

Immuno-electron Microscopy Characterization of Human Bone Marrow Stromal Cells with Anti-NGFR Antibodies

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ABSTRACT. Human bone marrow stromal cells have been examined with an immuno-electron microscopy technique in order to better define their structure and function in normal hematopoiesis. Bone marrow fragments from normal donors, after mild permeabilization and glutaraldehyde prefixation were labeled with the Me20.4 Mab, which recognizes the low-affinity nerve growth factor (NGFR) and was recently described as specifically identifying fibroblastic-like bone marrow stromal cells. Five nm gold immuno-conjugates served as markers. NGFR⁺ cells were showing either a star-shape, with long and convoluted dendritic projections, and branching with each other to form a complex system of lacunae upon which hematopoietic cells were arranged. Other NGFR⁺ cells had an elongated spindle-like morphology. NGFR⁺ dendrites were seen in close contact with each other and with the different hematopoietic cells, although definite junctions were never noticed. NGFR⁺ dendrites were also observed surrounding mature plasma cells, in close apposition with adipocytes or surrounding bone marrow sinusoids. These findings may give some clues about the function of the bone marrow stromal cells, which are known to be involved in the homing and recirculation of hemopoietic cells; in addition, the presence and distribution of NGFR in the bone marrow stroma may support the recent evidence of a co-stimulatory effect of NGF in early hematopoiesis.

Keywords: bone marrow, electron microscopy, nerve growth factor receptor, stromal cells.

INTRODUCTION

The bone marrow stroma, which has been extensively characterized by light and electron microscopy studies and by several studies of in vitro bone marrow cultures, is composed of fibroblastic reticular cells, endothelial cells lining sinusoidal spaces, adipocytes and mononuclear phagocytes (1,2,3). Bone marrow fibroblastic cells have been variously named according to their anatomical distribution as adventitial

reticular cells, perisinusoidal adventitial cells, periarterial adventitial cells and intersinusoidal reticular cells. They are poorly defined as far as their immuno-phenotypical characteristics as well as their origin (2,4). Furthermore, although a key role of these cells in supporting and regulating hematopoiesis has been suggested in a number of studies utilizing in vitro system with stromal cell lines or long term bone marrow cultures (5,6), the exact function of these cells still has to be clearly established.

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We have recently reported (7) that a monoclonal antibody directed against the low affinity nerve growth factor receptor (NGFR or p75^{NGFR}) (8,9) stains the majority of alkaline phosphatase⁺, collagen III⁺ and vimentin⁺ bone marrow cells, which are, on the other hand, negative for neural, endothelial and leukocyte markers, as demonstrated in double and triple immuno-histochemical labeling experiments. NGFR expression was in addition demonstrated at the m-RNA level by RT-PCR on bone marrow aspirates and cultures (7).

The ultrastructure of bone marrow stromal cells has been previously reported by numerous authors, although these observations were restricted to mice (10-13) and rat (11,14) models for technical reasons.

An anti-NGFR antibody (15,16,17), which specifically identifies fibroblastic-like bone marrow stromal cells, was utilized to immunolabel the human normal bone marrow stromal cells. This was achieved at the electron microscopic level, taking advantage of a novel tissue preparation procedure, which allows study of the fine details of the contacts among stromal cells projections and between stromal and hematopoietic cells.

MATERIALS AND METHODS

Sodium citrate anticoagulated bone marrow aspirates were obtained from 4 normal donors for allogeneic bone marrow transplantation. Bone marrow spiculae were picked up with thin-tips tweezers and resuspended into Hank's Balanced Salt Solution (HBSS).

After rinsing in PBS buffer for 10 min at room temperature (RT), the spiculae were prefixed in 0.2% glutaraldehyde in PIPES buffer for 35 min at RT, rinsed in PBS buffer and treated for 10 min with 1% glycine in PBS. The samples were subsequently incubated with PBS containing 1% BSA, 0.2% NaN₃, 10% decomplexed normal human AB serum, 0.001% Triton X-100 for 40 min at RT and incubated with the anti-NGFR Mab Me20.4

(Amersham International plc, Aylesbury, UK) diluted 1:200 in the above described solution for 2h at RT, rinsed in the same buffer, and finally incubated with goat anti-mouse Igs conjugated with 5 nm colloidal gold particles (GAM-G5, Janssen Pharmaceutica, Beerse, Belgium) at 4°C. Final rinsing in PBS/1%BSA/NaN₃ preceded fixation overnight with 2% glutaraldehyde in 0.1M cacodylate buffer at 4°C and washing in 0.1M cacodylate buffer.

The specimens were then postfixed in 1% OsO₄ in cacodylate buffer for 1h at 4°C. Dehydration was carried out using a graded ethanol series from 70% up to 100% and propylene oxide. After embedding in Araldite, ultrathin sections were obtained in a Reichert Jung ultramicrotome and counter stained with uranyl acetate and lead citrate. In addition, ultrathin sections from two samples were incubated with the Intense SE silver enhancement kit (Janssen Pharmaceutica, Beerse, Belgium) for 3 min. The grids were observed with a Philips CM10 transmission electron microscope at 80 KV. The 5 nm gold particles were observed at 20,000x and the labeling was finally drawn on a print of the same field with a thin marker.

RESULTS

Transmission electron microscopy study of the whole bone marrow spiculae after labeling with the Me20.4 Mab revealed the presence of numerous cells expressing the p75 Kd nerve growth factor receptor. The labeling was present throughout the bone marrow spiculae without gradient from the outer to the inner part of the fragments. The Me20.4 Mab was shown, in preliminary light microscopy experiments, to recognize a "fixation resistant" epitope: in particular, no difference was seen in the labeling between the frozen samples and the preparations fixed with buffered 0.2 % glutaraldehyde for up to 30 min.

The labeled cells were showing either a star-shape (Fig. 1a,b; 2a,b) or an elongated spindle-like morphology (Fig. 3a,b).

