Increased Proliferation of Bone Marrow-Derived Fibroblasts in Primitive Hypertrophic Osteoarthropathy With Severe Myelofibrosis

By Michéaël Fontenay-Roupie, Evelyne Dupuy, Eliane Berrou, Gérard Tobelem, and Marijke Bryckaert

Pachydermoperiostosis or primary hypertrophic osteoarthropathy (HOA), a rare congenital growth disorder of connective tissue, was first described in association with myelofibrosis in a patient with HOA. When cultured in vitro, patient bone marrow-derived fibroblasts displayed a high proliferative potential with a shortened doubling time (24 hours v 36 to 48 hours for normal fibroblasts). The role of platelet-derived growth factor (PDGF), previously implicated in the pathogenesis of secondary acquired myelofibrosis, was studied. HOA fibroblasts expressed an increased number of PDGF-BB binding sites (300,000 sites/cell v 200,000 sites/cell for normal fibroblasts) without any modification of affinity. The increased expression of PDGF-Rb appeared to result from an accelerated rate of PDGF-Rp resynthesis with normal kinetics of endocytosis. As a consequence, a several-fold increase of PDGF-Rb tyrosine kinase activity was observed. No autocrine mechanism of growth was suspected as neither spontaneous PDGF-Rb autophosphorylation nor mitogenic activity in HOA fibroblast-conditioned medium was detected. Patient serum and platelet lysates were less potent than controls in inducing [3H]thymidine incorporation into HOA fibroblasts. This was inconsistent with a paracrine mechanism of growth. In vitro, human serum or PDGF-BB were not more mitogenic for HOA than normal fibroblasts. High levels of cyclin D1, a putative oncogene, were detected in serum-deprived HOA fibroblasts. Cyclin D1 overexpression could be implicated in the accelerated growth of these cells. Our results suggest that the mechanism of fibroblastic proliferation observed in this case of myelofibrosis might differ from those reported in other acquired myeloproliferative syndromes and could be associated with an intrinsic abnormality of HOA fibroblast growth. © 1995 by The American Society of Hematology.

Primary OR idiopathic hypertrophic osteoarthropathy (HOA), also named pachydermoperiostosis, is a rare congenital disease characterized by a disorder of connective tissue growth.1,3 Manifestations in the relatives suggest autosomal dominant transmission.4 Although the secondary form of osteoarthropathy is associated with neoplastic or chronic disease, the primary form is of unknown origin and the pathogenesis uncharacterized. The clinical features of HOA affect the skin and the skeleton. The presence of digital clubbing and radiographic periostosis are the main diagnostic criteria for HOA. The sensitivity of these combined criteria is 86%, but the specificity has not been determined.5 Typically, the disease is recognized in young males, by the insidious development of thickening of the fingers and toes, clubbing of the terminal phalanges, enlargement of hands and feet, and cylindrical thickening of legs and forearms, involving the bones and the soft tissues. A progressive thickening of the skin of the face, forehead, and scalp is associated to a cutis verticis gyrata. Radiographic signs of bilateral and symmetrical periostosis such as a marked irregular periosteal ossification of the tibia and fibulas with thickening of the cortex are always observed.6 Modifications of vessels have also been reported in cases of HOA. The abnormalities associate diffuse endothelial hyperplasia with activated endothelial cells, thickening of the nasal membrane, and sclerosis with packing of collagen fibers.6 No specific biologic features could be identified, but some chromosomal abnormalities have been described.7

Extensive myelofibrosis, which consists of fibroblastic proliferation with increased collagen fiber deposition in the extracellular matrix, has been described in few cases of inherited HOA.8 Usually, myelofibrosis is a consequence of acquired myeloproliferative disorders such as primary myelofibrosis, essential thrombocytemia, polycythemia vera, and chronic myelogenous leukemia. It has been suggested that platelet-derived growth factor (PDGF) is involved in the progression of these fibrotic proliferative disorders.9,10 PDGF and PDGF-like molecules are secreted by megakaryocytes, platelets, smooth muscle cells, endothelial cells, and activated macrophages. In primary myelofibrosis and essential thrombocytemia, PDGF levels were high in the serum of patients whereas platelet PDGF contents were low.11-13 Therefore, fibroblastic proliferation stimulated by PDGF released from megakaryocytes, activated platelets, and/or inflammatory cells and matrix production through type III collagen secretion can be implicated in these diseases. Two receptor subtypes for PDGF have been identified. The α receptor subtype (PDGF-AA) binds either the A or B chain of PDGF, whereas the β type receptor (PDGF-Rb) binds only the B chain.14,15 Human bone marrow (BM)-derived fibroblasts mainly possess the β receptor subtype and consequently bind PDGF-BB.16 No abnormalities of PDGF receptors have yet been reported in these myelofibrotic disorders.

