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Angiogenesis from mononuclear cells in thrombi

Hans Jörg Leu[1], **Walter Feigl**[2], **and Martin Susani**[2]

[1] Institute of Pathology, Schmelzbergstr. 12, University of Zürich, CH-8091 Zürich, Switzerland
[2] Department of Pathology, Allgemeine Poliklinik der Stadt Wien, Austria

Summary. In organizing thrombi angiogenesis is not dependent on invasion of vasa vasora from the vascular wall. Mononuclear cells of the monohistiocytic system are always present within the clotted blood and are capable of differentiation into various types of mesenchymal cells, including endothelial cells. At first autolytic slits and clefts appear in the fibrinous superficial areas of the thrombus. They are gradually lined by spindle-shaped "pre-endothelial" cells that already possess immunohistological properties of endothelial cells but still resemble primitive mesenchymal cells ultrastructurally. Later these cells gain connection with each other by pseudopodia, overlapping and interdigitation until the channels in the fibrinous matrix are covered by an uninterrupted layer of cells. These cells are now characterized ultrastructurally by the appearance of specific endothelial organelles (Weibel-Palade bodies). Circulation within these channels begins from the blood stream. In addition, angiogenesis by sprouting of vasa vasora from the vascular wall occurs in those areas of the thrombus in contact with the vessel wall. In blood vessels with on unimpaired intimal layer, angiogenesis by invasion of capillaries occurs at an earlier date than capillary formation by mononuclear cells.

Key words: Thrombus organization – Angiogenesis in thrombi – Histogenesis of "pre-endothelial" cells in thrombi – Ultrastructural and immunohistological properties of "pre-endothelial" cells

Introduction

Angiogenesis plays an important role in a variety of conditions such as chronic inflammation, regeneration of tissue, organization of thrombi and emboli and vascularization of benign and malignant neoplasias. The idea that neoformation of capillaries occurs only by sprouting from already existing capillaries has lately been abandoned in favor of a more differentiated view. In benign vascular tumors, such as haemangiomas, sprouting of new vessels from surrounding capillaries seems to represent the main method of angiogenesis (Höpfel-Kreiner 1980). But Tsapogas et al. 1967 and Prathap 1972 have shown that angiogenesis also takes place in thrombi which have been experimentally separated from any contact with the vascular wall. They postulated that intra-thrombus capillaries develop from mononuclear blood cells either already present within the clotted blood or invading the thrombus from the surrounding blood stream. Bär et al. 1984 suggested that vascular tube formation may be confined to a single mononuclear cell in which a lumen develops by fusion of intracellularly generated vacuoles (Folkman and Haudenschild 1980). This route of vessel formation has been experimentally reproduced in vitro by Feder et al. 1983.

Human venous thrombi represent excellent and easily obtainable material for morphological studies of thrombus organization. The gradual steps of organization of thrombi and emboli are well known and the age of a thrombus can be established more or less accurately during the first twenty days (Stampfl 1962; Irniger 1963; Leu 1973; Doerr and Kayser 1977; Hofmann et al. 1980).

During the first 6 days spindle-shaped cells invade the thrombus from the vessel wall and form small capillaries. The first collagen fibers occur between 6 and 8 days. In central thrombus areas (distant from the vessel wall) organization sets in later (within 10 to 20 days). Mononuclear cells are pres-

Offprint requests to: H.J. Leu at the above address

ent in every thrombus. They seem to be activated already after 4 h to 6 h (Doerr and Kayser 1977) and appear in large numbers after about 4 days (Irniger 1963). Their nuclei are large and round. After 17 days they are gradually replaced by spindle-shaped cell elements (Irniger 1963). Spindle-shaped cells begin to occur from about the 14th or 15th day; they are found at first on the thrombus surface and later within slits and clefts which appear in the superficial thrombus areas. Still later the disintegrated thrombus surface and the slits and clefts are gradually lined by spindle-shaped cells with elongated nuclei which strongly resemble endothelial cells. They initially form a discontinuous and later an uninterrupted layer. Circulation of blood in these newly formed vascular channels occurs via connections with the blood stream in patent vessel segments around the thrombus and is not linked with vasa vasora (Stampfl 1962). The spindle-shaped cells are believed to derive from the mono-histiocytic system (Stampfl 1962; Wiener and Spiro 1962; Tsapogas et al. 1967; Stirling and Tsapogas 1969; Doerr 1970; Prathap 1972; Sevitt 1973). Under experimental conditions in which any contact with the vascular wall was excluded, one of us (Feigl et al. 1985b) has shown that these mononuclear cells may differentiate into almost any mesenchymal cell type and are also responsible for the formation of vascular tubes.

In our present investigation we intend to show the method of angiogenesis in thrombi and to determine the characteristics of the spindle-shaped "pre-endothelial" cells by electron microscopy and immunohistology.

