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Macrophages in normal human bone marrow and in chronic myeloproliferative disorders: an immunohistochemical and morphometric study by a new monoclonal antibody (PG-M_1) on trephine biopsies

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Summary. An immunohistochemical and morphometric study was performed on routinely processed trephine biopsies of the bone marrow in 30 normal individuals and in 90 patients with various subtypes of chronic myeloproliferative disorder. Using a new monoclonal antibody (PG-M_1) directed against a formalin-resistant epitope on macrophages and by employment of the Prussian blue reaction, quantitation of this cell population was feasible. Morphometric analysis revealed that the number of iron-laden macrophages represented only a fraction of the total number of histiocytic reticular cells. As could be expected, in polycythaemia rubra vera, no haemosiderin deposits were detectable, but the content of macrophages slightly exceeded that of the normal bone marrow. In chronic myeloid leukaemia 9 of 30 patients showed a significant increase in PG-M_1-positive reticular cell elements. These were consistent with pseudo-Gaucher cells, sea-blue histiocytes and intermediate cell types. Primary (idiopathic) myelofibrosis-osteomyelosclerosis was characterized by a significant increase in macrophages (25 of 30 patients). Involvement of macrophages in the complex mechanisms generating bone marrow fibrosis and angiogenesis and in bone remodelling (osteosclerosis) may be responsible for this finding.

Key words: Macrophages – Haemosiderin – Osteoclasts – Monoclonal antibody PG-M_1 – Bone marrow

Introduction

Macrophages (histiocytic-phagocytic reticular cells) of the bone marrow play a central role in generating the microenvironment essential for the control of haematopoietic tissue development (Dexter 1982; Allen and Dexter 1984; Strobel et al. 1986; Sullivan et al. 1989). Although the manifold functions of the mononuclear phagocyte system have been reviewed (Van Furth 1985; Johnston 1988; Foucar and Foucar 1990), the number of macrophages composing a normal myeloid stroma is virtually unknown. This lack of information does not refer to the iron-containing subpopulation of this cell lineage (Frisch et al. 1982, 1985). However, since haemosiderin deposits in bone marrow macrophages are mostly derived from degradation of senescent erythrocytes and may be further modified by anomalies of iron transport and utilization, this fraction is certainly not identical with the total number of phagocytic reticular cells. Furthermore, in chronic myeloproliferative disorders (CMPDs) experimental findings have indicated that changes occur in the number of macrophages and in the responsiveness of precursors (granulocyte-macrophage colony-forming cells) associated with therapeutic regimens (Nissen et al. 1988; Pelus and Vadhan-Raj 1988). The absence of reactivity in routinely prepared tissue sections has hampered the use of monoclonal antibodies specifically directed against cells of the monocyte-macrophage system. A recently described monoclonal antibody (PG-M_1) recognizes an epitope of an intracytoplasmic molecule, probably associated with lysosomal granules. PG-M_1 is selectively expressed by most, if not all, macrophages and is resistant to formalin fixation, decalcification by chelating agents and paraffin wax embedding. On the basis of its staining of CD68 transfectants, PG-M_1 belongs to the group of CD68 antibodies (Falini et al. 1992). However, it has to be emphasized that PG-M_1 differs from CD68-KP_1 (Pulford et al. 1989) by its non-reactivity with granulocytes and their precursor cells. Similar restrictions are evident when applying Mac 387 on bone marrow (Brandtzaeg et al. 1988). Following completion of this study PG-M_1 is now commercially available from Dako-Diagnostica, (Hamburg, FRG).

The aim of this work was to determine the number of macrophages in the normal bone marrow and in sev-

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eral subtypes of CMPDs like chronic myeloid leukaemia (CML), polycythaemia rubra vera (P. vera) and primary (idiopathic) myelofibrosis-osteomyelo-sclerosis (OMF).

Materials and methods

Bone marrow specimens from 30 individuals without haematological but with a borderline osteoporotic disorder served as controls for the normal state. Diagnostic assignments were made by accepted criteria for the establishment of CMPDs and included a total of 90 patients. Characteristics of patients enrolled in this study are listed in Table 1.