In the first case the cells had a central and irregular nucleus, a scanty perinuclear cytoplasm and an large number of dendritic projections, intermingled among the surrounding hemopoietic cells (Fig. 1a,b). In some cases, NGFR⁺ dendrites of two or more cells were interdigitating, forming a complicated system of lacunae, containing one or more hemopoietic cells (Fig. 2a,b).

The second typical morphology of NGFR⁺ cells was characterized by an oval or elongated nucleus with extremely ruffled dendrites at one or two poles of the cell (Fig. 3a,b). These cells were more isolated and sparse, making distant contacts through their dendrites with hematopoietic cells, with adipocytes and with sinusoidal spaces.

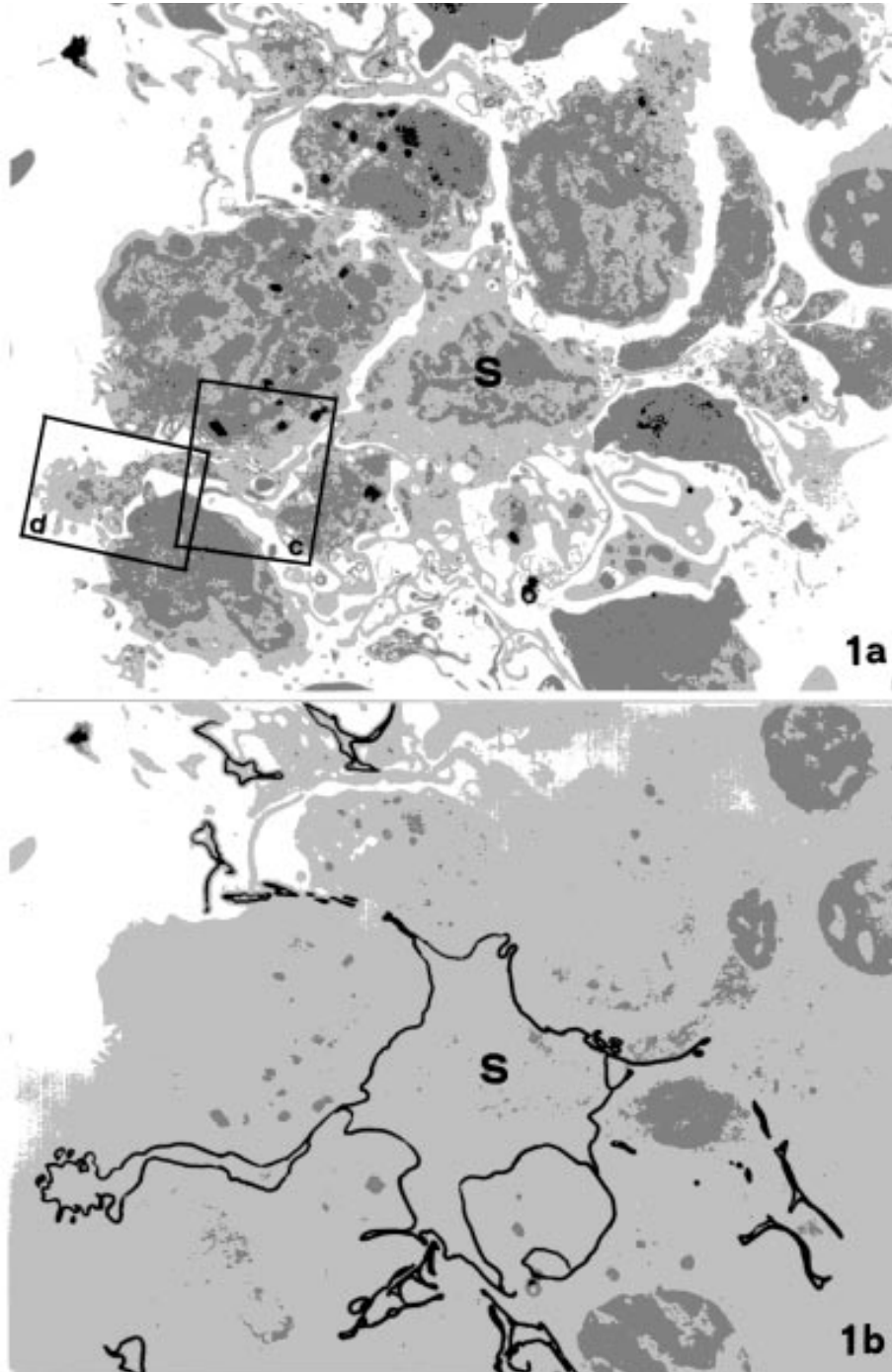


Fig. 1. a) A star-shaped NGFR⁺ stromal cell (S) surrounded by different hematopoietic cells. b) The labeling of the cell body and of the numerous dendrites is shown in the drawing. (Magnification, x 5000).