Materials and methods

Biopsy material of 15 cases with recent thrombosis of superficial leg veins was examined by light and transmission electron microscope. The entire occluded vein segments were removed by our surgical department mostly in connection with routine procedures for varicose veins. The clinical age of the thrombi, i.e. the time of onset of the clinical symptoms, was known. The cases included 10 males and 5 females of 14–74 years (mean age 48.9 years). The histological age of the thrombi varied between 8 and 20 days according to the criteria established by Irniger 1963 and Leu 1973, and correlated well with the clinical data. Special care was taken to examine those areas of the thrombus which were adjacent to its surface, bordering on the still circulating blood and without contact with the vascular wall (free floating thrombus tail). For light microscopic examination the material was treated by routine stains such as haematoxilin and eosin, van Gieson's stain and Weigert's elastin stain. Preparation for electron microscopic examination was performed according to the routine methods used at our institute: immediate fixation in phosphate-buffered glutaraldehyde, embedding in Epon 812, staining with uranyl acetate and lead citrate, examination on a Philips EM 201. The endothelial nature of cells was established by immunohistological examination with endothelial cell-specific reagents. The presence of factor VIII was established by the immunoperoxidase method, using a rabbit anti-factor VIII antibody purchased from Dakopatts in a 1:150 dilution. The antibody binding side was visualized with an avidin-biotin complex by an ABC-Vektastain kit (Vector Laboratories). The reaction substrate 3.3 diaminobencidine (DAB) was obtained from Serva Chemicals.

Ulex europaeus agglutinin type I binding was done with a biotinolated Ulex europaeus I agglutinin diluted 1:50 after blocking of endogenous peroxidase activity and preincubation with an avitin and biotin blocking reagent, following by peroxidase labeled avidin diluted 1:100. All chemicals were purchased from Vector Laboratories.

Results

In agreement with the clinically established age of not less than eight and not more than twenty days, all thrombi were in a medium stage of organization. Wall-adherent areas showed the typical invasion of fibroblast-like cells from the intimal layer of the venous wall. These cells were already surrounded by fine collagen fibers. Capillary formation had already set in. The greater part of the thrombus, however, was still unorganized and consisted of fibrin, clotted erythrocytes and scattered white blood cells such as granulocytes in various stages of degeneration and mononuclear cells with large round nuclei. Depending on the exact age of the thrombus, the areas adjacent to the free surface showed fragmentation still without or already with spindle-shaped cells lining the thrombus surface and frequently also the clefts between the fibrin fragments. The granulocytes were in different stages of degeneration showing pyknosis, karyolysis or cell necrosis. In thrombi of more than 12 days the mononuclear cells frequently contained vacuoles. With advancing thrombus age the number of intracellular vacuoles increased. Toward the twentieth day, vacuolated cells were rare and endothelium-lined capillaries containing one to several erythrocytes occurred in increasing numbers. Occasionally a large vacuole in a single cell contained one erythrocyte. The fibrinous masses between the capillaries were progressively replaced by a young mesenchymal tissue with a high amount of mitotic activity resembling that in a tumor tissue (Figures 1 and 2).

Electron microscopy revealed that the mononuclear cells in the early stages of thrombus organization were primitive mesenchymal cells without prominent characteristics. Their nuclei were large, round, with a small amount of dispersed chromatin. The cytoplasm contained only scarce organelles such as ribosomes, mitochondria and rough-surfaced endoplasmatic reticulum. In later stages of organization some of these cells showed some

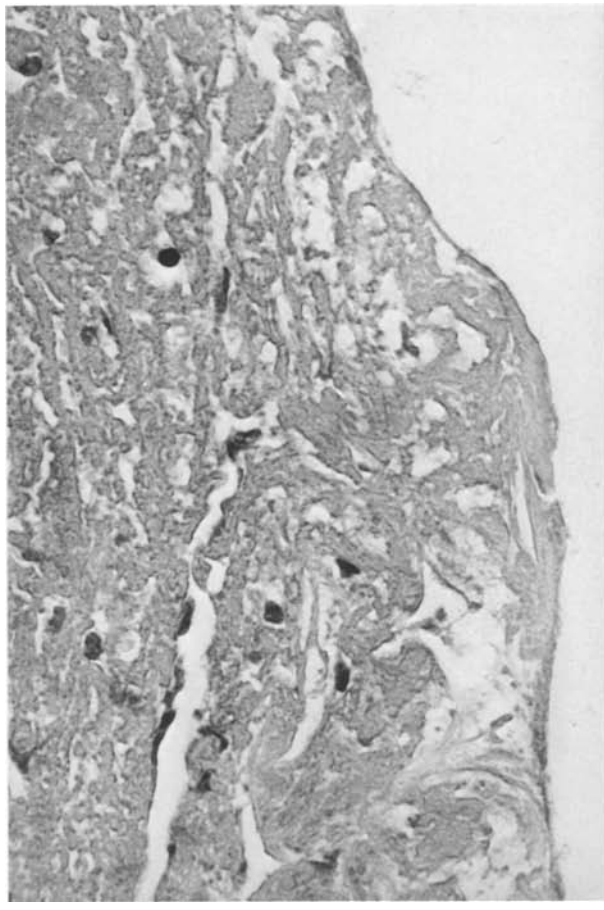

Fig. 1. Thrombus of 12–14 days. Fragmentation of the thrombus below the surface. H and E, 500 ×