Trephine biopsies of the bone marrow were performed from the posterior iliac crest on admission (Jamshidi and Swaim 1971). Fixation was done in an aldehyde solution for 12–48 h (2 ml 25% glutaraldehyde, 3 ml 37% formaldehyde, 1.58 g calcium acetate and distilled water per 100 ml) and further processing included decalcification for 3–4 days in 10% buffered EDTA, pH 7.4, paraffin wax embedding and employment of several staining methods including the Prussian blue reaction (Schaefer 1984). Before immunostaining with PG-M_1 all slides of the paraffin embedded marrow specimens were pre-digested with pronase (1 mg per 1 ml TRIS-buffered saline) for approximately 30 min at 37.5° C. Thereafter, samples were stained according to the alkaline phosphatase-anti-alkaline phosphatase technique using new fuchsin as the alkaline phosphatase substrate (Cordell et al. 1984; Stein et al. 1985). Negative controls were performed by applying only TRIS-buffer instead of the primary antibody.

Morphometric evaluation was performed by a manual optic planimeter (MOP-A-MO1-Kontron) using a standard programme set (Kontron software). Measurements included trephine biopsies with an artefact-free marrow area ranging between 3.9 and 8.8 mm^2 following immunostaining with PG-M_1 and the Prussian blue reaction. Total count for macrophages per square millimetre was obtained at 500× by calculation of the evaluable marrow area and the total number of the corresponding positively reacting cells. In this context it should be emphasized that, because of the extensive ramification and cluster formation, only cell elements containing a definite nucleus (haematoxylin as nuclear counterstain) were taken into consideration (Fig. 1b, c). In order to avoid an erroneous confusion with small mononucleated osteoclasts, no elements deployed along the endosteal border were considered (Fig. 2d). Statistical analysis included the U-test after Mann-Whitney (Fisher 1972) with a level of significance of $P<0.05$.

Table 1. Characteristics of patients enrolled in this study

	n	Sex (male/female)	Age (median, years)
Normal bone marrow	30	13/17	57
CML	30	17/13	49
P. vera	30	13/17	60
OMF	30	11/19	64

CML, Chronic myeloid leukaemia; P. vera, polycythaemia rubra vera; OMF, primary (idiopathic) myelofibrosis-osteomyelosclerosis

Results

Survey of normal bone marrow tissue stained with the monoclonal antibody PG-M_1 showed numerous positive cells within the myeloid stroma (Fig. 1a). These cells revealed a conspicuous stellate or spider-like aspect with multiple slender processes and contained a medium-sized lobulated nucleus with a dispersed chromatin pattern (Fig. 1b). Frequently a close association with erythropoietic islets was detectable (Fig. 1c). Following the Prussian blue reaction, on gross calculation significantly lower numbers of macrophages were present exhibiting coarse deposits of haemosiderin. In CMPDs strikingly variable amounts of positively reacting phagocytic reticular cells, either by PG-M_1 or by the Prussian blue reaction, were disclosed (Fig. 1d–f). The results of our morphometric evaluation are summarized in Table 2. A significant difference between the number of macrophages identifiable by immunostaining and histochemistry (Prussian blue reaction) was evident. In the normal bone marrow about 50% of the macrophages displayed storage of iron compounds, whereas in CMPDs this percentage was considerably less pronounced and reached zero in P. vera. In the latter disorder a slight increase in these cell elements could be encountered, since 7 patients exhibited a significant accumulation of macrophages in comparison with the control group. CML showed a ten-

Table 2. Morphometric analysis of the macrophage compartment per square millimetre bone marrow (means, standard deviations and ranges; abbreviations – see Table 1) by the monoclonal antibody PG-M_1 and the Prussian blue reaction

	PG-M_1 (mm^2)	Prussian blue-haemosiderin deposits – (mm^2)	Adipose tissue %	Megakaryocytes (mm^2)	Argyrophilic fibres ($ix10^2/mm^2$ haematopoiesis)
Normal bone marrow	30±12 11–53	16±13 1–37	49.5±13.8	15±3	16±5
CML	45±18 20–85	9±3 0–19	0.9±2.4	49±34	33±27
P. vera	39±12 21–70	0	11.9±7.9	55±8	22±10
OMF	74±13 35–113	3±7 0–35	18.0±12.4	52±18	94±33

These data are shown in comparison with the relative amount of adipose tissue, total number of megakaryocytes (per square millimetre) and density of argyrophilic fibres (intersections $-i\times10^2$ per square millimetre haematopoiesis), partially derived from previous studies (for references see Thiele and Fischer 1991)

