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Megakaryocytes and sinus walls in primary osteomyelofibrosis: transendothelial migration as revealed by three-dimensional reconstruction of serial sections following sequential double-immunostaining

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Abstract Using sequential double-immunostaining and a newly-developed three-dimensional (3D-) reconstruction technique on serially cut sections from bone marrow trephines, we studied the transmural passage of megakaryocytes through the sinus wall. Biopsies derived from patients with primary (idiopathic) osteomyelofibrosis were exposed to monoclonal antibody against type IV collagen to delineate the sinus walls and also the frequently thickened basement membrane. Staining with the primary antibody was followed by Y2/51 (CD61) to identify all elements of megakaryopoiesis. In most instances serial sectioning and 3D-reconstruction revealed an amoeboid shape of megakaryocytes and a tandem-like arrangement in close spatial contact with the abluminal surface of the sinus wall. Preceded by formation of cytoplasmic processes, straight penetration of entire megakaryocytes through gaps in the sinus walls into the lumen was seen. Where collagen deposits apparently presented a barrier, a mole-like tunnelling through the basement membrane material (type IV collagen) was recognizable. Our findings are in keeping with the assumption that megakaryocyte locomotion is an essential requirement for normal thrombocytogenesis.

Key words Megakaryocytes · Sinus wall · Transmural migration · 3D-reconstruction · Double-immunostaining

Introduction

There is increasing evidence that the conspicuous subendothelial localization of megakaryocytes in the bone marrow provides the microenvironment necessary for the normal maturation of this cell line. In addition, this peculiar topography may serve to keep these large polyploid cells at an optimal site for platelet-shedding or/and delivery of whole megakaryocytes into circulation (Lichtman et al. 1978; Tavassoli and Aoki 1989; Young 1989; Thiele et al. 1991). Although the very close spatial and functional relationships between megakaryocytes and endothelial cell layer are amply documented, there is hardly any information about details characterizing the passage of these large cells through the sinus wall (Becker and DeBruyn 1976; Tavassoli and Aoki 1981). That this occurs is documented by the very old observations of megakaryocytes in the peripheral blood (Whitby 1948; Melamed et al. 1966; Maniatis 1969; Hansen and Pedersen 1978; Pedersen 1978) and in a variety of other tissues, particularly lungs (Smith and Butcher 1952; Scheinin and Koivuneimi 1963; Pedersen 1974, 1978). Lack of knowledge about transmural migration originates firstly from the fact that in the normal bone marrow and even under experimental conditions megakaryocytes are infrequently recognized penetrating the endothelium. Secondly, both components (sinus wall and megakaryocytic cytoplasm, including small intraluminal projections) are difficult to identify properly at a light microscopic level using routine stains. Finally, the salient point is that histotopographical interactions between these two features are partially obscured by two-dimensional visualization.

To evaluate the morphological features characterizing transendothelial migration of megakaryocytes, we had to select certain bone marrow disorders. These needed to show distinctively developed sinusoidal vessels with transmural passage of haemopoietic cells as a frequently detectable event. For this reason, we disregarded normal bone marrow and reactive lesions. Idiopathic (autoimmune) thrombocytopoenia was unsuitable for this evaluation, since in this condition sinusoidal vessels were not always well delineated by a (thickened) basement membrane and intravascular penetration of megakaryocytes was only infrequently observed. We used bone marrow trephine biopsies derived from patients with chronic myeloproliferative disorders (CMPDs), mainly from so-called primary (idiopathic) osteomyelofibrosis (OMF). The latter condition is characterized by a significant increase in medullary vessels including not only a prominence and distention of sinus, but also remarkable intravascular haemopoiesis (Georgii et al. 1980; Burkhardt et