Studies of fibroblast cell cycle regulation, under various conditions including serum-deprivation, stimulation by growth factors, and senescence have shown the involvement of cyclins in the progression through the growth factor-dependent part of the G1 phase. One of the G1 cyclins, cyclin D1 is serum-inducible in normal fibroblasts and deregulated in senescent fibroblasts.17,18 Additionally, the PRAD1 gene which encodes the cyclin D1 is rearranged in benign parathyroid adenomas and overexpressed in malignant tumors such as lymphomas suggesting its oncogenic potential.19-21 Thus,
the expression of cyclin D1 appears to be a marker of the proliferative state of cells.

In this study, we report a case of primary HOA with severe myelofibrosis. HOA BM-derived fibroblasts had an accelerated rate of proliferation, a significant increase in the number of PDGF-BB receptors, and an enhanced PDGF-induced phosphotyrosine kinase activity. Neither autocrine stimulation of HOA fibroblast growth nor paracrine stimulation by platelets were detected. In view of the evidence for increased cyclin D1 protein expression in serum-deprived fibroblasts, these results suggest the existence of an intrinsic abnormality in the HOA fibroblast cell cycle.

MATERIALS AND METHODS

Case report. A 46-year-old man was referred for severe anemia. He presented a primary hypertrophic osteoarthropathy identified during childhood. He has had a synoviothecese of his left knee and a synovectmy of his right knee for bilateral hydrarthrosis. Physical examination showed cutaneous thickening of the hands, feet, and forehead with deep furrows extending to the scalp, clubbing of the fingers, and enlargement of the soft tissues and bones of the extremities. The patient’s father also presented digital clubbing. X-ray examination of the long bones showed major periostosis with cortical thickening and widening of the shafts. Neither hepatosplenomegaly nor tumoral syndrome were found by physical examination or computed tomographic scans. The case was unusual in that there was a BM failure. Investigations showed that the patient had a bicipitenna with severe normochromic agregenerative anemia (hemoglobin [Hb] 5.4 g/dL, red cell [RBC] count 2.06 10^{12}/L, low reticulocyte count 30.2 10^{10}/L) and moderate thrombocytopenia (platelets 100 10^{10}/L). The white blood cell (WBC) count (4.7 10^{10}/L) was normal. No abnormality of RBC morphology was observed. Several assays of BM aspirates were unsuccessful and no cytogenetic study was obtained. BM iliac biopsy specimens showed a severe myelofibrosis with an accumulation of collagen and reticulin fibers without bone handling, osteoclastic reaction, and osteomyelosclerosis. Biologic investigations for inflammation were negative.

Cell culture and conditioned media. After informed consent, BM was obtained by iliac biopsy from the patient and, during hip surgery, from femoral apophysis of volunteer donors. Five donors (age ranging from 30 to 55 years) were used during the time course of the study. Bone samples were treated with collagenase and infused several times with α minimum essential medium (αMEM) supplemented with 20% of fetal calf serum (FCS), 2 mmol/L L-glutamine, 50 μM penicillin, and 50 μg/mL streptomycin. Fibroblasts were then harvested, washed twice, and seeded in 25-cm² flasks at 37°C in 5% CO₂/95% air. All the experiments were performed with cells that had undergone fewer than five passages.

Conditioned media were prepared from subconfluent cell cultures of either patient or normal fibroblasts, maintained in 20% FCS, and then deprived of serum for 48 hours. Cells supernatants were collected, concentrated about eight times, and then tested for their mitogenic activity on normal fibroblasts.

Proliferation assays. For cell-counting experiments, fibroblasts were seeded at 25,000 cells per well in 12-well plates in αMEM + 20% FCS. Cells were incubated for 36 hours and then deprived of serum for 48 hours in Ham’s F12 medium (JBio, Les Ulis, France) and then deprived of serum for 48 hours in Ham’s F12 medium (JBio, Les Ulis, France) + 0.5% of bovine serum albumin (BSA; Sigma, St Louis, MO). The growth rates of patient and control fibroblasts from three different donors were determined after serum reintroduction (10%) by cell counting from day 0 to day 5 and the doubling time was calculated.