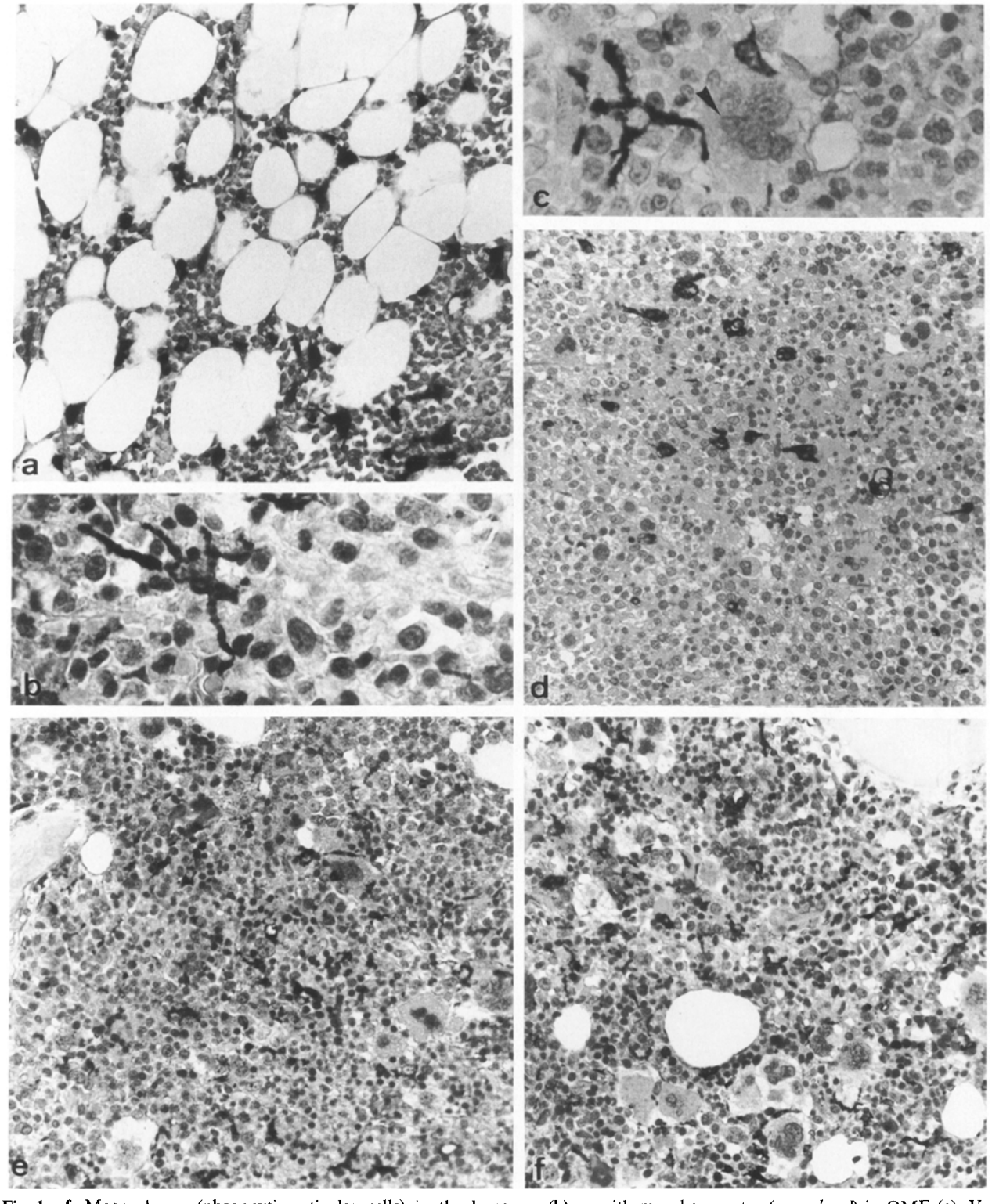

Fig. 1a–f. Macrophages (phagocytic reticular cells) in the bone marrow labelled with monoclonal antibody PG-M_1. Survey of normal bone marrow (**a**) with stellate appearance of macrophages revealing a close association with nests of erythro- and normoblasts (**b**) or with megakaryocytes (*arrowhead*) in OMF (**c**). Various aspects of macrophages in chronic myeloid leukaemia (CML) (**d**), polycythaemia rubra vera (P. vera) (**e**) and osteomyelofibrosis (OMF) (**f**). **a**, **d–f** ×220; **b**, **c** ×560

dency for an increase in PG-M_1-positive reticular cells in 9 of the 30 patients under study (Fig. 1d). These 9 cases were identical with those patients who revealed so-called sea-blue histiocytes and pseudo-Gaucher cells within their bone marrow (Fig. 2a, b). In OMF (Fig. 1f) a conspicuous association of large stellate macrophages with megakaryocytes or degenerative forms of this lineage, so-called naked (pyknotic) nuclei, was a frequent finding (Fig. 1c). In advanced stages of this disorder characterized by collagen fibrosis as well as accompany-

