenotype in the latter (20). The diagnostic criteria for IMF and ET are less precise, but other possible causes for the hematologic abnormalities in these patients were excluded in each case.

Preparation of the Mononuclear Cell Lysates. Platelet-poor blood obtained by differential centrifugation (21) was centrifuged on a Ficoll/Hypaque gradient to obtain mononuclear cells free of erythrocytes and granulocytes. The mononuclear cells were centrifuged twice through 2% bovine serum albumin to reduce residual platelet contamination and in some experiments were also depleted of T lymphocytes by the E (erythrocyte) rosette technique (22). The final cell pellet was washed twice, resuspended in 3–5 ml of phosphate-buffered saline (140 mM NaCl/1 mM KH_2PO_4/4 mM Na_2HPO_4, pH 7.4) and sonicated to complete lysis as

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Abbreviations: PV, polycythemia vera; IMF, idiopathic myelofibrosis; ET, essential thrombocytosis; PDGF, platelet-derived growth factor; αMEM, alpha-modified Eagle's medium; PBMNC, peripheral blood mononuclear cell(s); FCS, fetal calf serum.

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determined by light microscopy. The sonicate was centrifuged at 11,000 × *g* for 20 min at 4°C, heated for 30 min at 56°C, and recentrifuged at 31,000 × *g* for 30 min at 4°C. The supernatant was filter-sterilized (0.22 μM) and stored at −70°C. In some experiments, the sonicate was centrifuged at 100,000 × *g* for 60 min, but the results were not different.

Mitogenic Assay. BALB/c 3T3 cells (clone A31, American Type Culture Collection) were maintained in alpha-modified Eagle's medium (αMEM, GIBCO) and 10% fetal calf serum (FCS; KC Biological, Lenexa, KS). The cells were subcultured every 3 to 4 days and discarded after eight passages. For assay, 1.5×10^5 cells in 1.5 ml of αMEM and 5% FCS were added to a 35-mm culture dish and allowed to grow for 4 days at which time they reached confluence and became quiescent. The medium was then replaced with 1.5 ml of αMEM containing 0.5% FCS and the sample to be tested. Control cultures received either 0.3 ml of whole blood serum prepared as previously described (21) or 0.5% FCS in a total volume of 1.5 ml αMEM. After 24 hr at 37°C in a humidified air/5% CO_2 atmosphere, the cells were harvested with 0.5% trypsin/0.2% EDTA, and the cell number was determined visually in a hemocytometer. Four dishes were counted for each experiment point.

In some experiments cell proliferation was monitored by the incorporation of [^{3}H]thymidine (specific activity 25 Ci/mMol; 1 Ci = 37 GBq; Research Products International, Mt. Prospect, IL), which was added at a concentration of 1 μCi per dish 16 hr after exposure of the confluent-quiescent 3T3 cells to the test samples. Eight hr later, the cells were harvested for determination of acid-precipitable radioactivity by liquid scintillation counting.

Transforming Assay. A suspension of 5×10^3 normal rat kidney fibroblasts (NRK-49F, American Type Culture Collection) in 1 ml of αMEM/10% FCS/0.3% agar (Difco) was layered over a prehardened layer of 1 ml of 0.5% agar containing the samples to be tested for transforming activity. After one week of incubation at 37°C in a humidified air/5% CO_2 atmosphere, colonies larger than 3000 μm^2 were counted visually (8).

Bio-Gel P-100 Gel Filtration. Cell lysates (3–5 mg of protein) were made 1 M in acetic acid and applied to a Bio-Gel P-100 column (1.2 × 90 cm) equilibrated with 1 M acetic acid at a flow rate of 9 ml per hr at 4°C. Fractions of 2 ml were collected, pooled in groups of five fractions, lyophilized, redissolved in phosphate-buffered saline, and assayed for protein content (23) and mitogenic and transforming activity.