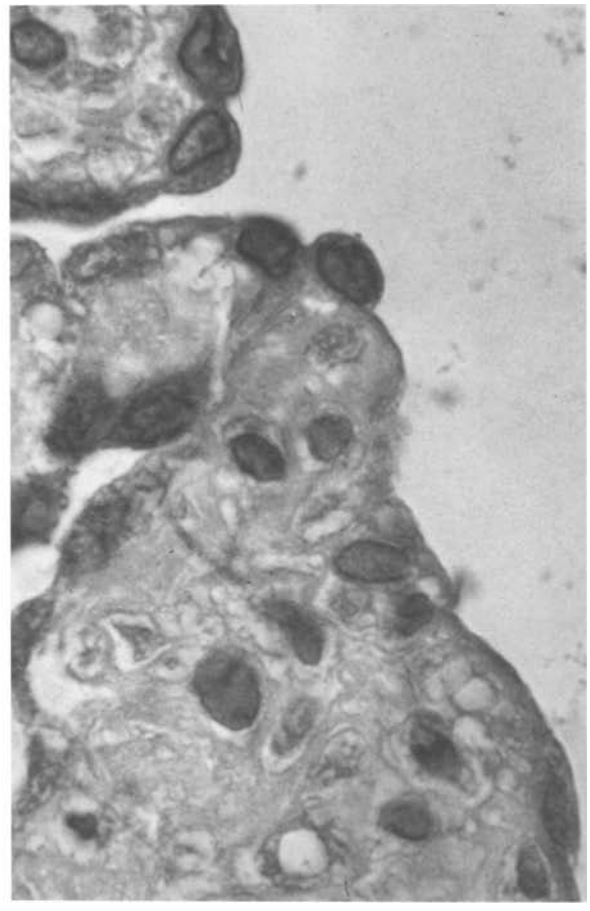

Fig. 2. Irregular surface of a thrombus of 14–18 days. Singular lining endothelial cells. Ulex europaeus agglutinin, 1000 ×

characteristics of smooth muscle cells such as actin type filaments with densities and attachment points. No micropinocytotic vesicles were present. Other cells resembled macrophages and contained siderosomes as well as a large amount of dilated endoplasmatic reticulum. Autolytic vacuoles appeared first around the nucleus. In some of the cells several vacuoles seemed to become confluent. Single large vacuoles occasionally contained one erythrocyte or a large erythrocyte fragment. Some of the vacuoles were not lined by a membrane, but in others a membrane separated the lumen from the cytoplasm. Vacuoles were also found in cells with characteristics of smooth muscle cells. However, these vacuoles never contained erythrocytes (Figures 3–6).

Slits and clefts in the fibrin appeared early. Many of them were not lined by a continuous cell layer but bordered by fibrin, erythrocytes in necrobiosis, necrotic granulocytes and mononuclear cells. Occasionally spindle-shaped cells of fibroblast-like appearance were observed along the surface of the slits. These cells contained a few organelles, mostly rough-surfaced endoplasmatic reticulum, microfilaments and mitochondria. They were unconnected with each other. Their surface was often irregularly shaped and showed villi and pseudopodia. In later stages these cells were more numerous and at several areas were in contact with each other. They progressively gained connections by overlapping and interdigitating pseudopodia until the slits and clefts were covered by a uninterrupted lining of endothelium-like cells. Specific organelles of endothelial cells were absent at first, but in later stages Weibel-Palade bodies were frequently seen. In these vascular channels, erythrocytes of normal shape intermingled by white blood cells indicated the existence of a circulating blood stream. In cross sections the participation of more than one endothelial cell in the formation of blood vessels was indicated by the presence of one or several intercellular spaces. Around the 20 day

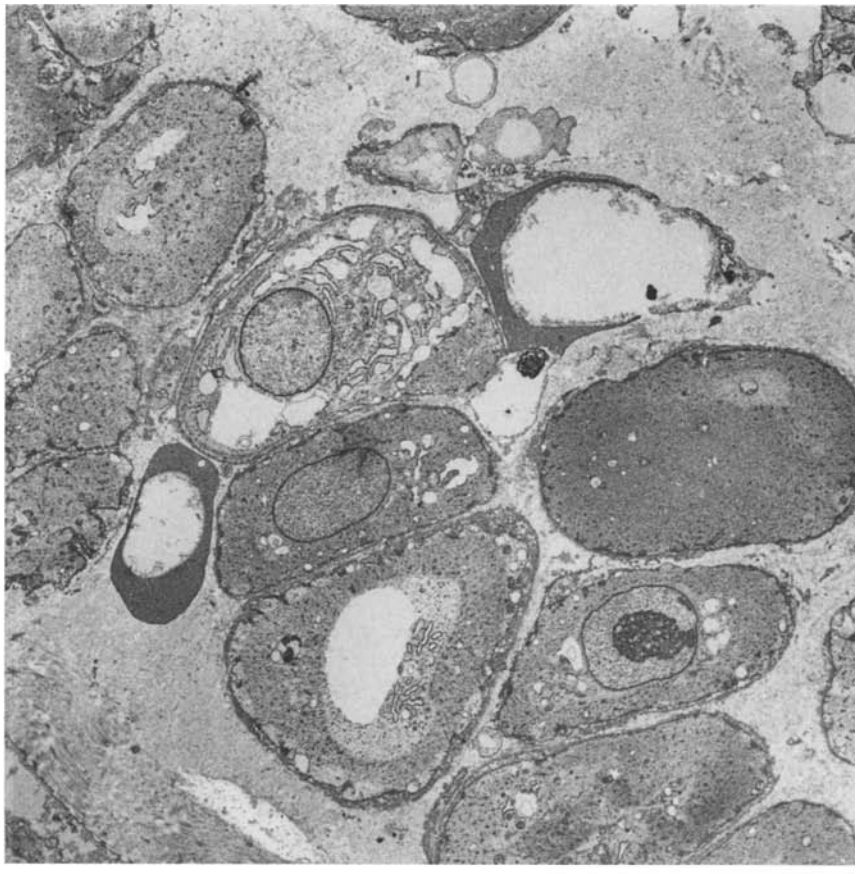

Fig. 3. Smooth muscle cells and macrophage-like cells in organizing thrombus undergoing vacuolar degeneration. Phosphate-buffered glutaraldehyde, 3350×