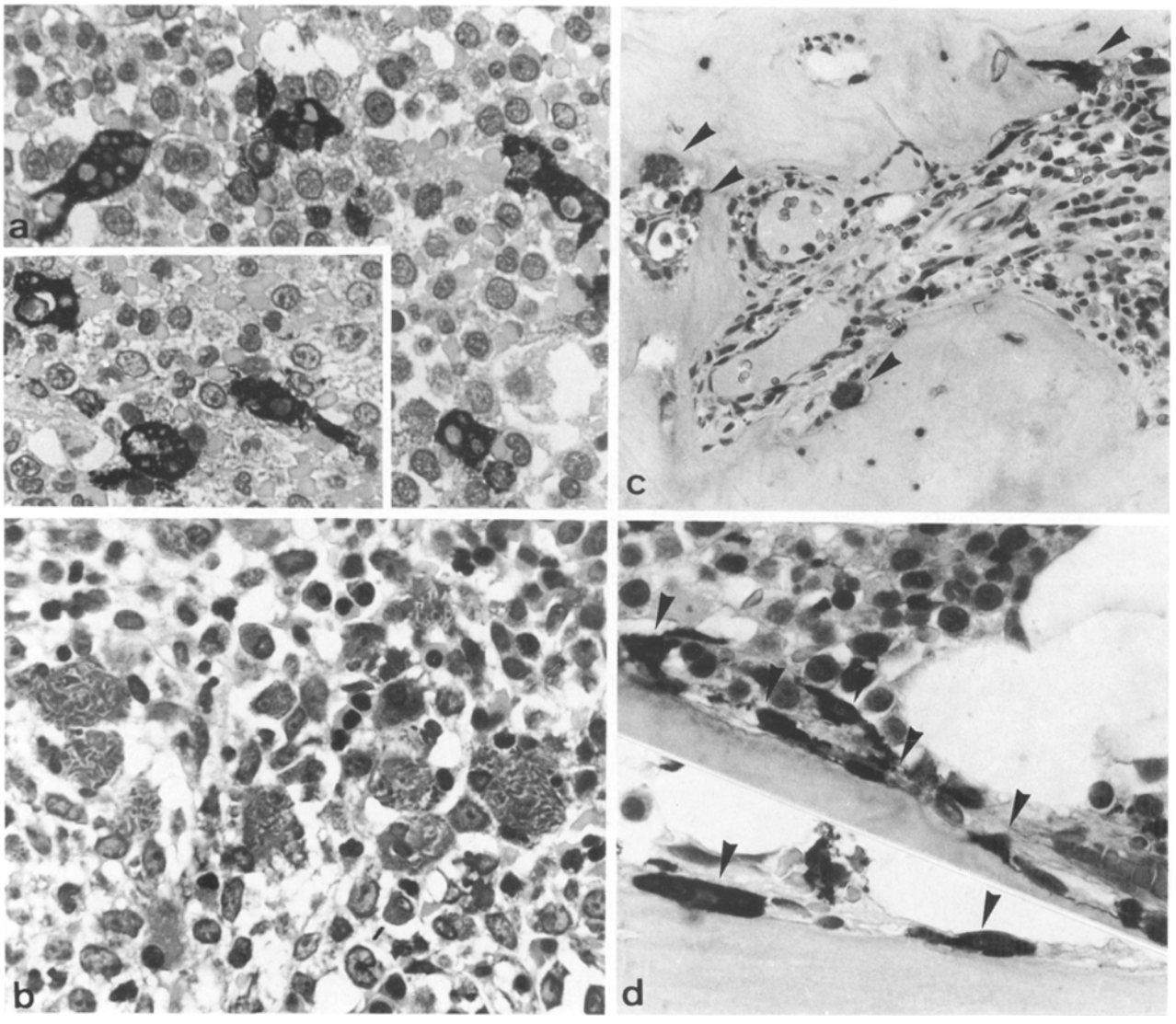

Fig. 2a–d. Pseudo-Gaucher cells in CML identified by the monoclonal antibody PG-M_1. These cells show inclusions and apparently phagocytosis of haemotopoietic elements (**a**), but hardly a fibrillar cytoplasmatic structure (**a**, *inset*). In comparison with the periodic acid-Schiff reaction an abundant finely striated, onion-skin-like pattern of the cytoplasm (**b**) is disclosed. In OMF large multinucleated osteoclasts (*arrowheads*) lying at the newly formed (endophytic) bone are labelled (**c**). Frequently, large (**d**) and small uninucleated elements are detectable deployed along the endosteal border of the woven bone (*arrowheads*). These ill-defined elements are possibly pre-osteoclasts and small osteoclasts and/or their marginal sections (**d**, *inset*). **a**, **b**, **d** and *inset* × 560; **c** × 220

ing osteosclerosis with bone remodelling and vascular proliferation, an increased number of positively stained large osteoclasts were observed (Fig. 2c). There were also small mono-nucleated osteoclastic elements localized at the endosteal border (Fig. 2d). In OMF 25 patients clearly exceeded the upper limit for the normal values of macrophages (Table 2). This implied a significant increase in the number of histiocytic reticular cells in comparison with the controls and the other subtypes of CMPDs. However, no correlation between the number of macrophages and bone marrow cellularity or amount of adipose tissue could be found (Table 2). Moreover, computation of different variables like megakaryocytes and density of argyrophilic fibres (Table 2) failed to reveal a significant relationship with the number of macrophages in the various CMPDs under study.

Discussion

We have documented the total number of macrophages within the bone marrow in the normal state and in various subtypes of CMPDs. PG-M_1-positive phagocytic reticular cells significantly exceeded the number of haemosiderin-containing macrophages. Our findings concerning iron-storing macrophages (Table 2) in the normal