Reversed-Phase HPLC. Lyophilized fractions from the Bio-Gel P-100 column were dissolved in a minimal volume of 0.1% trifluoroacetic acid containing 10% (vol/vol) acetonitrile and passed through a 0.45-μM Millex-GV filter (Millipore). Samples (0.1 to 0.25 ml) were applied to a Vydac TP-C_4 column (1.0 × 2.5 cm; Separations Group, Hesperia, CA) equilibrated with 0.1% trifluoroacetic acid/acetonitrile 9:1. After 5 min (flow rate, 2 ml/min), a gradient of 10–50% acetonitrile (1%/min) was started, and the column was washed with 65% acetonitrile before returning it to the initial conditions. The chromatography system consisted of a Varian model 5560 chromatograph and CDS-402 data station. Proteins were detected by absorbance at 205 nm; peaks were collected manually and dried using a Speed Vac concentrator (Savant).

NaDodSO$_4$/PAGE. Electrophoresis was done according to the method of Laemmli (24) using a 12.5% separating gel and 5% stacking gel, and proteins were visualized by silver staining (25). To study the behavior of individual proteins, the stained bands were excised from the gel and electroeluted using a Trans-Blot cell (Bio-Rad) (26).

RESULTS

Mitogenic Activity of Mononuclear Cell Sonicates. Sonicates of peripheral blood mononuclear cells (PBMNC) from 22 normal individuals failed to reverse the quiescent state of confluent BALB/c 3T3 cells as determined by an increase in their cell number. By contrast, PBMNC sonicates prepared from twelve patients with PV stimulated cell growth (Fig. 1). Similar results were obtained with PBMNC sonicates from three patients with IMF and one patient with ET, whereas PBMNC sonicates from five patients with chronic myelogenous leukemia (Fig. 1), sonicates from the PBMNC of B-cell chronic lymphocytic leukemia and acute myelogenous leukemia, as well as K-562 and HL-60 cell sonicates failed to induce a response (data not shown). PBMNC sonicates from patients with secondary erythrocytosis behaved like normal PBMNC sonicates (Fig. 2).

To further localize the source of the mitogenic activity, we fractionated normal and PV PBMNC into T-lymphocyte and non-T-lymphocyte mononuclear cell populations. For these experiments, the effect of the PV and normal PBMNC sonicates on 3T3 proliferation was measured by incorporation of [^{3}H]thymidine into DNA. Fig. 2 shows that following fractionation, mitogenic activity was found in the T-lymphocyte-depleted mononuclear cell population of PV PBMNC at low concentrations of protein, whereas at high concentrations of protein a low level of activity was found in normal T-lymphocyte-depleted mononuclear cells. Neither normal nor PV T-cells had mitogenic activity (data not shown).

Transforming Activity of Mononuclear Cell Sonicates. T-lymphocyte-depleted mononuclear cell sonicates from patients with PV also induced anchorage-independent colony formation in soft agar by fibroblasts from normal rat kidney in a dose-dependent fashion (Fig. 3). Similar activity could not be detected in T-lymphocyte-depleted mononuclear cell sonicates prepared from normal individuals (Fig. 3). Peripheral blood T-lymphocyte sonicates from either PV patients or normal individuals also failed to promote soft agar colony formation by normal rat kidney fibroblasts (data not shown).

Physicochemical Properties of Mitogenic and Transforming Factors. The mitogenic and transforming activities in PV PBMNC sonicates were acid-stable and protease-sensitive. The mitogenic activity was resistant to heating at 100°C for 5 min, was resistant to 40 mM dithiothreitol and 4 M guanidine

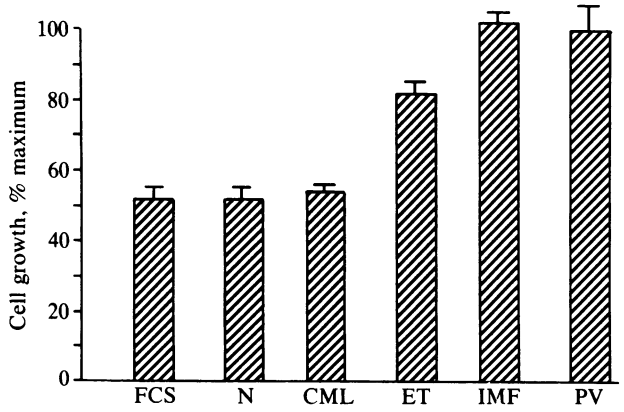

FIG. 1. Effect of 200 μg of PBMNC sonicates or 0.5% FCS on the growth of quiescent BALB/c 3T3 cells expressed as a percentage of the maximum response obtained with whole blood serum (0.3 ml per dish). Data represent results (mean ± SD) from 22 normal individuals, 12 patients with PV, 5 patients with chronic myelogenous leukemia (CML), 3 patients with IMF, and 1 patient with ET. N, Normal. The proliferative effects of PV, IMF, and ET sonicates were significantly different from normal sonicates ($P < 0.001$), whereas CML sonicates were not significantly different. Similar results (not shown) were obtained with 100 μg of protein.