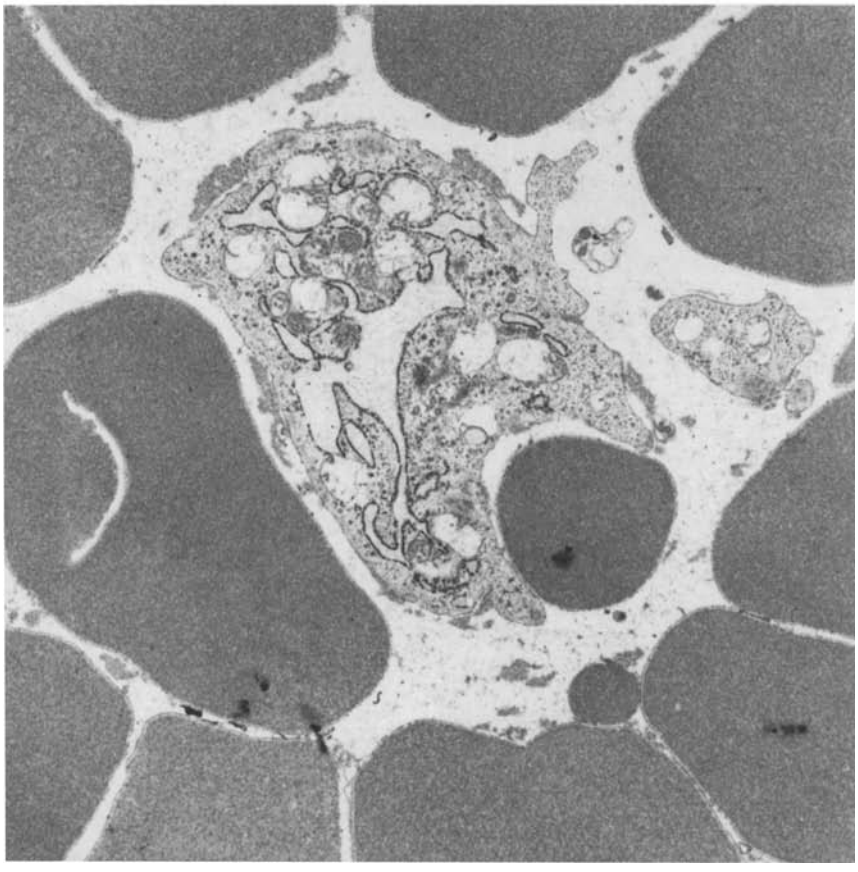

Fig. 4. Macrophage-like cell in an organizing thrombus. Phagocytosis of an erythrocyte fragment by pseudopodia. Phosphate-bufferered glutaraldehyde, 7550×

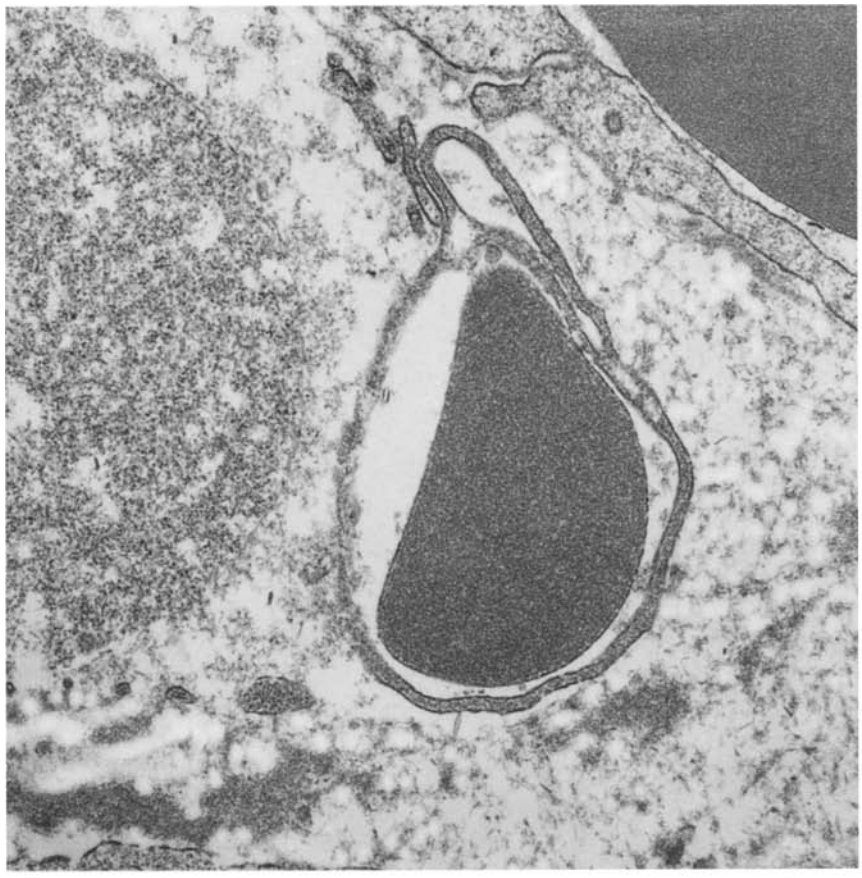

Fig. 5. Erythrocyte fragment surrounded by pseudopodia of a macrophage-like cell in an organizing thrombus. Phosphate-buffered glutaraldehyde. 3350 ×