The mitogenic responses of HOA and control fibroblasts to increasing amounts of conditioned media from patient and normal fibroblast cultures (0% to 100%), PDGF-BB (0 to 15 ng/mL), human serum (0% to 15%), platelet lysates obtained by freezing and thawing of washed platelets, and platelet-poor plasma (PPP) (0% to 15%) were assayed by measuring incorporation of [methyl-3H]thymidine ([3H]Tdr). Cells were seeded in 12-well plates and grown until subconfluence. After serum-deprivation for 48 hours, they were incubated for 48 hours in the presence of mitogen and 1 μCi/mL [3H]Tdr (Amersham, Les Ulis, France). Trichloroacetic acid-precipitable radioactivity was counted using a Betaplate Kontron counter (Kontron, Montigny-le-Bretonneux, France). Results were expressed as percentage of [3H]Tdr incorporation relative to a control without growth factor. Control data were represented by the mean ± SEM of means of three independent experiments using cells from at least two different donors, except for PPP mitogenic activity where the experiment shown was representative of two experiments using cells from two different donors. Control serum and PPP used were a pool of eight age-matched donors. For platelet lysates, results were expressed as equivalents of nanograms (ng, ng/mL) of PDGF-BB per 10⁷ platelets, in comparison with a standard curve of PDGF-BB, and the control value represented mean ± SEM of means of four determinations from four age-matched donors.

Quantitative determination of β-thromboglobulin (β-TG) in platelet lysates. β-TG levels in platelet lysates, prepared as previously described, were determined using an enzyme-linked immunosorbent assay (Diagnostics Stago, Asnières-sur-Seine, France).

PDGF-BB receptor assay and downmodulation experiments. PDGF-BB was obtained from Amersham Laboratories (specific activity 75,000 cpd/mg). The binding of [125I]PDGF-BB to BM-derived fibroblasts was assayed in 12-well plates as previously described. Briefly, confluent cells were incubated for 3 hours at 4°C in binding buffer (20 mmol/L phosphate-buffered saline [PBS] containing 0.9 mmol/L CaCl₂, 0.5 mmol/L MgCl₂, and 0.1% BSA, pH 7.4) with 1 ng/mL of [125I]PDGF-BB and increasing concentrations of unlabeled PDGF-BB (1 to 400 ng/mL). After five washes, cell-associated radioactivity was determined using a gamma counter. Nonspecific binding was evaluated by addition of a 400-fold excess of unlabeled PDGF-BB.

PDGF-BB receptor assay and downmodulation experiments. To evaluate the rate of PDGF-BB receptor resynthesis after downmodulation, patient and control fibroblasts were preincubated with 1, 10, or 50 ng/mL of PDGF-BB for 0 to 48 hours at 37°C. Cells were washed with fresh medium and [125I]PDGF-BB binding (1 ng/mL) was performed for 5 minutes at 37°C. After 5 washes, cell-associated radioactivity was determined using a gamma counter. Non-specific binding was evaluated by addition of a 400-fold excess of unlabeled PDGF-BB.

Immunoprecipitation and Western blotting experiments. Fibroblasts were seeded in 10-cm² dishes and deprived of serum for 48 hours. A monoclonal antiphosphotyrosine antibody, 4G10 (UBI, Lake Placid, NY), was used for immunoprecipitation experiments from either unstimulated or PDGF-BB–stimulated (25, 50, 75, and 100 ng/mL, 5 minutes at 37°C) fibroblasts. After washing, cells were lysed by incubation in 1 mL of lysis buffer (20 mmol/L HEPES pH 7.40, 145 mmol/L NaCl, 5.4 mmol/L KCl, 1.8 mmol/L CaCl₂, 2 mmol/L MnCl₂, 20 mmol/L ZnCl₂, 50 mmol/L NaF, 10 mmol/L Na pyrophosphate, 1.5 mmol/L NaVO₃, 10 mmol/L EDTA, 2 mmol/L EGTA, and a mixture of protease inhibitors [Sigma]: 1 mmol/L PMSF, 10 μmol/L aprotinin, 5 μmol/L leupeptin, 20 μg/mL benzamidine, 10 μg/mL soybean trypsin inhibitor) for 30 minutes in ice. After centrifugation at 11,000 ×g for 15 minutes at 4°C, cell lysates were immunoprecipitated using 4 μL of 4G10 antiphosphotyrosine antibody. Immunoprecipitated tyrosine phosphoproteins were collected on protein A-Sepharose beads (Sigma), separated on a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto a nitrocellulose membrane, and revealed with...
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Fig 1. Growth curve. BM-derived fibroblasts were plated in αMEM + 20% FCS at a density of 25 x 10^3 cells/well. After a 48-hour serum starvation, control (C) and patient (P) cells were restimulated (day 0) with 10% FCS and cell counting was performed every day. The results were means ± SEM of four independent experiments using control fibroblasts from three different donors (**P < .005; *P < .05).

the same antibody and the iodinated protein A system as previously described.18

For Western blotting experiments, subconfluent fibroblasts plated in 6-well plates were deprived of serum for 48 hours. After two washes in PBS pH 7.4, fibroblasts were lysed in 50 mmol/L TRIS pH 7.40, 150 mmol/L NaCl, 0.5% NP-40 plus protease inhibitors. Aliquots of 50 μg of protein were separated on 11% SDS-PAGE and electrotransferred for Western blotting with a rabbit-antihuman cyclin D1 antibody (1 μg/mL; UBI). Specific labeling was shown using a goat-antirabbit polyclonal antibody coupled to horseradish peroxidase (dilution 1:10,000) (Amersham) and the ECL* system (Amersham).