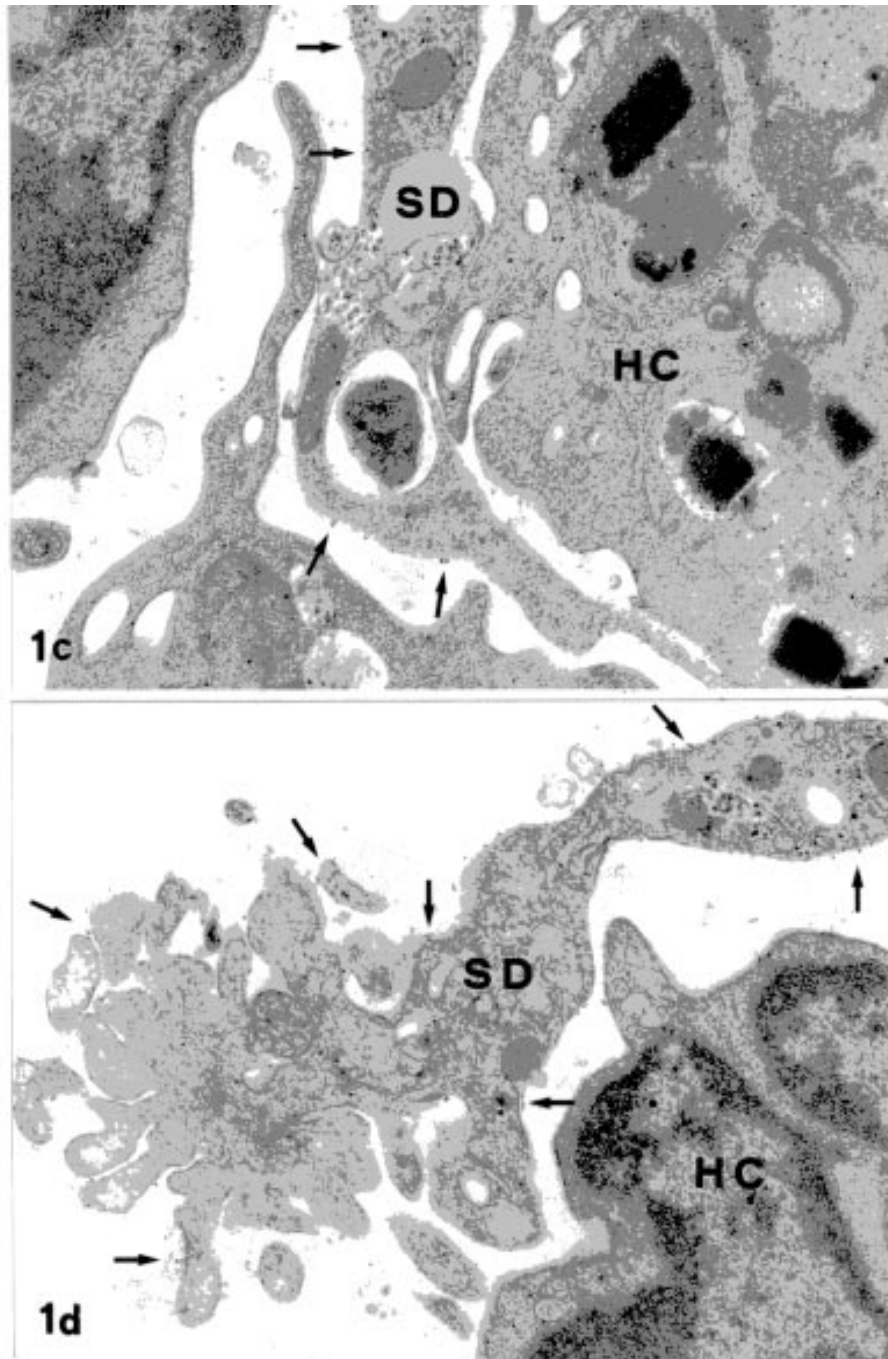


Fig. 1c, d. Details of the labeling (arrows) with 5 nm silver enhanced gold particles and of the contacts between the stromal cell dendrites (SD) and hemopoietic cells (HC). The dendrites contain mitochondria, vesicles of different size and glycogen particles and never form clear-cut junctions with the surrounding cells. (Magnification, x 29400).