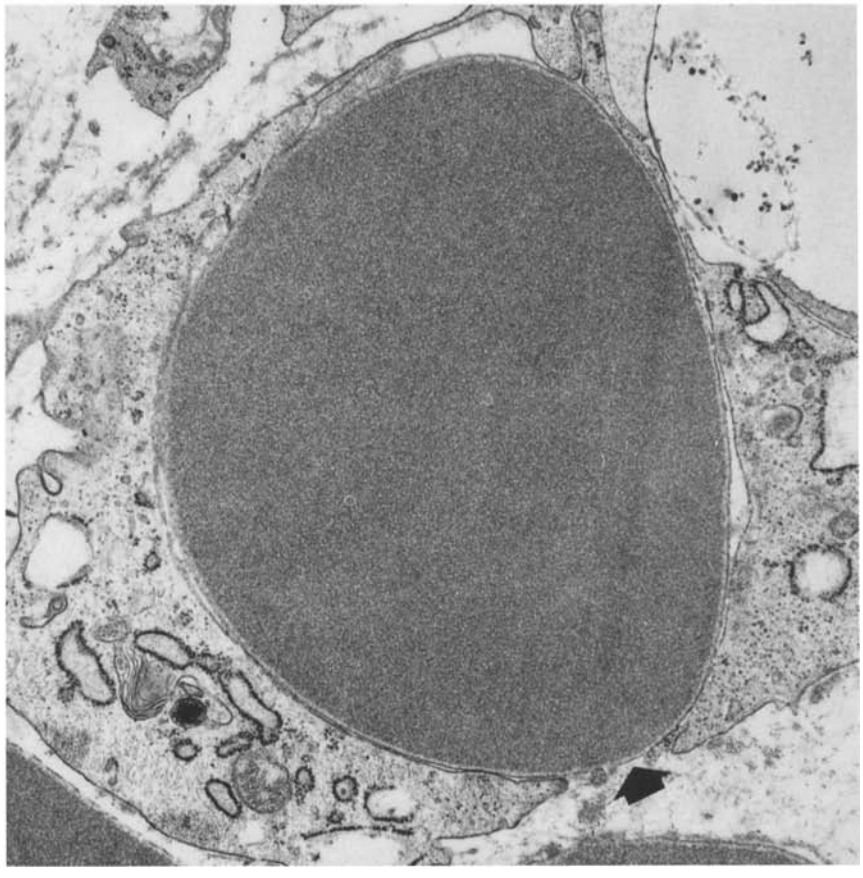

Fig. 6. Vascular tube consisting of two endothelial cells, containing one seemingly intact erythrocyte. The tube is not yet entirely closed, the intercellular space (*arrow*) is still open. Phosphate-buffered glutaraldehyde. 17300 ×