Statistical analysis. Data were expressed as the mean of at least three determinations. Comparison of the means were tested by the Mann-Whitney t-test for paired values.

RESULTS

High proliferative potential of HOA fibroblasts. BM fibroblasts (BMF) were seeded in αMEM + 20% FCS to study their proliferation in vitro. In primary cultures, HOA fibroblasts reached confluence by day 2, whereas control cells reached confluence by day 5, but their saturation densities were similar (20 x 10^6 cells/cm² v 18.5 x 10^6 cells/cm² for patient and control BMFs, respectively). Thus, patient fibroblasts exhibited density-dependent inhibition of growth.

The calculated doubling time was 24 hours for HOA fibroblasts and 36 to 48 hours for normal fibroblasts, depending on the sample. HOA fibroblasts did not display morphologic abnormalities such as transformed foci (data not shown).

Increased number of [125I]PDGF-BB binding sites on HOA fibroblasts. To evaluate the role of PDGF in HOA myelofibrosis, [125I]PDGF-BB binding at 4°C was determined on patient BMF in comparison with control BMF. Specific [125I]PDGF-BB binding to HOA fibroblasts saturated at 15 ng/10^6 cells of radioligand. In contrast, normal fibroblast binding sites were saturated with 10 ng/10^6 cells of ligand. In both cases, nonspecific binding was less than 25% of total binding (Fig 2A).

Scatchard analysis indicated a significantly larger number of binding sites in patient (n = 300,000 sites/cell) than control BMF (n = 200,000 sites/cell) (P < .025). There was no difference in the affinity constant (kdcontrol = 0.5 x 10^-7 mol/L) (Fig 2B). Thus, HOA fibroblasts expressed 1.5-fold times more PDGF-Rβ receptors. Whether PDGF-BB binding sites were functionally normal was further examined.

Accelerated PDGF-BB receptor recycling in HOA fibroblasts. Dereguation of PDGF-R endocytosis and resynthesis might play a role in the accumulation of receptors at the cell surface. Therefore, the kinetics of PDGF receptor downmodulation and resynthesis was studied. [125I]PDGF-BB binding experiments were performed for 5 minutes at 37°C after preincubating BMF for 0 to 48 hours with 1, 10, or 50 ng/mL of unlabeled PDGF-BB. [125I]PDGF-BB binding was 0.5 ng/10^6 cells for HOA fibroblasts and 0.34 ng/10^6 cells for control fibroblasts, respectively, at time 0 (Fig 3, A and B). This represents a 1.47-fold higher [125I]PDGF-BB binding for HOA fibroblasts than control fibroblasts, consistent with data from saturation experiments. After 3 hours of incubation in the presence of unlabeled PDGF-BB, [125I]PDGF-BB binding was reduced by 30% and 23% with 1 ng/mL of PDGF-BB and by 48% and 41% with 10 ng/mL in patient and control BMFs, respectively. In the presence of 50 ng/mL PDGF-BB, [125I]PDGF-BB binding decreased to 0.2 ng/10^6 cells and 0.15 ng/10^6 cells for patient and control fibroblasts, respectively, without any significant difference between the two types of cells. No difference in receptor downmodulation was detected.

In the presence of 10 ng/mL PDGF-BB, the same kinetics of PDGF-Rβ resynthesis was observed for both types of BMF. Interestingly, in the presence of 50 ng/mL PDGF-BB, [125I]PDGF-BB binding was significantly higher in HOA fibroblasts after 48 hours of incubation (0.3 ng/10^6 cells) than after 3 hours (0.2 ng/10^6 cells) (P < .05), whereas no modification of [125I]PDGF-BB binding was observed in normal fibroblasts. The PDGF-Rβ synthesis was stimulated in the presence of high concentrations of PDGF-BB and a 1.5-fold increase of [125I]PDGF-BB binding was observed at
48 hours for only HOA, but not control fibroblasts. This result suggests an accelerated rate of PDGF-R/β recycling in HOA fibroblasts.