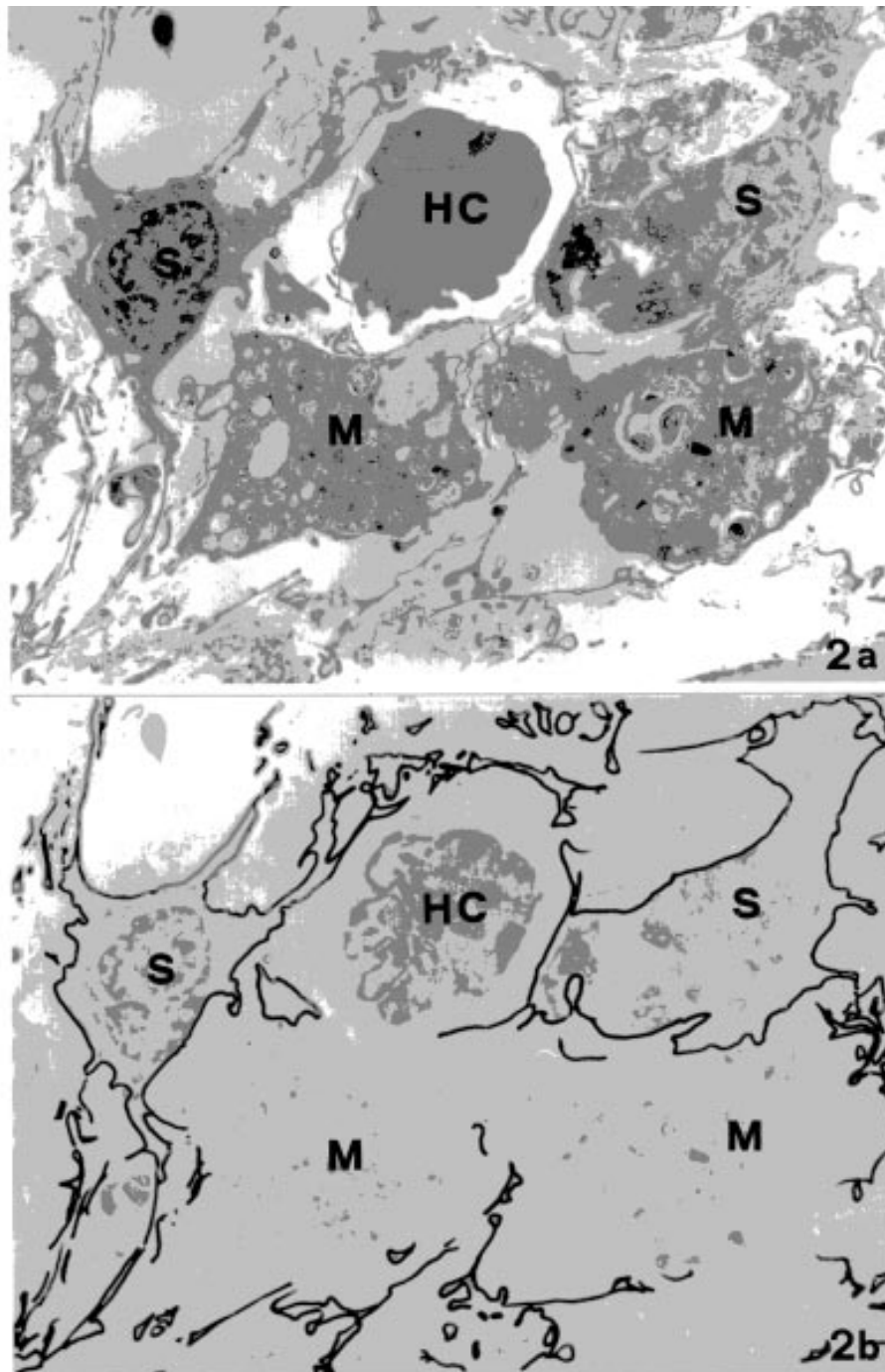


Fig. 2. a) Two labeled NGFR⁺ stromal cells are seen interdigitating together with numerous dendrites and forming a lacunar system containing an immature hemopoietic cell (HC) and two macrophages (M). **b)** The labeling is shown in the drawing. (Magnification, x 4600).

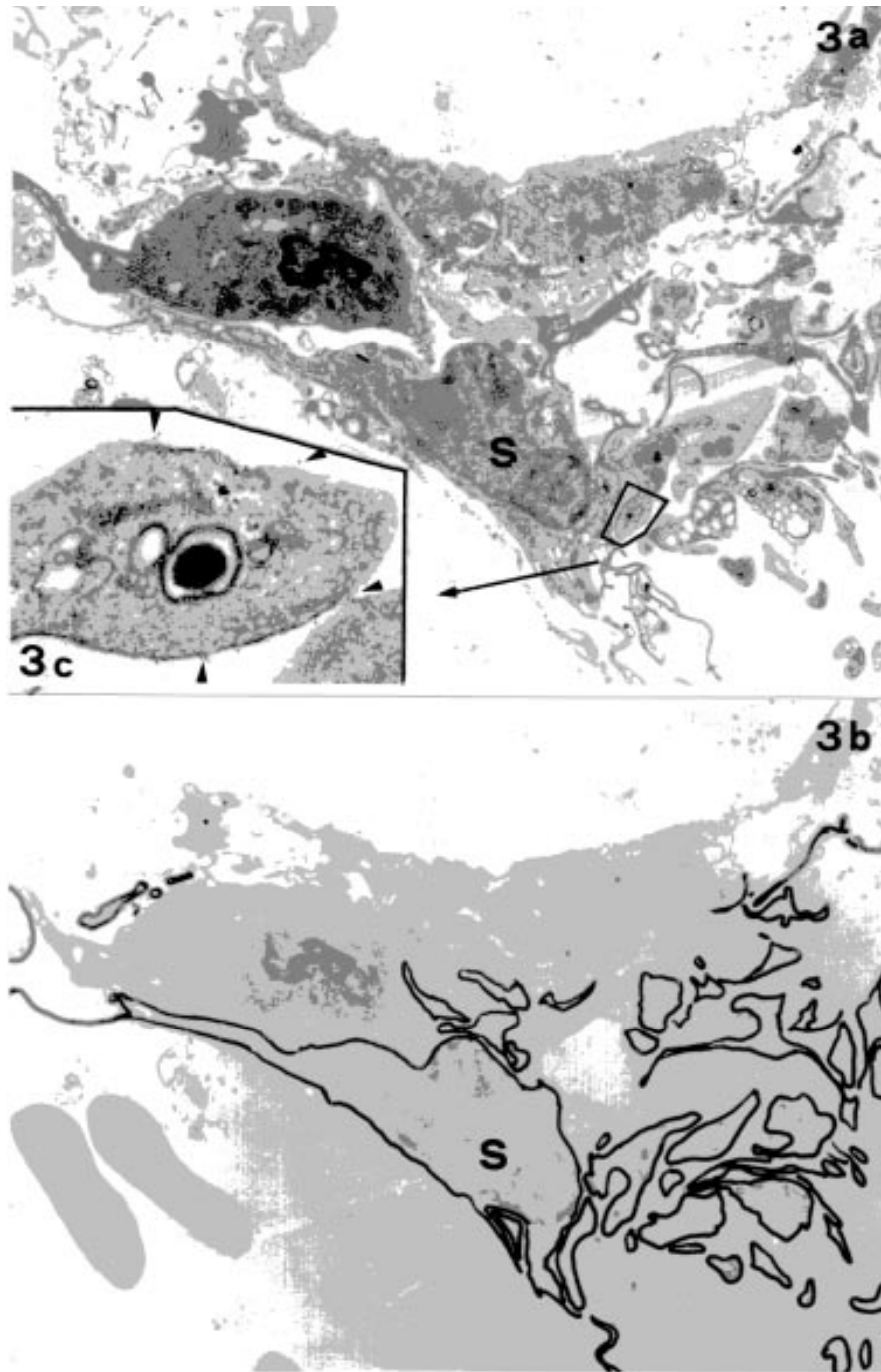


Fig. 3. a) A NGFR⁺ stromal cell (S) with extremely ruffled dendrites at the two poles of the cell. **b)** The labeling of this cell is shown in the drawing. (Magnification, x 6600). **c)** In the inset a higher magnification of a NGFR⁺ dendrite is visible; the labeling with 5nm gold particles (arrowheads) and the details of the cytoplasm, containing vesicles and a coarse core granule, can be observed. (Magnification, x 43700)