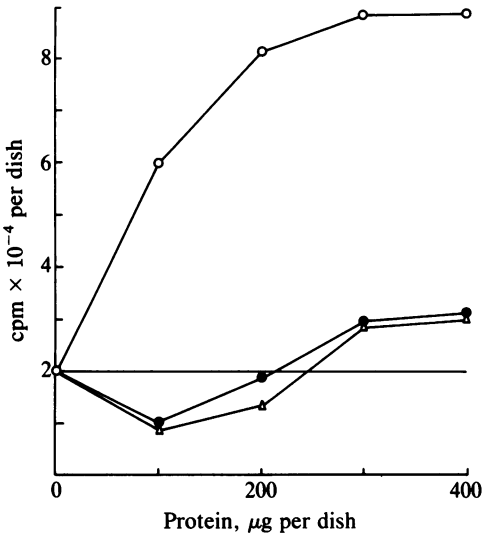

FIG. 2. Effect of T-lymphocyte-depleted PBMNC sonicates on DNA synthesis of quiescent BALB/c 3T3 cells. Data represent the results from six patients with PV (○), six normal individuals (●), and one patient with secondary erythrocytosis (△). The straight line represents the effect of 0.5% FCS.

hydrochloride, and was not inhibited by poly(L-lysine) (4 kDa, 100 μg/ml) or protamine (100 μg/ml). Transforming activity in the sonicates was abolished by exposure to heat or reducing agents.

Isolation and Characterization of Mitogenic and Transforming Proteins. Gel filtration of PV PBMNC sonicates on Bio-Gel P-100 yielded two major peaks (I and II), each containing mitogenic and transforming activity (Fig. 4). Both peaks were found to be heterogeneous when subjected to reversed-phase HPLC. The mitogenic activity from Bio-Gel P-100 peak I eluted in two peaks on HPLC: one at 36.2 min, the second at 37.0 min (Fig. 5). Transforming activity was found in the 34.9-min peak of the same Bio-Gel eluate.

Chromatography of the Bio-Gel peak II on reversed-phase HPLC yielded five different peaks (9.9, 11.2, 17.8, 18.0, and 21.6 min) that had both mitogenic and transforming activity (Fig. 6).

Each reversed-phase HPLC peak with transforming or mitogenic activity was further analyzed by $NaDodSO_4$/PAGE. Under nonreducing conditions, the 36.2-min peak exhibited two major protein bands with apparent molecular masses of 12 and 13 kDa (data not shown). Following electroelution, both protein bands retained mitogenic activity. The 37.0-min peak under nonreducing conditions was more heterogeneous—exhibiting multiple bands (data not shown). After electroelution, mitogenic activity was associated only with the 12-kDa protein. Under reducing conditions, all three mitogenic proteins from the Bio-Gel peak I migrated as a single band with an apparent molecular mass of 13 kDa (Fig. 5).

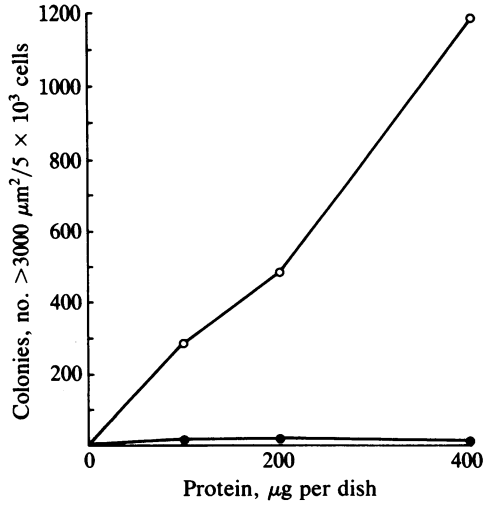

FIG. 3. Effect of T-lymphocyte-depleted PBMNC sonicates on anchorage-independent colony formation in soft agar by normal rat kidney fibroblasts. Data represent pooled results from nine patients with PV (○) and seven normal individuals (●).