**Increased tyrosine phosphorylation induced by PDGF-BB.** Then we investigated the PDGF-R transduction pathway to examine the consequences of an increased number of PDGF-BB receptor using antiphosphotyrosine immunoprecipitation experiments. The pattern of phosphotyrosine proteins (pp180, pp145, pp85, pp72-74, pp60) observed in response to PDGF-BB was similar in HOA and control fibroblasts, but the intensity of the signal was higher in patient

**Fig 2.** [¹²⁵I]PDGF-BB binding. Confluent control (C) and HOA (O) fibroblasts were incubated for 3 hours at 4°C with [¹²⁵I]PDGF-BB (75,000 cpm/ng) in the presence of increasing amounts of unlabeled ligand (0 to 400 ng/mL). Nonspecific binding determined in the presence of a 400-fold excess of unlabeled PDGF-BB was subtracted. (A) Saturation data are expressed as PDGF-BB bound in nanograms per 10⁶ cells and are representative of three independent experiments using control fibroblasts from three different donors. (B) Scatchard analysis.

**Fig 3.** PDGF receptor downmodulation and resynthesis. Confluent (A) control and (B) HOA fibroblasts were preincubated with 1 ng/mL (circles), 10 ng/mL (squares), or 50 ng/mL (triangles) of PDGF-BB for 0 to 48 hours at 37°C. After washing, binding of [¹²⁵I]PDGF-BB (1 ng/mL) was performed for 5 minutes at 37°C. Results are expressed as cell-associated [¹²⁵I]PDGF-BB in nanograms per 10⁶ cells and are the mean ± SEM of three independent experiments in duplicates with control fibroblasts from three different donors.

BMFs. A pp180 phosphoprotein band previously shown to be the autophosphorylated form of the PDGF receptor increased in intensity in response to increasing concentrations of PDGF-BB. The pp180 was clearly detectable in HOA fibroblasts in response to a low dose of PDGF-BB (25 ng/well), whereas only a weak signal was seen with control BMFs (Fig 4A). Quantification by densitometric scanning showed that PDGF-R phosphorylation in response to PDGF-BB was significantly higher in HOA fibroblasts than control fibroblasts (1.6- to 3.4-fold increase depending on the PDGF-BB concentration) (Fig 4B). Interestingly, no phosphorylation was observed in either patient or control unstimulated fibroblasts, suggesting that there was no autocrine stimulation of PDGF-R/β tyrosine kinase activity. The increased tyrosine phosphorylation of PDGF-R without spontaneous autophosphorylation and the absence of abnormal phospho-
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Fig 4. Tyrosine phosphoprotein immunoprecipitation. After a 48-hour period of serum deprivation, control (lanes 1, 2, 3, 4, and 5) and HOA (lanes 6, 7, 8, 9, and 10) fibroblasts were either unstimulated (lanes 1 and 6) or treated for 5 minutes at 37°C by PDGF-BB (25 ng/well: lanes 2 and 7; 50 ng/well: lanes 3 and 8; 75 ng/well: lanes 4 and 9 or 100 ng/well: lanes 5 and 10). Fibroblast lysates were immunoprecipitated with 4G10 monoclonal antiphosphotyrosine antibody and analyzed by a 7.5% SDS-PAGE. After electrotransfer, tyrosine phosphoproteins were detected by Western blotting using the same antibody. Results are representative of two experiments using control fibroblasts from two different donors. (A) Autoradiography. (B) Densitometric scanning.

protein in response to PDGF-BB was consistent with the recruitment of an increased number of normal PDGF-BB binding sites at the cell surface of HOA fibroblasts.

Similar mitogenic activity of HOA and control fibroblast conditioned media. Autocrine secretion of growth factors was investigated by determining the mitogenic activity of conditioned medium from patient and control fibroblasts. Concentrated cell culture supernatant (0% to 100%) either from patient or control fibroblasts did not induce any [3H]-TdR incorporation into normal fibroblasts compared with the unstimulated control (Fig S).

In view of the absence of spontaneous PDGF-R autophosphorylation, this suggested that autocrine secretion of growth factors could not account for the proliferative potential of HOA fibroblasts.

Identical PDGF-BB mitogenicity in HOA and control fibroblasts. To evaluate the effects of an increased PDGF-BB receptor number and tyrosine kinase activity on HOA fibroblasts, the mitogenicity of exogenously added PDGF-BB (0 to 15 ng/mL) was assayed in vitro by [3H]-TdR incorporation (Table 1). PDGF-BB (15 ng/mL) induced similar increase in the rate of DNA synthesis in HOA (1.343% ± 221%) and in control fibroblasts (1.277% ± 274%) compared with unstimulated HOA and control fibroblasts, respectively. In these conditions, HOA fibroblasts retained their high proliferative potential as confirmed by the absolute values of [3H]-TdR incorporation.

Decrease or absence of mitogenic activity in patient serum, platelet lysates, and PPP. Paracrine secretion of growth factors was investigated by testing patient serum, platelet lysate, and PPP for their mitogenic activity on HOA and control fibroblasts.