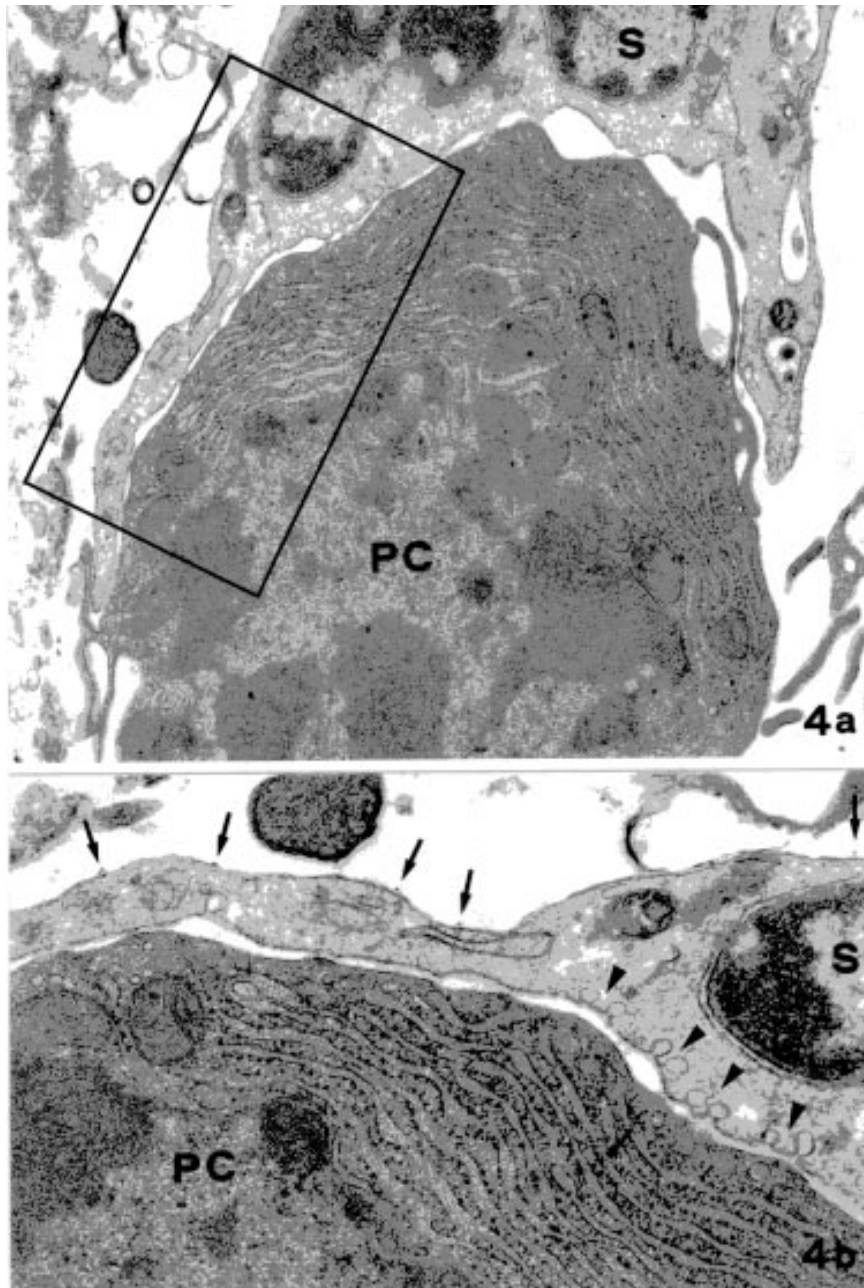


Fig. 4. a) A NGFR⁺ stromal cell (S) branching a mature plasma cell (PC). (Magnification, x 23300) **b)** At higher magnification the stromal cell cytoplasmic membrane is labeled with 5 nm gold particles (arrows); numerous pinocytotic vesicles are also visible in the cytoplasmic portion facing the plasma cell (arrowheads). (Magnification, x 42000)

In both cases a small nucleus was present and euchromatin was more abundant than heterochromatin, which was scattered in small clusters near the nuclear envelope (Fig. 1a, 2a, 3a). The scanty cytoplasm contained a well developed Golgi apparatus, a large amount of smooth and rough endoplasmic reticulum in the perinuclear

region, many mitochondria and vacuoles. The dendritic projections mainly contained some vesicles, some cisternae of endoplasmic reticulum and isolated mitochondria (Fig. 1c,d; 3c). The immuno-gold labeling was weak but present all over the cell outline, with some enhancement along the dendrites (Fig. 1c,d; 3c).

The NGFR⁺ dendrites were seen surrounding all the different hemopoietic cell types: myeloid, erythroid, lymphoid as well as the immature ones (Fig. 1a; 2a; 3a); in some cases the NGFR⁺ dendrites were in close contact with the outer membrane of hemopoietic cells, although junctions were never observed (Fig. 2 a,b).

NGFR⁺ dendrites were seen branching mature plasma cells (Fig. 4a): in all the examined sections the NGFR⁺ dendrites were in close contact with the plasma cell membrane, with many pynocytotic vesicles in the NGFR⁺ dendrites facing the plasma cell membrane (Fig. 4b).

NGFR⁺ cells were also seen completely surrounding adipocytes (Fig. 5a); the thin cytoplasmic layer of these cells was always in close apposition, but with no junctions, with both the NGFR⁺ cell bodies (Fig. 5a,c) and/or dendrites (Fig. 5a,b).

NGFR⁺ dendritic projections from stromal cells were also focally surrounding bone marrow vascular spaces (Fig. 6a,b): however, NGFR⁺ dendrites were always separated from endothelial cells by a variable space generally filled with extracellular matrix proteins (Fig. 6a,b).