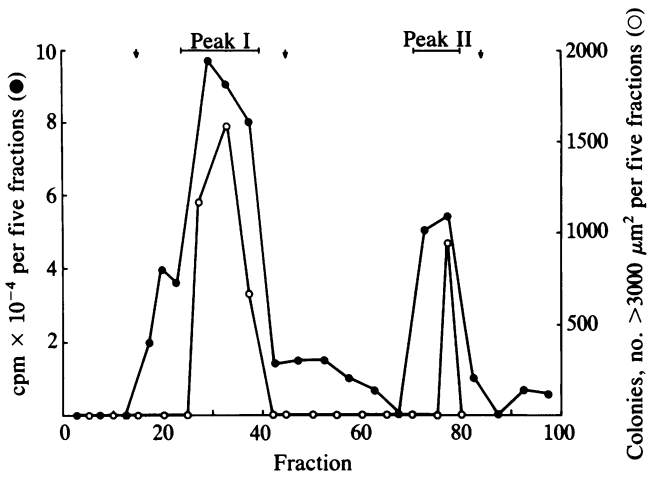

FIG. 4. Gel filtration on Bio-Gel P-100 of a T-lymphocyte-depleted PV PBMNC sonicate. The samples (3–5 mg of protein) were made 1 M in acetic acid and applied to a 1.1 × 85-cm column equilibrated in 1 M acetic acid. The flow rate was 9 ml/hr, and 2-ml fractions were collected and pooled in groups of five for assay of mitogenic (●) and transforming (○) activity. The arrows indicate the elution of bovine serum albumin, lysozyme, and insulin respectively, which were employed as molecular mass markers.

The 34.9-min peak had one major protein band with an apparent molecular mass of 17 kDa on $NaDodSO_4$/PAGE. Transforming activity was retained under nonreducing conditions upon electroelution (Fig. 5). However, under reducing conditions, the protein migrated in two bands (one with a molecular mass of 14 kDa, and the other with a molecular mass of 3 kDa) and had no transforming activity (data not shown).

All five reversed-phase HPLC components of Bio-Gel peak II migrated as a single band with an apparent molecular mass of 65 kDa under both reducing and nonreducing conditions (Fig. 6). Following electroelution, each of the five proteins exhibited equivalent mitogenic and transforming activities under nonreducing conditions, but transforming activity was lost on reduction, although no change in molecular mass was detected (data not shown).

Relationship of Mitogenic and Transforming Activities to PDGF and EGF. Column fractions were assayed for PDGF-like activity by Russell Ross in a PDGF radioreceptor assay (27) and by PDGF antibody neutralization. The Bio-Gel peak I contained PDGF-like activity that was neutralized by the PDGF antibody. The Bio-Gel peak II, however, had no PDGF-like activity. Neither material from Bio-Gel peaks I and II nor the active reversed-phase HPLC fractions competed with epidermal growth factor when tested in a radioreceptor assay (28) (data not shown).

Mitogenic and Transforming Activity in Medium Conditioned by PV PBMNC. To determine if PBMNC from patients with PV released mitogenic or transforming factors, the PBMNC were cultured *in vitro* in serum-free medium for 15 days. Medium was collected every 2 days and concentrated 250-fold before assay, but neither mitogenic nor transforming activity was detected.