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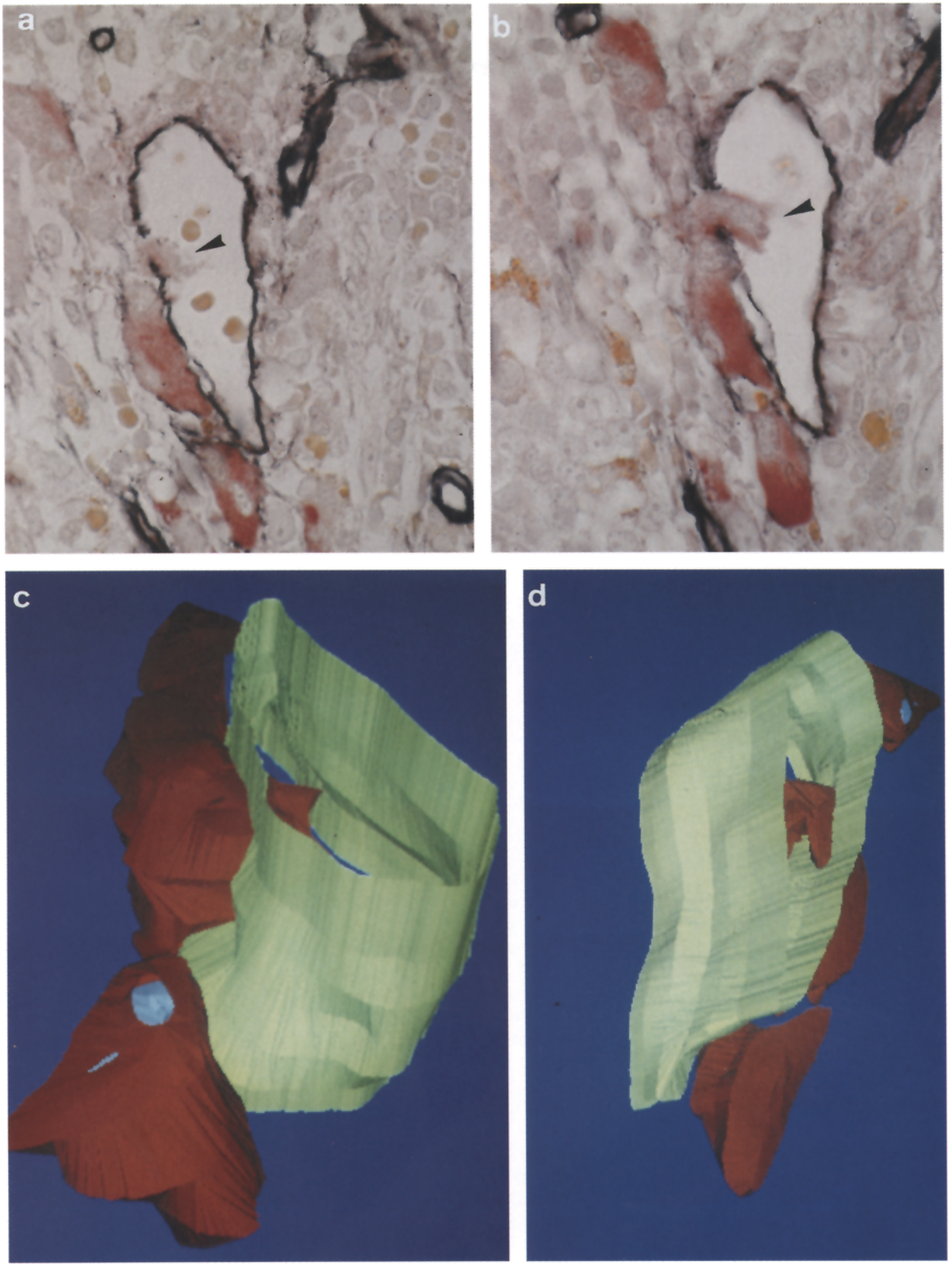

Fig. 1 Two-dimensional overview of a bone marrow sinusoidal vessel with attached megakaryocytes following double-immunostaining (**a, b**) and resulting computer-generated three-dimensional (3D-) reconstruction (**c, d**). In a tandem-like fashion several megakaryocytes are lying at the abluminal surface of a well delineated and distended sinus (**a, b**). In section number 6 the sinus wall reveals a small gap through which a fluffy portion of megakaryocyte cytoplasm extends into the lumen (**a** – *arrowhead*). Section number 8 shows a transmural penetration spear-headed by the nucleus (**b** – *arrowhead*). These features are visualized by solid rendered computer-based 3D-reconstruction (**c, d**): *yellow tones,* sinus wall; *red tones,* megakaryocytes (cytoplasm); *grey tones,* cross-sectioned nuclei of megakaryocytes. × 350