DISCUSSION

The bone marrow microenvironment is composed of macrophages, endothelial cells, adipocytes and fibroblastic-like stromal cells (1,3,4,13,18). These latter had been extensively characterized: histochemically, on the basis of the reticulum silver and endogenous alkaline phosphatase staining, and immunophenotypically, for the presence of various surface markers like vimentin, collagen III and CD13 (7,11,19). The fibroblastic-like stromal cells seem to play a key role in the homing of stem cells, in the regulation of differentiation as well as in the bone marrow egress of the hemopoietic cells of different lineages (10,12).

The ultrastructure of the bone marrow microenvironment had been clearly established in early papers in which a network of reticular cells

surrounding hemopoietic cells and branching with the endothelial sinusoidal spaces and adipocytes has been described (13,14,18). The majority of these studies were however done on mouse or rat bone marrow, fixed by perfusion to get an optimal tissue preservation, which was further hampered by the presence of calcified tissues.

We describe here a technique which allows, for the first time, to perform immuno-electron microscopy studies of the whole bone marrow. The single bone marrow fragments, generally obtained in anticoagulated bone marrow aspirates, but still representative of the bone marrow tissue even after having lost its connections with the calcified bone framework, were picked up with thin-tweezers and subjected to a mild prefixation. This procedure allows to maintain both the cohesiveness of the cells within the spicula and a good preservation of the fine structural morphology of all the different components of the bone marrow (the hemopoietic and stromal) with their naturally occurring interactions. In addition, this technique, which included a mild permeabilization with Triton X100 and the use of relatively small gold particles (5 nm), permitted the penetration of immuno-labeling throughout the fragments. Taking advantage of an antibody directed against the low affinity NGF receptor (16,17), shown to be the only stromal cell restricted marker (7), we were able to confirm these observations at the ultrastructural level.

One of the most striking findings in our immuno-labeled samples is the extremely developed dendritic morphology of the NGFR⁺ cells, with small dendritic fragments, which can be observed far away from the stromal cell body. The labeled dendrites are forming complex systems of lacunar spaces, but gap junctions at contact between stromal cells as described by Watanabe in the murine bone marrow are never observed (10,13). The hemopoietic cells are generally completely surrounded by the NGFR⁺ dendrites, but somewhat floating in these lacunar

spaces, while direct contacts among hemopoietic stromal membranes are rarely observed. The hemopoietic cells appear therefore to come in contact with stromal cells on a few spots along their surface, contacts consisting of a close apposition of the two membranes with no recognizable junctions. This finding of a somewhat loose association between stromal and hemopoietic cells may be artifactual, resulting from the tissue processing and permeabilization, which was however necessary to improve accessibility of the immuno-labeling. It could also indicate that the hemopoietic cells interact with the stromal cells by a variable number of dendrites, and that the number of cell contacts may play a role in stromal cells control of homing and recirculation of hemopoietic cells within the

bone marrow. Serial section studies could of course provide a better evaluation of the contact areas on hemopoietic cells at differentiation stages. Co-localization at contact points of adhesion molecules could also be feasible with immuno-gold techniques at the EM level. A role of extracellular matrix proteins, like fibronectin, collagen and proteoglycans and of a number of adhesion molecules, to anchor and present growth factors to hemopoietic cells has been suggested in numerous publications (10,14,20,21). The loose connections we found in the hemopoietic islets suggest that these regulatory interactions are not taking place at the stromal-hemopoietic cell contacts, but probably in the extracellular matrix which fills lacunar spaces of the stromal cell network.

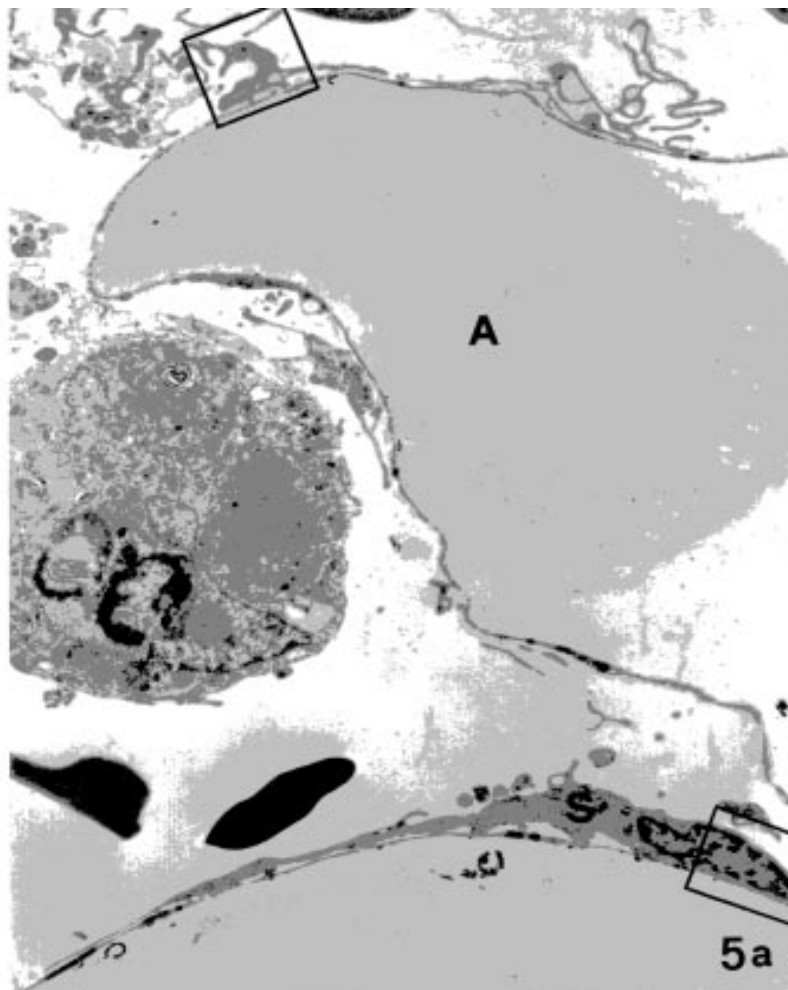


Fig. 5. a) Two closely adjacent fat-laden adipocytes (A): both cells are completely surrounded by NGFR⁺ dendrites. An elongated NGFR⁺ stromal cell (S) is also seen completely lying on the adipocyte surface. (Magnification , x 5000).