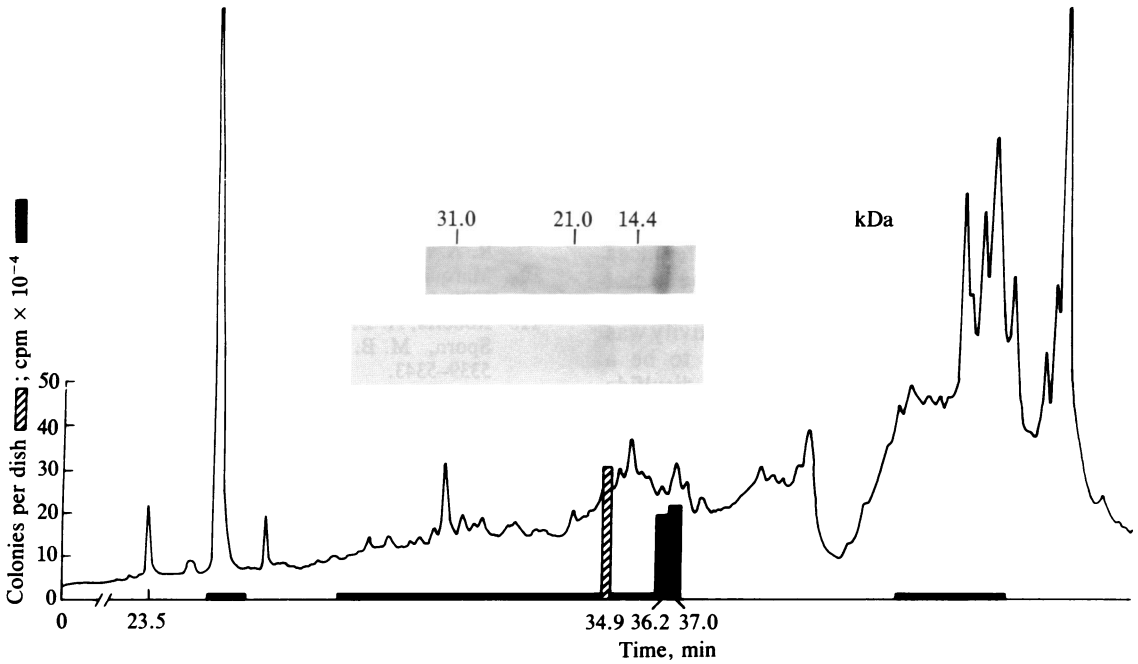

FIG. 5. Reversed-phase HPLC of Bio-Gel P-100 peak I. Only the peaks eluting at 36.2 min and 37.0 min, respectively, had mitogenic activity (■), and only the peak eluting at 34.9 min had transforming activity (▨). (*Inset*) NaDodSO$_4$/polyacrylamide gel under reducing conditions (*Upper*) of the 36.2-min and 37.0-min peaks and under nonreducing conditions (*Lower*) of the 34.9-min peak.

DISCUSSION

In PV, IMF, and ET, a clonal abnormality in a multipotent progenitor cell results in the uncontrolled production of phenotypically normal hematopoietic cells. An interesting feature of these disorders is the dominance of hematopoiesis by the abnormal clone with concomitant suppression of normal progenitor cell proliferation (29); as a consequence, the circulating formed elements of the peripheral blood are derived from the abnormal clone (1). In the present study, we took advantage of this clonal dominance by using PBMNC to search for growth-promoting factors that might be responsible for the autonomous hematopoiesis in PV, IMF, and ET.

Sonicates from PBMNC of patients with PV, IMF, and ET promoted the proliferation of quiescent-confluent 3T3 cells as determined by cell counting, whereas sonicates of PBMNC from normal individuals, patients with an expanded erythrocyte mass due to hypoxia, patients with chronic myelogenous leukemia, acute myelogenous leukemia or B-cell chronic lymphocytic leukemia, as well as cell sonicates from two leukemic cell lines, K-562 and HL-60, did not promote this proliferation. When incorporation of labeled thymidine was employed as a measure of cell proliferation, a low level of mitogenic activity was seen with high concentrations of normal- or secondary-erythrocytosis PBMNC sonicate protein. The nature of this activity and its relationship to that observed in PV PBMNC sonicates remains to be established. PV PBMNC sonicates, but not normal PBMNC sonicates, also contained transforming activity; whether transforming activity is present in IMF or ET PBMNC sonicates has not been determined.