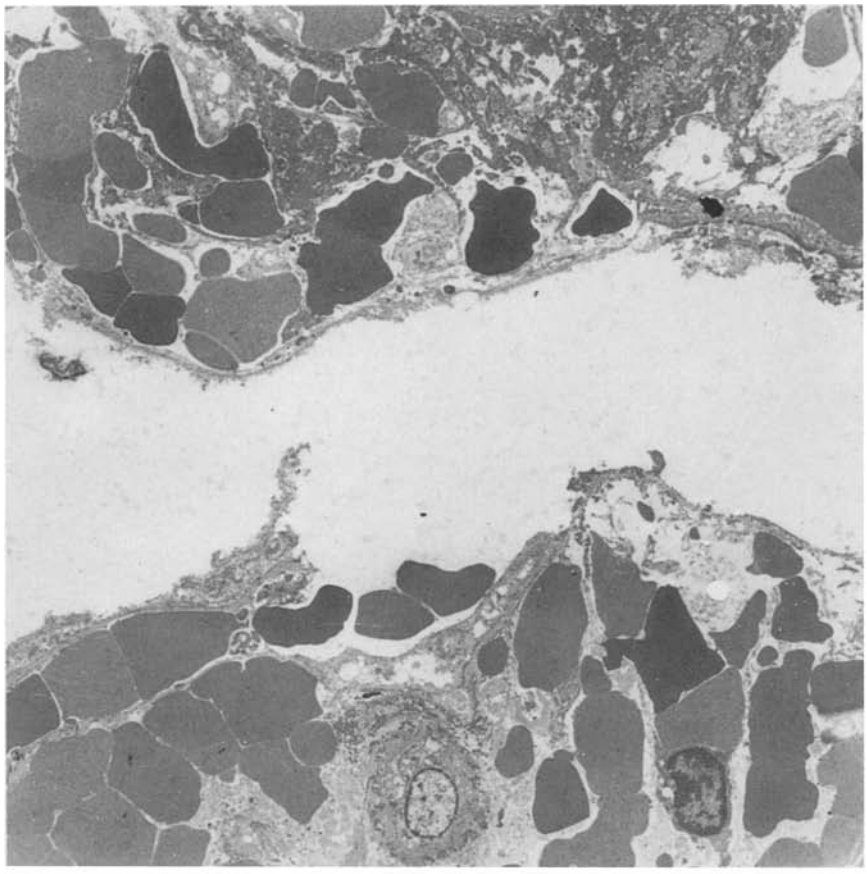

Fig. 7. Unorganized thrombus with slits and clefts without endothelial lining, filled by erythrocytes and fibrin. Phosphate-buffered glutaraldehyde. 2280 ×

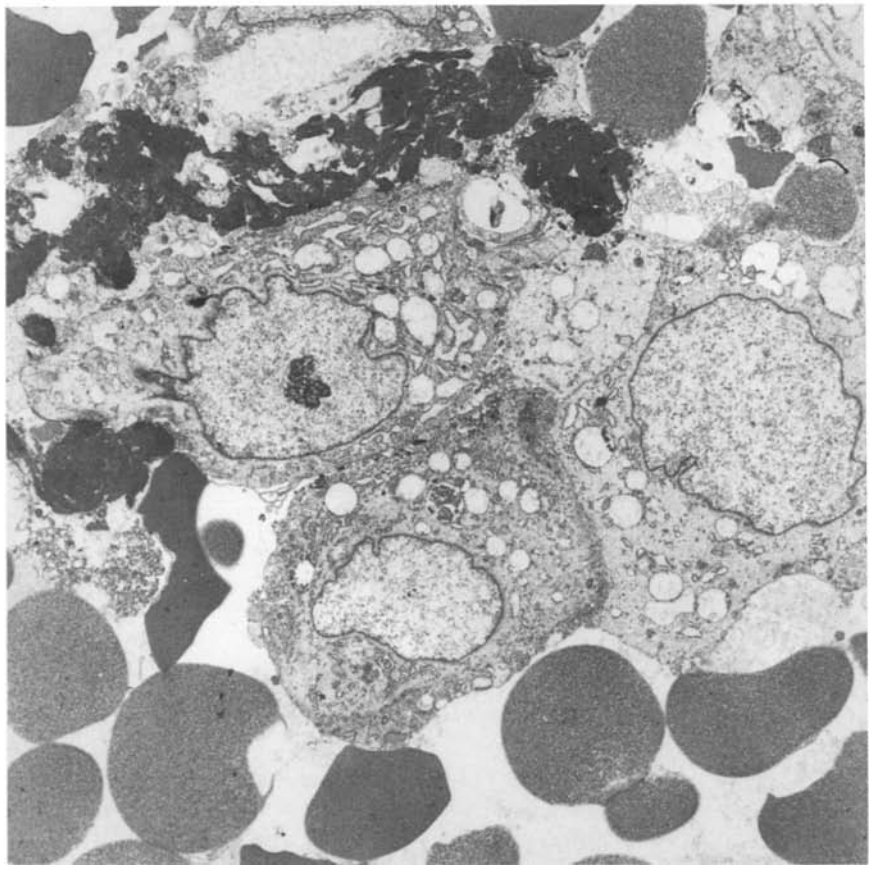

Fig. 8. Mononuclear cells with the characteristics of primitive mesenchymal cells bordering a cleft in an unorganized thrombus. The cleft is filled by erythrocytes and erythrocyte fragments. Phosphate-buffered glutaraldehyde. 3350 ×