In the presence of patient and control sera, a dose-dependent induction of DNA synthesis was observed. But, in normal fibroblasts, [3H]-TdR incorporation, expressed as percentage of unstimulated control, was significantly lower in the presence of patient serum than in the presence of a pool of control sera (1.737% ± 316% v 2.846% ± 219% with 10% patient and control serum, respectively, P < .05) (Fig 6A). The same difference was found for HOA fibroblasts where patient and control sera stimulated DNA synthesis by 1.464% ± 300% and 2.227% ± 315%, respectively (P < .05) (Fig 6B). However, the percentage of [3H]-TdR incorporation relative to controls without serum was similar for HOA and normal fibroblasts, especially in the presence of the different concentrations of patient serum. For example, in the presence of 15% patient serum, DNA synthesis was stimulated by 1.783% ± 336% and by 2.165% ± 236% in HOA and normal fibroblasts, respectively (P > .05). Thus, the proliferative response of HOA fibroblasts to serum addition did not overcome the proliferative response of normal fibroblasts. In contrast, absolute values showed the higher proliferative potential of HOA fibroblasts that incorporated fourfold more [3H]-TdR than control fibroblasts in the absence of serum (2.102 cpm ± 702 v 483 cpm ± 55).

Because the lower mitogenicity of patient serum could be caused by thrombopenia (75 × 10⁶ platelets/L at the moment of the BM biopsy), we determined the mitogenic activity of patient platelet lysates in comparison with four volunteer
donors. Platelet lysates obtained after successive freezings and thawings were assayed for their mitogenic effect on normal fibroblasts. The HOA platelet lysate mitogenic activity (16 eq.ng/10^6 platelets ± 4) was significantly lower than the control mean (57 eq.ng/10^6 platelets ± 5) (Table 2). The level of platelet β-thromboglobulin, a specific α-granule protein, was also determined. Platelet β-TG was strictly normal suggesting the absence of platelet activation in this disease (Table 2).

Finally, patient and control PPPs were tested for their mitogenicity. In the presence of control PPP, a threefold to fourfold increase of [3H]TdR incorporation compared with unstimulated fibroblasts was observed in normal and HOA fibroblasts. No DNA synthesis was detected in the presence of unstimulated fibroblasts was observed in normal and HOA fibroblasts. No DNA synthesis was detected in the presence of control PPP (Fig 7, A and B). In contrast, plasma β-TG was found similar in patient PPP (36 μg/mL) and in a series of controls (32 ± 6 μg/mL).

Thus, patient serum and platelet lysates displayed decreased mitogenicity and patient PPP did not contain any detectable mitogenic activity. These results are in favor of the absence of paracrine stimulation of growth implicating platelets as a source of PDGF and the blood flow as the recipient of secreted PDGF.

**Upregulation of cyclin D1 in HOA fibroblasts.** Cyclin D1 is an oscillating protein. Its biosynthesis is induced as fibroblasts enter G1 phase and then decreases during S and G2 phases, thus cyclin D1 is a marker for progression through G1. Our results shown above suggested the existence of a primary abnormality of HOA fibroblasts leading to a shortened doubling time. To investigate the possible deregulation of the cell cycle in HOA fibroblasts, we looked for the expression at the protein level of the mitogen-inducible cyclin D1. Cyclin D1 expression was appreciated in serum-deprived fibroblasts by immunoblotting (Fig 8A). We found that cyclin D1 expression was two-fold higher in HOA fibroblasts than in normal fibroblasts (Fig 8B). Thus, the accelerated rate of proliferation of HOA cells correlated with the overproduction of cyclin D1 in the absence of mitogen.

### DISCUSSION

The reported case presented the characteristic features of primary HOA with clinical manifestations including digital clubbing and radiographic periostosis. Primary HOA was confirmed by the insidious development of the lesions in the patient, digital clubbing in the patient’s father, and the absence of either tumors or pulmonary diseases. This severe form of primary HOA was characterized by an intensive myelofibrosis as has been previously described in some cases. The absence of hepatosplenomegaly, abnormal RBC morphology, and erythromyelosclerosis were inconsistent with primary myelofibrosis. Clinical and hematologic data were not consistent with myeloproliferative syndromes as neither splenomegaly nor immature granulocytes could be detected in the circulating blood. These hematologic abnormalities have been described in congenital osteopetrosis where the medullary cavities of bones are slowly obliterated by the cortical bone. However, the other clinical features of the reported case are not associated with congenital osteopetrosis.