bone marrow confirmed previously described frequencies ($16 \pm 10/mm^2$) calculated for this cell population (Frisch et al. 1982, 1985). The same applied for the remarkable absence of iron-staining capacity of the myeloid stroma in patients with P. vera (Ellis and Peterson 1979). Particularly in this group it was evident that there is a gap between the number of haemosiderin-laden reticular cells and the iron-depleted macrophage population

(Table 2). The slightly increased number of macrophages in CML could be related to the presence of pseudo-Gaucher cells, so-called sea-blue histiocytes and intermediate cell types. This peculiar cell population has been reported to occur frequently (about 20%) in patients with CML (Albrecht 1966; Gerdes et al. 1969; Kattlove et al. 1969; Lee and Lawrence 1971; Dosik et al. 1972) and the increased appearance seemed to be associated with a more favourable prognosis (Albrecht 1972; Kelsey and Geary 1988; Thiele et al. 1991). When comparing the various subtypes of CMPDs a striking increase in the number of macrophages was noticeable in OMF (Table 2). Structural changes in the trabecular bone (osteosclerosis) are characteristic and diagnostic of lesions in this disorder. It is tempting to speculate that the increase in macrophage population could be related to the significantly increased frequency of osteoclasts, which is closely associated with the peculiar osseous remodelling (Thiele et al. 1989a). Although the origin of osteoclasts is still under discussion, most authors at present favour an origin from the cells of the monocyte-macrophage system (Burger et al. 1985; Zheng et al. 1991). Experimental findings in cultured haematopoietic tissues suggest that monocyte-macrophage-type cells were the source of osteoclasts and pre-osteoclasts (Ash et al. 1980; Burger et al. 1982; Loutit and Nisbet 1982). Multiple interactions between the monocyte-macrophage system and osteoclasts may have a considerable impact on the evolution of disease features in OMF and are assumed to be enhanced in this disorder.