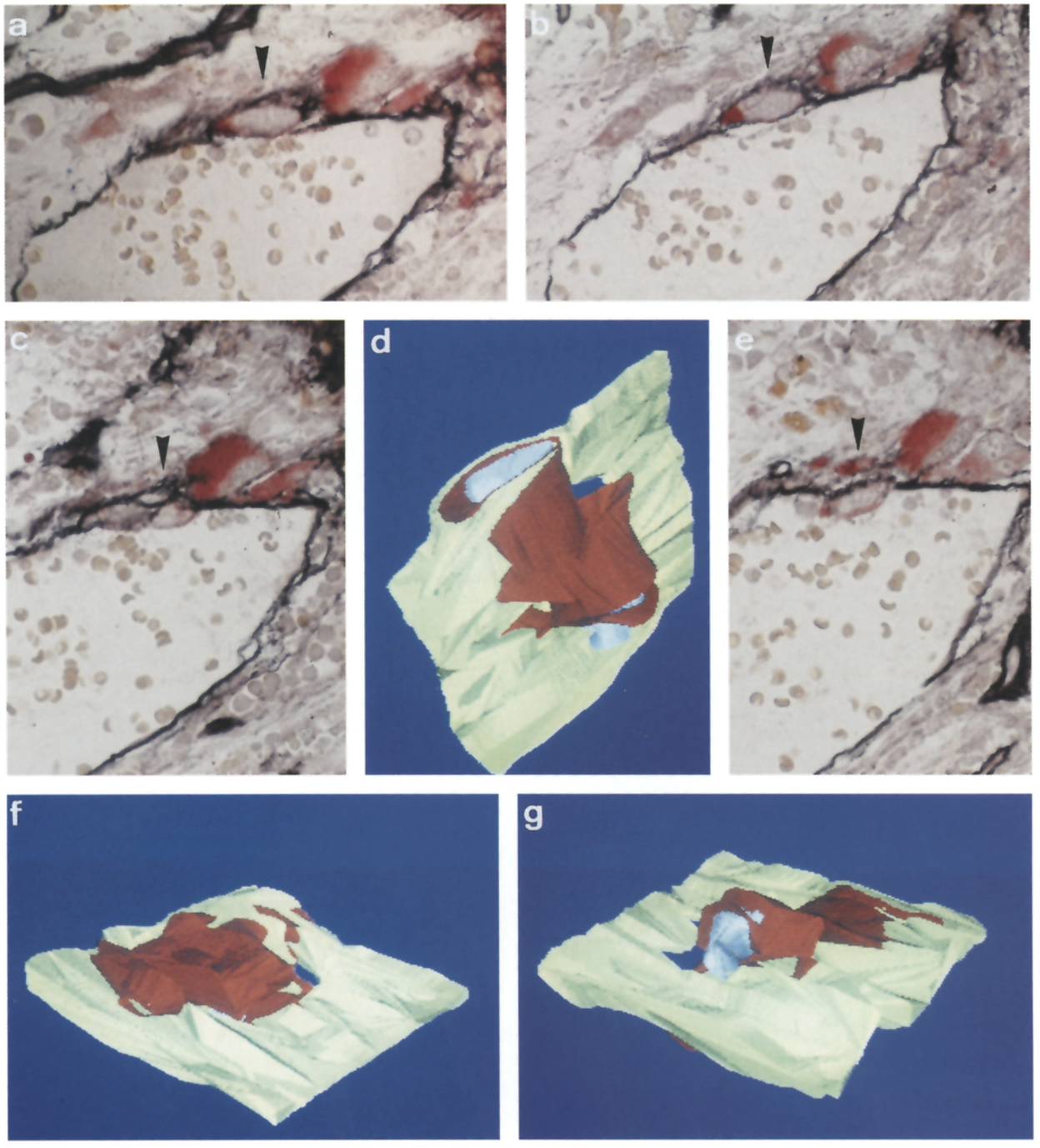

Fig. 2 Two-dimensional survey of a portion of a bone marrow sinus with transmural passage of a megakaryocyte (**a–c, e**) and resulting computer-generated 3D-reconstruction (**d, f, g**). Section-numbers 7 and 9 show a part of three megakaryocytes of which one reveals a close attachment to the basement membrane of the sinus wall (**a, b**). In section 11 only a small fragment of the nucleus is visible apparently surrounded by type IV collagen-positive material (**c**) and a later section (number 13) reveals a partial trans-endothelial migration of this megakaryocyte attached to the luminal surface of the sinus wall (**e**). Major aspects of megakaryocyte penetration through the sinus wall are exhibited by solid rendered computer-based 3D-reconstruction (**d, f, g**) which displays a tunnelling of this cell through the thickened basement membrane of the sinus wall in a mole-like fashion (**d**): *yellow tones,* sinus wall; *red tones,* megakaryocytes (cytoplasm); *grey tones,* cross-sectioned nuclei of megakaryocytes. × 550

al. 1984; Wolf and Neiman 1985; Burkhardt 1988; Thiele et al. 1989). Moreover, sequential double-immunostaining with a monoclonal antibody against type IV collagen enabled a clear-cut identification of the sinus wall (Thiele et al. 1992). In addition, another antibody against platelet-glycoprotein IIIa (Y2/51-CD61) facilitated the recognition even of small portions or extensions of megakaryocyte cytoplasm and immature precursors (Gatter et al. 1988). Following serially cut sections from trephine biopsy specimens a computer-aided three-dimensional