We studied the growth in vitro of BM-derived fibroblasts from this case of myelofibrosis of unusual origin. These

<table>
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<th>Table 1. PDGF-BB Mitogenic Activity</th>
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*Increasing concentrations (0-15 ng/mL) of PDGF-BB with 1 μCi/mL [3H]TdR were added to serum-deprived fibroblasts for 48 hours at 37°C. Results are expressed in percentage of [3H]TdR incorporation relative to a control without growth factor and are the mean ± SEM of four independent experiments using control fibroblasts from three different donors. Absolute values in cpm/well from one representative experiment are indicated in parentheses.*
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HOA fibroblasts presented an accelerated rate of proliferation with a shortened doubling time (24 hours vs 36 to 48 hours for normal fibroblasts). This result was unexpected as the proliferation of BMFs from myeloproliferative syndromes, including chronic myelogenous leukemia, polycythemia vera, or essential thrombocythemia was thought to be similar to that of normal fibroblasts. The colony-forming unit-fibroblasts or CFU-Fs in primary myelofibrosis have a normal growth rate, a similar doubling time, and the same phenotype as normal CFU-Fs, and myelofibrosis-derived marrow fibroblasts have the same properties in vitro than normal marrow-derived fibroblasts such as anchorage and serum dependence and contact inhibition of growth.26,27 Fibroblastic proliferation is also considered as a secondary event because fibroblasts are not deriving from a totipotent hematopoietic stem cell,28 even if they are thought to support hematopoiesis.27 In contrast, only one study reported an increase of [3H]Tdr incorporation in chronic myelogenous leukemia fibroblasts in response to human serum, but there was no significant difference in the cell count after a 4-day stimulation.29 In vivo, myelofibrosis-derived fibroblasts synthesize increased levels of laminin and collagen type I and III, which are normally produced by these cells.30,31 As expected, HOA fibroblast cultures were able to generate high quantities of extracellular matrix compared with control cultures.

Packaging abnormalities of PDGF in megakaryocytes and platelets and/or increased secretion of PDGF from activated platelets have been suspected in the development of myelofibrosis. Thus, the role of PDGF in this case of myelofibrosis was studied. We found that HOA BM-derived fibroblasts exhibited a significant increase of PDGF-BB receptor number without any modification of binding affinity. Moreover, PDGF-R resynthesis was faster after a 48-hour incubation in the presence of high concentrations of exogenous PDGF-BB. An increase of PDGF-R autophosphorylation and phosphotyrosine proteins without additional bands in response to PDGF-BB was observed that correlated with the increased number of sites. These results suggest that: (1) increased PDGF-Rβ recovery might account for the increased number of sites; (2) the unchanged affinity of PDGF-BB binding sites and the normal pattern of phosphoproteins in response to PDGF-BB were in favor of the expression of a functionally normal PDGF-Rβ in HOA fibroblasts. This type of deregulation phenomenon of PDGF receptor expression has never been described in myelofibrotic disorders. In contrast, the fibroblastic proliferation of benign origin occurring in other tissues during inflammatory process can display an increase in PDGF-Rβ expression, as described in synovitis.32 A marked expression of PDGF-Rβ was detected on smooth muscle cells in atherosclerotic plaques and rejected kidneys.33 The induction of PDGF-Rβ would render inflammatory cells more responsive to growth stimulation by PDGF in vivo. Thus, autocrine or paracrine secretion of PDGF may

![Graph](image)

**Fig 6.** Serum mitogenic activity. Exponentially growing (A) control and (B) HOA fibroblasts were serum-deprived and stimulated with increasing concentrations (0% to 15%) of either control or patient serum in addition to 1 μCi/ml [3H]Tdr for 48 hours at 37°C. Results are expressed in percentage of [3H]Tdr incorporation relative to a control without growth factor and are means ± SEM of three independent experiments using control fibroblasts from three different donors (**P < .025; *P < .05).**

Table 2. Platelet Lysate Mitogenic Activity and β-Thromboglobulin Levels

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<thead>
<tr>
<th>Platelet Lysates</th>
<th>Mitogenic Activity (μg of PDGF-BB/10⁸ platelets)</th>
<th>β-TG Levels (μg/10⁸ platelets)</th>
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<td>Control</td>
<td>57.0 ± 5.5</td>
<td>2.8 ± 0.7</td>
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<tr>
<td>Patient</td>
<td>16.0 ± 4.0</td>
<td>4.4 ± 1.0</td>
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Platelet lysates were assayed for their mitogenic activity and β-thromboglobulin (β-TG) contents. DNA synthesis was appreciated by [3H]Tdr incorporation in control fibroblasts from two different donors. Results are expressed as equivalents of nanograms of PDGF-BB in ng/10⁸ platelets and represented the means ± SEM of four determinations for the patient and the four donors. β-TG levels were determined using an enzyme-linked immunosorbent assay (Diagnostica Stago) and results represented the means ± SEM of four independent experiments in duplicates.
account for the formation of proliferative lesions. Recent evidences for a transient upregulation of PDGF-R α and β in human foreskin fibroblasts in response to PDGF-BB[^24] led us to investigate the possible involvement of PDGF-BB either in an autocrine or paracrine mechanism of growth stimulation in HOA fibroblasts.