Using PV PBMNC sonicates for further analysis, mitogen-

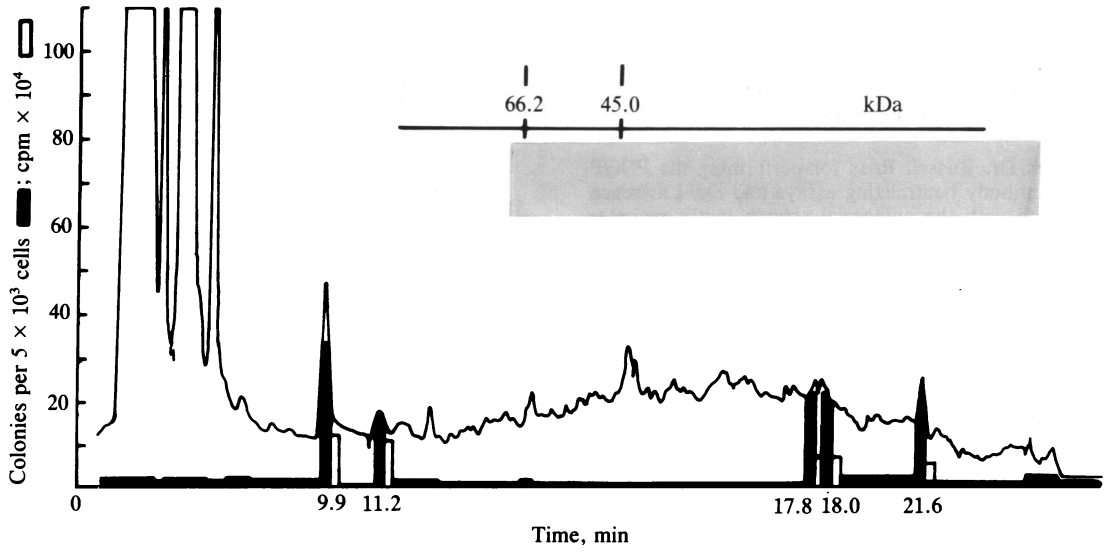

FIG. 6. Reversed-phase HPLC of Bio-Gel P-100 peak II. Of all the peaks assayed, five eluting at 9.9, 11.2, 17.8, 18.0, and 21.6 min, respectively, were found to have both mitogenic (□) and transforming (■) activity. As shown above the reversed-phase HPLC absorbance profile, with NaDodSO$_4$/polyacrylamide gel under nonreducing conditions, all five active fractions migrated as a single peak.

ic activity was found in proteins with molecular masses of 13 and 65 kDa, and the latter had transforming activity as well. The heterogeneity exhibited by the 13-kDa protein on reversed-phase HPLC and $NaDodSO_4$/PAGE may reflect differences in glycosylation or phosphorylation. Although the 13-kDa protein competed with PDGF in a radioreceptor assay and shared antigenic characteristics with it, it differed from PDGF—not only with respect to its molecular mass but also because it retained activity in the presence of dithiothreitol, poly(L-lysine) or protamine (30). These differences are not surprising because the c-*sis* protooncogene is not usually expressed in transformed hematopoietic cells (31).

In addition to the 65-kDa protein, transforming activity was also present in a 17-kDa protein that appeared to be a heterodimer of 14- and 3-kDa subunits linked by disulfide bonds. Like other transforming proteins, these were acid-stable but were inactivated by heat and reducing agents (8). The 65-kDa protein also had mitogenic activity, a property shared by other transforming proteins (32). Although the PV PBMNC transforming proteins did not compete with epidermal growth factor and are clearly not platelet-derived, they have not been further characterized.

Both mitogenic and transforming proteins were present in the T-lymphocyte-depleted fraction of PV PBMNC, but the cell or cell types responsible for their production have not been identified. Macrophages are known to secrete mitogenic factors that stimulate the proliferation of fibroblasts (33, 34). Although our sonicates were derived from a cell population containing monocytes, it is unlikely for several reasons that the proteins we identified represent macrophage-derived growth factors. First, production and secretion of macrophage-derived growth factors by circulating monocytes requires prior adherence or activation (33, 34), but no such activity was identified in medium conditioned by prolonged *in vitro* incubation of PV PBMNC. Second, macrophage-derived growth factors are inactivated by heating or reducing agents in contrast to the mitogenic factors in PV PBMNC sonicates (34).

The production of mitogenic and transforming proteins by neoplastic cells is well documented, but these factors have only been identified in cultured human or animal cell lines. The data reported here provide evidence for the production of such factors by freshly explanted, clonally derived human hematopoietic cells. The mitogenic and transforming proteins in these cells share functional characteristics with proteins found in transformed cell lines but differ with respect to their physical characteristics. Although a causal role for these proteins in the autonomous cell proliferation that characterizes PV, IMF, and ET has not been established, the presence of these proteins suggests a possible explanation for the hematologic abnormalities in these disorders.

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