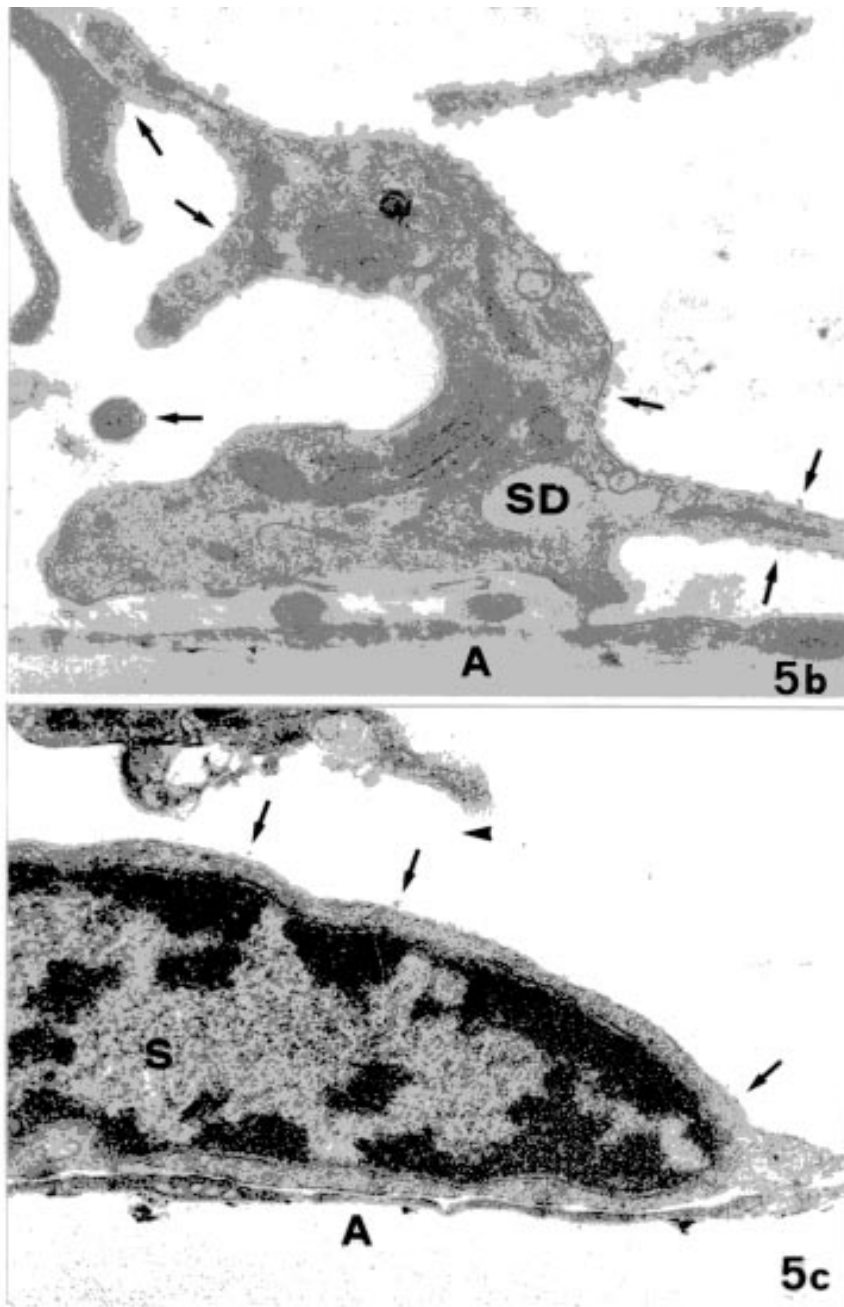


Fig. 5 b) A ruffled NGFR⁺ dendrite (SD) in close contact with the cytoplasmic membrane of an adipocyte (A): the opposite membranes are not connected by junctions but kept separated by a thin layer of extracellular matrix. (Magnification, x 40000)
c) Details of the labeling (arrows) of the stromal cell body (S) as well as of the contact between the stromal cell and the cytoplasmic body of an adipocyte (A). (Magnification, x 34000).