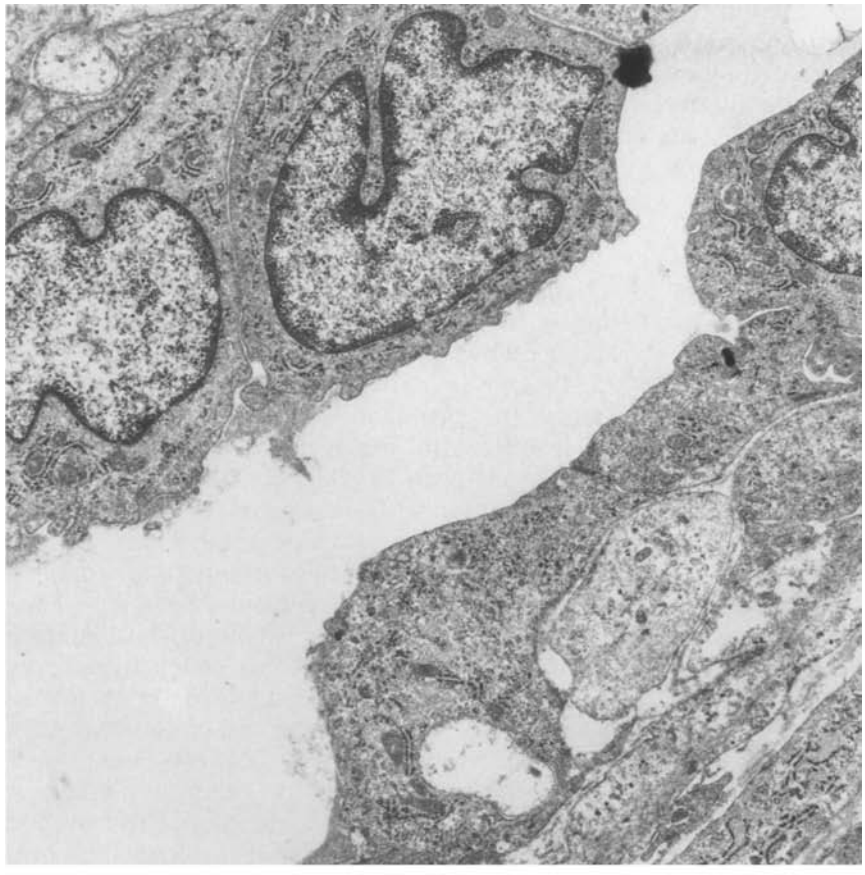

Fig. 9. Slit in the fibrinous matrix of an unorganized thrombus. The slit is lined by primitive mesenchymal cells without the ultrastructural characteristics of endothelial cells. Phosphate-buffered glutaraldehyde. 7550 ×

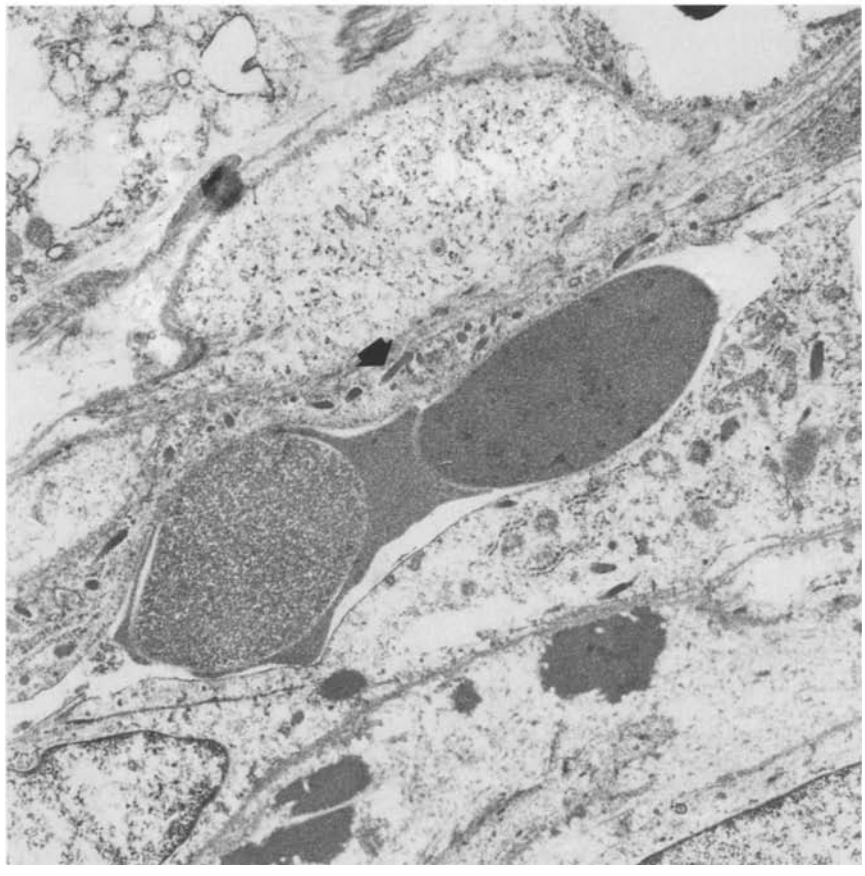

Fig. 10. Primitive vascular channel in unorganized thrombus. It already contains erythrocytes and is lined by endothelial cells with specific organelles (Weibel-Palade bodies). Phosphate-buffered glutaraldehyde. 7550 ×

small capillaries of typical structure, consisting of two or three endothelial cells and occasionally surrounded by a pericyte were frequently observed in cross sections whereas single vacuolated cells could no longer be found (Figures 7–10).

The cases in a more advanced stage of organization showed densely packed clefts lined by endothelial cells with an intensive staining for factor VIII associated antigen and Ulex europaeus agglutinin I (typical for mature endothelial cells). In an earlier stage of organization we found a loose matrix with rather immature histiocytic cells and intermingled capillary sprouts and cell lined spaces containing erythrocytes. In these areas the immunohistochemical and lectin binding sites were unevenly distributed: there was strong reaction with the Ulex europaeus agglutinin, only partial or weak reactions with factor VIII associated antigen, and transitions from a strong cytoplasmatic to a delicate membrane associated staining pattern. The vascular spaces filled with erythrocytes often contained lining cells that were negative for factor VIII, whereas adjacent cell clusters may be strongly positive for this antigen.

These results indicate that recanalisation and revascularisation of a thrombus is not performed by mature endothelial cells but by a rather immature cell population. Typical endothelial cell staining properties develop during the organization process.