(3D-) reconstruction was employed to elucidate the spatial appearance of megakaryocyte entry into the sinus lumen.

Materials and methods

After informed consent, representative trephine biopsies of the bone marrow (mean size 15× 1.3 mm) were performed from the posterior iliac crest (Jamshidi and Swaim 1971) in ten patients (four men, six women; median age 66 years) presenting with the generally accepted criteria for the diagnosis of OMF. Fixation was carried out in a low-concentrated (1.5%) aldehyde solution for 12–48 h (2 ml 25% glutardialdehyde, 3 ml 37% formaldehyde, 1.58 g anhydrous calcium acetate and distilled water per 100 ml). Further processing included decalcification for 3 days in 10% buffered ethylenediaminetetracetic acid, pH 7.4, paraffin embedding and employment of several staining reactions, including Giemsa, periodic acid-Schiff, naphtol-AS-D-chloroacetate esterase, Perl's blue reaction, and the silver impregnation method after Gomori (Schaefer 1984). For this study serial sections (15–34) with a constant thickness of 5 µm were cut from each paraffin block. These were very carefully mounted on glass slides to ensure that the techniques used were standardized to reduce significant tissue distortion.

Sequential double-immunostaining was performed by using the biotin–streptavidin method (Guesdon et al. 1979) followed by the alkaline-phosphatase-anti-alkaline-phosphatase (APAAP) technique (Cordell et al. 1984). After predigestion with pronase (1 mg per 1 ml distilled water) for approximately 20 min at 37.5° C serial sections were incubated with the primary antibody directed against type IV collagen (dilution 1: 50) for 18–24 h at 0–4° C. All antibodies and the following reagents have been purchased from Dako Diagnostika, Hamburg, Germany. Following several washes in TRIS-buffered saline the slides were incubated with a biotinylated rabbit anti-mouse monoclonal antibody (dilution 1: 200) for 1 h. After another washing step we incubated the sections with streptavidin labelled AP (dilution 1: 300) again for 1 h. The AP was visualized with nitro-blue-tetrazolium and the slides rinsed in distilled water. After completion of immunostaining with the first antibody against type IV collagen, sections were incubated with the second antibody CD61 (Y2/51; anti-platelet glycoprotein IIIa) for 68–72 h (dilution 1: 15) at 0–4° C. Thereafter, the slides were incubated with a rabbit anti-mouse monoclonal antibody (dilution 1: 50) for 30 min followed by incubation with the APAAP complex for 1 h (Cordell et al. 1984). The incubation with the rabbit anti-mouse antibody and the APAAP complex was repeated for 30 min each. The AP was detected by the new fuchsin method (Stein et al. 1987) and finally, nuclear counterstaining with Mayer's haematoxylin was carried out.

Following sequential double-immunostaining, for 3D-reconstruction sections were digitalized (magnifications from 350× to 550×) using a VIDAS image analysis system (Zeiss, Germany). Contours of stained sinus walls and megakaryocytes were traced manually and alignment of consecutive sections was performed with the help of the video hardware based on specific structural landmarks such as trabecular bone structure as internal references. A recently-introduced fast and graphically interactive software package was used for computer-based 3D-reconstruction which is applicable on low-cost personal computer facilities (Salisbury and Whimster 1993; Kvasnicka et al. 1994). The system allows reconstructions from any serial image in solid model form using a fast triangulation algorithm for surface generation. Branching of complex contours was solved interactively by employment of a section description language. High quality and photorealistic representation was provided by applying modern rendering methods with Phong-shading (Phong 1975) and ray-tracing techniques (Foley and Van Dam 1982). The ray-traced computer models were viewed from different perspectives and photographed from the graphics screen. Further details of this method have been described elsewhere (Kvasnicka et al. 1994).