Increase in macrophages in OMF may also be due to the involvement of the mononuclear-phagocyte system in the complex mechanisms generating bone marrow fibrosis (McCarthy 1985) as well as in angiogenesis (Hasselbalch 1990). In myelofibrotic conditions, because of an intrinsic stem cell defect (Adams et al. 1988), megakaryocytes undergo an abnormal maturation which causes a pronounced death rate. Senescent megakaryocytes (naked or pyknotic nuclei) are phagocytosed and degraded by macrophages (Radley and Haller 1983). The number of these degenerative elements was significantly increased in the advanced fibro-osteosclerotic stages of OMF (Thiele et al. 1989b). This process of an enforced break-down of megakaryopoiesis results in a release of various growth factors (Assoian et al. 1984; Kimura et al. 1988) into the myeloid environment. Amongst these, platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β) endothelial cell growth factor (ECGF) and finally factor 4 have been reported (Burstein et al. 1984; Castro-Malaspina 1984; McCarthy 1985; Roberts et al. 1986; Miyazono et al. 1987; Kimura et al. 1988; Martyre 1991). TGF-β and PDGF are mitogenic to fibroblasts and therefore stimulate collagen type I and III synthesis (Castro-Malaspina 1984; Roberts et al. 1986; Kimura et al. 1988; Terui et al. 1990). In an adverse reaction the activity of the enzyme collagenase, which is produced by the monocyte-macrophage is inhibited by factor 4 (Burstein et al. 1984; Castro-Malaspina 1984; Martyre 1991). The imbalance between collagen synthesis and degradation could be responsible for an accumulation of fibres in the mesenchymal compartment of the bone marrow. In this context it is noteworthy that in OMF, increase in fibre density is not correlated with an increase in megakaryocyte number (Thiele et al. 1989b) or, as shown in this study, by a corresponding accumulation of macrophages. In fact, the increased numerical density of histiocytic (phagocytic) reticular cells in OMF involved cases with early reticular fibrosis and patients with late fibro-osteosclerotic changes alike. Moreover, activation of the monocyte-macrophage system is not only mediated by defective megakaryocytes but also by the secretion of growth factors (Tzeng et al. 1985). Evolution of fibro-osteosclerotic changes in the myeloid stroma of patients with OMF is accompanied by vascular proliferation (Reilly et al. 1985; Charbord 1986; Hasselbalch 1990; Lisse et al. 1991). Angiogenesis is supposed to be modulated by a complex process particularly involving TGF-β (Roberts et al. 1986; Hasselbalch 1990) and ECGF (Miyazono et al. 1987). A schematic survey of major interactions between macrophages and target cells as well as the mesenchymal compartment is shown in Fig. 3. In P. vera a tendency to develop myelofibrosis (post-polycythaemic myeloid metaplasia) is a well-known feature in the so-called spent phase of the disease process (Ellis et al. 1986). Therefore, the increase in macrophages in several patients with this disorder may also reflect the above-mentioned mechanisms. Since the functions of macrophages are intimately associated with important changes in the myeloid stroma (Castro-Malaspina 1984; Hasselbalch 1990) and with the regulation of haematopoiesis by direct cell-to-cell contact with myeloid and

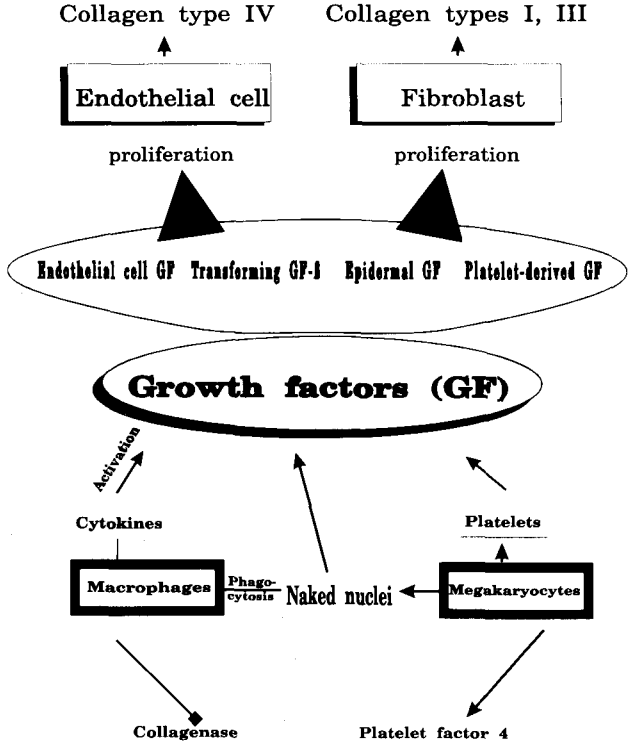

Fig. 3. Possible interactions between macrophages, megakaryocytes, fibroblasts, and endothelial cells for the development of myelofibrosis and angioneogenesis in OMF

erythroid precursors and by production of various cytokines (Van Furth 1985; Johnston 1988; Foucar and Foucar 1990), quantitation of this important cell population was warranted.

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