First, neither a spontaneous autophosphorylation of PDGF-R in the absence of exogenously added growth factor nor mitogenic activity in conditioned medium was detected. Thus, we could not find any argument for a correlation between the increased expression of PDGF-Rβ and an autocrine secretion of PDGF-BB. Presumably, therefore, the pathogenesis of this disease that affects both the BM and the skin differs from that of benign tumors of the skin where the PDGF-B chain is coexpressed with a high expression of PDGF-Rβ[^24].

Second, the possibility of a paracrine mechanism of growth was investigated. We found that the mitogenic activity of patient serum and platelet lysates were lower than the control values and that patient PPP was not mitogenic. But neither platelet activation nor α-granule storage disease could be suspected because platelet and plasma levels of β-thromboglobulin were normal. As PDGF represents the main mitogen in platelets, we suggest that a specific defect of PDGF biosynthesis and storage in platelets might exist. Alternatively, PDGF could be normally produced but abnormally trapped by target cells such as fibroblasts, which were shown to express a higher number of PDGF-BB receptors than normal fibroblasts. Because PDGF has a short half-life in serum[^35], a rapid clearance by HOA fibroblasts could lead to the absence of biologically detectable concentrations of PDGF-BB. In contrast, in other fibroblastic proliferations,
the serum mitogenicity is often found increased. For example, in the Gray platelet syndrome and hairy cell leukemia, increased amounts of PDGF are released from megakaryocytes and platelets that lack storage α-granules resulting in a decrease of platelet mitogenic activity.\textsuperscript{36,37} In myeloproliferative syndromes, it has been shown that platelets contain decreased amounts of PDGF or β-thromboglobulin in association with an increase of PDGF plasma levels.\textsuperscript{11,12,38,39} In the idiopathic pulmonary fibrosis, an increased PDGF release from macrophages has been shown to account for the fibroblast proliferation.\textsuperscript{40} Thus, the mechanism of HOA fibroblast proliferation that occurs without an increase of serum mitogenicity could be different from those of acquired fibrosis.

However, we cannot exclude the role of an intramedullary secretion of PDGF-BB, especially from endothelial cells known to accumulate PDGF-BB in their conditioned medium.\textsuperscript{41} Continuous PDGF-BB release in the BM microenvironment either from platelets or endothelial cells might account for the insidious development of myelofibrosis. Electron microscopic studies of dermal endothelium in HOA have identified several features of endothelial cell activation with an increased intracellular trafficking.\textsuperscript{4} However, such abnormalities have never been described for intramedullary endothelium. Thus, the role of the endothelium in the development of myelofibrosis could not be ascertained.

In our in vitro conditions, HOA fibroblasts appeared to be PDGF-BB receptor overexpressing cells, but neither PDGF-BB nor human serum seemed to account for the increased DNA synthesis in vitro. The capability of HOA fibroblasts to proliferate at double the rate of the control suggests a primary abnormality of cell cycle regulation. This hypothesis was reinforced by studying the levels of a potential oncogene: we found that the amount of cyclin D1 in HOA cells was high in the absence of mitogen. This correlates with the fourfold increase of [\textsuperscript{3}H]Tdr incorporation in HOA cells when serum-deprived. HOA fibroblasts of passages 2 to 4 were tested and gave the same result. The role of cyclin D1 upregulation in the shortened duration of the cell cycle in HOA fibroblasts was suspected; in fact, constitutively overexpressed cyclin D1 in Rat-1 fibroblasts was previously shown to decrease the duration of G1 phase, but a compensatory lengthening of S and G2-M phases leading to a normal doubling time was observed. Saturation density was enhanced in these cells, whereas we found it normal in HOA fibroblasts.\textsuperscript{42,43} Thus, cyclin D1 overexpression might be the consequence rather than the cause of the abnormal cell growth which features are an accelerated rate of proliferation without loss of contact inhibition. Two explanations could account for these results: (1) the accumulation of cells in S phase correlating with an increased number of cyclin D1 expressing cells; (2) the absence of a negative feedback mechanism that normally regulates cyclin D1 expression.

The proteins implicated in the regulation of cyclin D1 expression are not yet known. However, inhibitors of cyclin D/cyclin-dependent kinase complexes have recently been identified.\textsuperscript{44} Deletions of the gene of one of these proteins have been found in many primary tumors.\textsuperscript{45} The absence or inactivation of a cell cycle inhibitor could contribute to an accelerated rate of proliferation. Therefore, the constitutively high levels of cyclin D1 associated with this fibroproliferative disorder might reflect the existence of a primitive abnormality of HOA fibroblastic growth.

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Increased proliferation of bone marrow-derived fibroblasts in primitive hypertrophic osteoarthropathy with severe myelofibrosis

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