Results

Following sequential double-immunostaining bone marrow specimens in OMF were characterized by a prodigious proliferation and distention of sinusoidal vessels. These showed walls thickened by type IV collagen deposits. Frequently, tandem-like arranged megakaryocytes were deployed along their abluminal surface (Figs. 1a, b; 2a–c, e). Occasionally, serial sections revealed either a straight penetration (Fig. 1b) or an uncertain passage of megakaryocytes (Fig. 2a–c,e) through the sinus wall. 3D-reconstruction yielded an insight into details of this phenomenon: transmural migration was usually preceded by fluffy portions or tentacle-like processes of cytoplasm. These cytoplasmic projections were apparently pushed forward into the lumen by tightly squeezed nuclear lobules (compare Fig. 1b with Fig. 1c, d). Since several amoeboid-shaped megakaryocytes were attached to each other in a row (Fig. 1c), passage into the distended sinus lumen was assumed to occur in a tandem-like fashion. There may be occasions, where a thickened sinus wall prevents easy access to the lumen. Under these circumstances the megakaryocyte was forced to penetrate the basement membrane and endothelial layer by tunnelling its way like a mole. 3D-reconstruction (Fig. 2d, f, g) revealed this modification of straight transmural penetration; in suitable models it showed major portions of megakaryocyte cytoplasm surrounded by type IV collagen deposits (compare Fig. 2c with 2d).

Discussion

There is general consent that various components of the sinus wall and its surroundings provide a microenvironment which is necessary for the normal development and maturation of megakaryocytes and that transendothelial migration may be a manifestation of an increased thrombocytopoiesis (Lichtman et al. 1978; Tavassoli and Aoki 1989; Young 1989). Generally, passage of whole megakaryocytes through the sinus wall and intraluminal localization may be considered to represent a physiological process closely associated with the biological behaviour of this cell lineage (Whitby 1948; Smith and Butcher 1952; Scheinin and Koivuneimi 1963; Melamed et al. 1966; Maniatis 1969; Pedersen 1974, 1978; Hansen and Pedersen 1978).

Previous studies have indicated that this peculiar feature of intraluminally localized megakaryocytes may be particularly expressed in certain subtypes of CMPDs which were accompanied by a high platelet count and prominent vascular proliferation, like OMF (Georgii et al. 1980; Burkhardt et al. 1984; Wolf and Neiman 1985; Burkhardt 1988; Thiele et al. 1989, 1992). The striking compartmentalization of marrow megakaryocytes that typically lie at the abluminal (interstitial) surface of the sinus endothelium has been emphasized following ultrastructural evaluation (Becker and DeBruyn 1976; Tavassoli and Aoki 1981, 1989; Thiele et al. 1991). In this po-

sition megakaryocytes develop cytoplasmic extensions, which are sent into the lumen. Following experimental studies and analysis of this phenomenon in human bone marrow derived from patients with CMPDs, two types of cytoplasmic projections were described by electron microscopy: many short organelle-free and also a few extended processes containing numerous platelet components (Tavassoli and Aoki 1989; Thiele et al. 1991). The long projections showing all the organelles of megakaryocyte cytoplasm were interpreted to present either presumptive platelets (pro-platelets) or, alternatively, to indicate the beginning of megakaryocyte migration into the sinus lumen (Tavassoli and Aoki 1989; Thiele et al. 1991). In keeping with our findings, at least some of these processes should be regarded as features initiating the transmural penetration of whole megakaryocytes (Fig. 1a–d). However, in comparison with electron microscopy the relative merits of 3D-reconstruction consist not only in the demonstration of an amoeboid shape of megakaryocytes covering the abluminal surface of the sinus wall in a tandem-like fashion as a discontinuously arranged adventitial layer, but also in facilitating the illustration of certain details of an obviously impaired passage through those sinus walls, when thickened by collagen (type IV) deposits of basement membrane (Fig. 2a–g). In OMF megakaryocytes somehow manage to enter bone marrow sinuses by penetration, spearheaded by a nuclear lobule surrounded by a veil-like portion of cytoplasm. This peculiar intrasinusoidal migration underlines the importance of megakaryocyte locomotion as an essential part of its biological behaviour. Access of megakaryocytes into circulation and release of their platelets directly into the sinusoidal-vascular lumen of the bone marrow and various other organs (Lichtman et al. 1978; Pedersen 1978; Young 1989) is a basic pathomechanism allowing undisturbed function of the megakaryocyte-thrombocyte cell lineage. It is understandable that in OMF megakaryocytes force their way through the barrier of a thickened basement membrane by oblique tunnels like a mole, in order to preserve some aspects of their function.

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