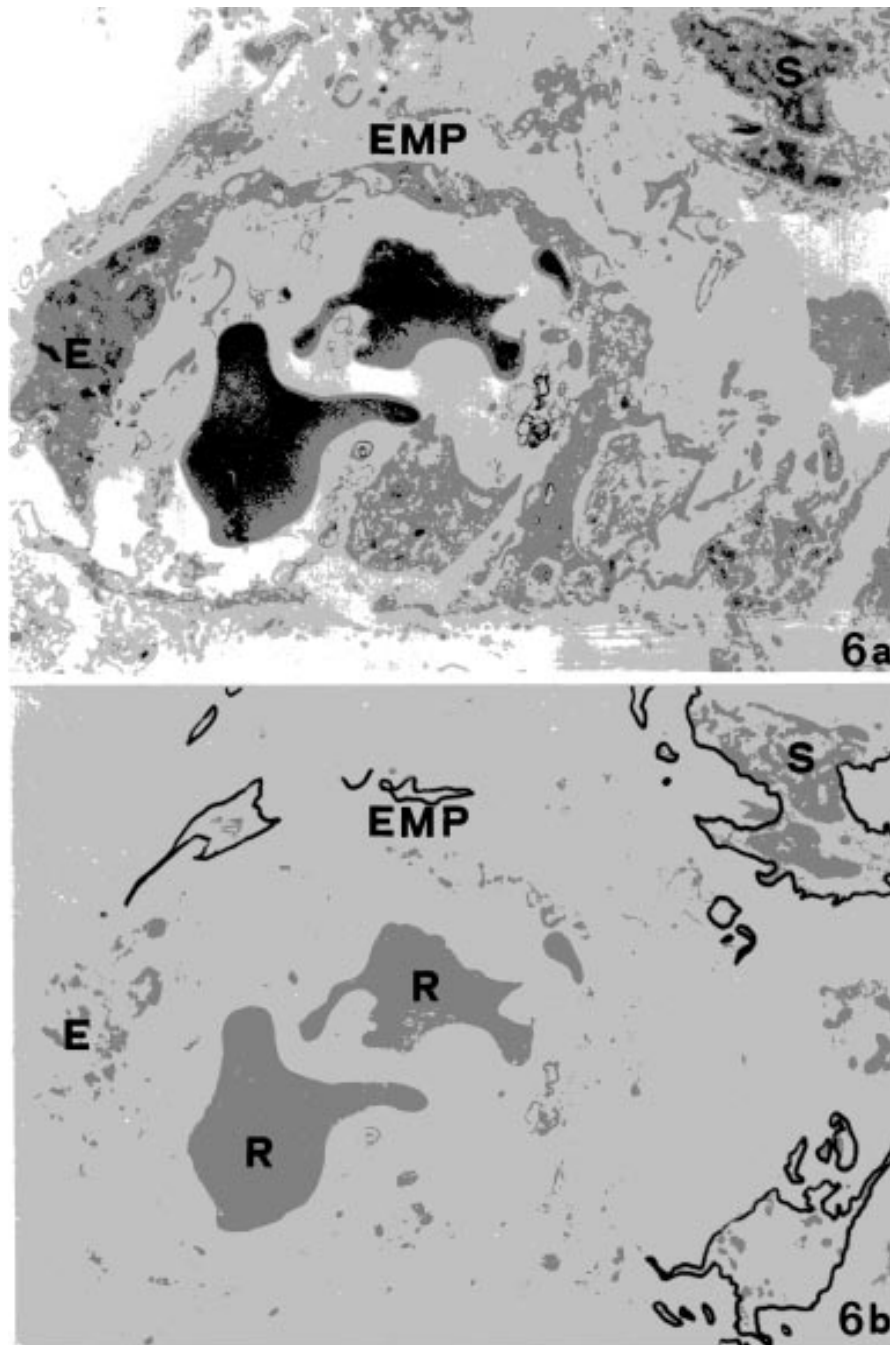


Fig. 6. a) A small bone marrow vessel, containing two reticulocytes (R) and a fragment of a phagocytic cell, surrounded by small NGFR⁺ dendrites. (Magnification, x 6700) **b)** The labeling of the stromal cell body (S) and of the numerous dendrites "branching" the vessel is shown in the drawing. It can be noticed that the space between the stromal cell dendrites and the endothelial cell (E) lining the vessel is wide and completely filled with extracellular matrix proteins (EMP). (Magnification, x 6700).

The close association between bone marrow reticular cells and adipocytes have been already described (14,18). In our observations the cytoplasmic membrane of the adipocytes is completely surrounded by thin NGFR⁺ dendrites. An important role of these cells in interconnecting adipocytic cells with the stromal network and with the vascular spaces is implied. These findings also suggest a possible regulatory function of stromal cells in bone marrow adipogenesis. Numerous observations in the literature support the notion that adipocytes may derive from progressively fat laden reticular cells (14,18). However, no labeling of adipocyte membranes has ever been observed in our samples. This finding could either suggest that the NGFR expression is lost during adipocytic differentiation or that these cells may derive from a NGFR⁻ precursor, or not necessarily from a bone marrow reticular cell.

The frequent association of NGFR⁺ stromal cells with bone marrow plasma cells has never been reported in previous ultrastructural studies. Stromal cells generally embrace plasma cells completely and make numerous contacts with them: in addition, a large number of pinocytotic vesicles are frequently present along the stromal cell membrane facing the plasma cell. NGFR labeling of these cells permitted to exclude their endothelial origin, as previously reported (7) In addition these findings may indicate some role in antibody processing by stromal cells. On the other hand NGF has been shown to induce B-cell proliferation and differentiation, while NGFRs have been described on tonsillar and peripheral blood B-cells (22) and on follicular dendritic cells (FDC) in secondary lymphoid follicles (15). The possibility that NGF may interact with NGFR on the surface of stromal cells is particularly interesting, when considering the effects of NGF on lymphoid cells and on cell survival, as recently demonstrated at least in the neuronal system (23). The expression of NGFR on stromal cells may indicate a more key role of this receptor in regulating hemopoiesis, especially on the basis of the recent demonstration that NGF induces

autophosphorylation of the TrkA proto-oncogene product and could also induce proliferation and differentiation of hemopoietic precursors in association with other hemopoietic growth factors (24,25).

Our immuno-EM study confirms the previously described (10,13,14,18) close relationship between stromal cells and bone marrow sinusoids, which were always seen surrounded by stromal cell bodies or dendrites. Nevertheless, in all our samples a wide space, filled with filamentous extracellular matrix, was seen between stromal cell dendrites and endothelial cells: this is consistent with our previous immuno-histochemical observation of an interrupted layer of collagen IV and laminin present between stromal and bone marrow endothelial cells (7). Direct contacts have never been seen by immuno-EM in contrast to what reported by Weiss, who described stromal like junctions between reticular adventitial cells and endothelium in the rat bone marrow (14). Our EM observations rather suggest a role of NGFR⁺ stromal cells in branching and interconnecting the bone marrow endothelial sinusoidal spaces with the hemopoietic tissue. On the other hand, our findings do not provide direct evidence of a regulatory function of NGFR⁺ cells on sinusoidal spaces in the bone marrow.

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