Discussion

Endothelial cells and pericytes are believed to derive from a common angiogenic lineage (Spence and Rubinstein 1975), from interstitial fibroblasts (Crocker et al. 1970; Höpfel-Kreiner 1980) or from macrophages (Polverini and Leibovich 1984). The mechanism of blood vessel formation has been explained to occur in different ways: by sprouting of surrounding capillaries with formation of solid cell buds in which a lumen develops by dilation of intercellular spaces, or by formation of capillaries from mononuclear cells. This may occur by clustering of cells with a central lumen developing from intercellular spaces between adjacent cells (Bolck 1952; Crocker et al. 1970), by tube formation confined to a single cell with fusion of intracellularly generated vacuoles into a lumen (Clark and Clark 1939; Bremer 1958; Rosai et al. 1976; Folkman and Haudenschild 1980; Bär et al. 1984; Llombart-Bosch 1982), or by formation of uncovered slits and clefts in the fibrin by autolytic fibrinolysis and progressive lining of these spaces by mononuclear cells until an uninterrupted endothelial lining has developed (Leu 1973).

The second group of mechanisms are independent of pre-existing capillaries. Tube formation from a single cell is believed to occur initiated by the formation of autolytic vacuoles within a mononuclear cell, the conversion of this vacuole to a tube and the connection of this tube to a similar one in another mononuclear cell (Folkman and Haudenschild 1980; Feder et al. 1983; Bär et al. 1984). Our present findings do not support this theory. Our examinations indicate the existence of single cells with angiogenic capacities. Primitive vascular channels develop in a thrombus without any connection with the vasa vasora of the vascular wall. Although granulocytes are present from the beginning of thrombus formation, mononuclear cells become numerous around the 4 day. They persist until the thrombus is completely organized and are able to differentiate into mesenchymal cells of different type and function (Feigl et al. 1985a, b) including the formation of endothelial cells within the fibrinous matrix. The turn-over time of endothelial cells is long. Folkman and Haudenschild (1984) consider it to be measured in years. Formation of vascular tubes from single mononuclear cells is doubtless one possible method of angiogenesis and at first sight our findings seem to support the theory of tube formation from intracellular vacuoles. Vacuolization of mononuclear cells begins at about the twelfth day of thrombus organization when necrobiosis of mononuclear cells seems to be much less conspicuous than that of granulocytes. The mononuclear cells are integrated into a young and proliferative mesenchymal tissue with a high mitotic activity. These observations may speak in favor of continuous development of mononuclear single cells into tubular structures, however, electron microscopy reveals findings that contradict this opinion. The vacuoles appear first in the perinuclear area of fibroblast-like or macrophage-like mesenchymal cells. Small vacuoles are empty, larger vacuoles contain floccular material, erythrocyte fragments or entire erythrocytes, and are lined by a membrane. Many of the vacuole-containing cells are in different stages of necrobiosis. Vacuoles may also be observed in cells with characteristics of smooth muscle cells but in these cells they do not contain erythrocytes or erythrocyte fragments. We believe that the occurrence of erythrocytes within single mononuclear cells is no proof of three-dimensional tube formation but evidence of erythrocyto-phagocytosis by macrophage-like cells. The short life time of extravasated erythrocytes is well-known. It seems im-

possible that a single erythrocyte in a cell vacuole could survive more than a few hours. The connection of several similar cells to form a tube would require much more time to gain access to the circulating blood stream than a few hours. Furthermore, many of the cells in vacuoles are not intact erythrocytes but erythrocyte fragments, a further indication of erythrocytophagocytosis. The occurrence of small capillaries built of two or three endothelial cells in cross section at the time of disappearance of mononucleated cells with vacuoles is not proof of a connection of these two events. These capillaries consist of endothelial cells that contain specific organelles and show presence of factor VIII. How can their formation be convincingly explained? It is a fact that slits and clefts in the fibrinous matrix of every thrombus occur between the 10th and 14th day. They are probably induced by auto-fibrinolysis and appear first in the superficial areas below the surface of the thrombus adjacent to the blood stream. This leads to the characteristic fragmentation of the thrombus surface. Also quite early (around the 14th to 16th day), occasional and later numerous spindle-shaped cells begin to line the surface of the thrombus and the walls of the clefts. They can already be identified as endothelial cells by factor-VIII-antigen and ulex europaeus agglutinin. Later these cells develop contacts with each other and are linked by pseudopodia, interdigitation and overlapping. Only after a more or less complete lining of the clefts is formed and only when this cannel is in connection with the circulating blood outside the thrombus do erythrocytes begin to flow in and circulate. The primitive capillaries then grow (probably by sprouting) further into the thrombus matrix, until the entire thrombus is vascularized. In addition to the capillaries, spindle-shaped fibroblast-like cells (probably also derived from the mononuclear cells) occur between the capillaries and fill up the matrix. They produce collagen fibers. At last the entire fibrinous matrix is replaced by a richly vascularized mesenchymal connective tissue.

We may assume that the mononuclear cells derive from the mono-histiocytic system. Mononuclear phagocytes are mobile and long-lived. They are present within the tissues, ready for a prompt response if necessary, whereas a secondary wave is brought to the site of action by the circulation (Cohn 1986) from the bone marrow replicative pool (Cohn 1986). These cells possess the capacity to differentiate into mesenchymal tissue components.

In conclusion our findings support the assumption that angiogenesis in thrombi is independent of pre-existing capillaries in the vascular wall, that mononuclear cells of the mono-histiocytic system are capable of neoformation of capillaries and that this vessel formation happens by gradual lining of slits and clefts within the fibrinous matrix of the thrombus.

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