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STRUCTURAL FEATURES OF ISOLATED, FRACTIONATED BONE MARROW ENDOTHELIUM COMPARED TO SINUS ENDOTHELIUM IN SITU

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Abstract

Structural features of isolated, fractionated rat bone marrow endothelium were compared to those of marrow sinus endothelium in situ. Marrow endothelium was purified, first by density gradient sedimentation on Percoll and then subjected to centrifugal elutriation. Using antifactor VIII antibody staining (indirect immunofluorescent method), preparations of greater than 50% purified endothelium were obtained. By SEM, these cells were about 10 μm in size and showed smooth surface and numerous invaginations. These features were also observed in the in situ endothelium obtained by perfusion-fixation and freeze-cracking. In addition, in situ endothelium displayed numerous hemopoietic cells in migration through the endothelium. By TEM, isolated endothelium showed numerous vesiculations, giving the cell sponge-like appearance. This corresponded to numerous intracellular vesicles in sinus endothelium in situ, reflecting high magnitude of fluid and molecular transport across the endothelium. Weibel-Palade bodies were not seen in either form of the endothelium, despite the positive reaction for factor VIII-related antigen. This finding suggested that the cell, while possessing factor VIII -related antigen, does not store this protein.

Key Words: Bone marrow, endothelium, cell fractionation, elutriation, density gradient sedimentation, marrow sinuses, sinus endothelium, isolated endothelium, Factor VIII staining, electron microscopy.

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Introduction

Interactions involving bone marrow endothelium are critical to several aspects of hemopoiesis. Production of a colony-stimulating activity by endothelium has been documented (14) and it has been shown that the production of this activity is regulated by a factor produced by monocytes (4). However, regulatory function of endothelium is not limited to granulopoiesis. Endothelium can also regulate erythropoiesis through elaboration of a burst-promoting activity (23) as well as modulation of mixed colony formation (3).

Within the bone marrow, endothelium also operates as a regulator of cell egress (1,2,5-8, 17,18) as well as in trapping and homing of stem cell and other progenitor cells (12,20). This regulation forms the basis of the concept of bone marrow-blood barrier (18,19).

Because purified preparations of marrow endothelium have been difficult to obtain, most studies on endothelial regulation of hemopoiesis have been done with endothelial cells from other sources. These endothelial cells may actually not be representative of marrow endothelium because of considerable heterogeneity in the structure and function of endothelia. We have recently developed a method to obtain relatively purified preparations of marrow endothelial cells (10). In the present study, we compare ultrastructural features of these isolated, fractionated endothelial cells with those of marrow sinus endothelium in situ.

Materials and Methods

Sprague-Dawley rats, 200-250 g, and New Zealand white rabbits 1-2 kg were used throughout these studies.

Isolation of marrow endothelium

To obtain isolated purified marrow endothelial cells, femoral and tibial marrow tissue was removed and placed in a test tube containing 2-3 ml of buffered collagenase solution as described elsewhere (15). Marrow tissue was then incubated for 45 min. at 37°C to obtain single cell suspensions which were then centrifuged thrice at 350 g for 5 min. The cell pellet was then resuspended in phosphate-buffered saline (PBS, pH 7.4) to a final concentration of 2-4 x 10^8 cell per ml. The cells were then subjected to a discontinuous density gradient centrifugation sedimentaion, using 1.04 and

1.06 g/ml of Percoll (3 ml each). Cells were overlayered on these gradients and centrifuged at 400 g for 30 min. at 4°C. This led to the appearance of three cellular fractions. The fraction at the interface of the two gradient solutions proved to contain the majority of endothelial cells as shown by factor VIII positivity using the indirect immunofluorescence technique (9). This fraction was collected and washed thrice in PBS by centrifugation at 350 g for 5 min. and then resuspended in PBS to a final concentration of 0.2-1.2 x 10^8 cells/ml.

Five ml of this sample was then subjected to centrifugal elutriation using standard (4.2 ml) chamber and a JE-6B elutriation rotor in a J2-21 centrifuge. The rotor speed was adjusted to 613 g and the flow rate was increased stepwise from 10 to 20 ml/min. (12.5, 15 and 17.5). The suspending fluid (PBS containing 0.2% bovine serum albumin, BSA) was collected from the efferent tube into 4 fractions of 300 ml each. The cells in each fraction were concentrated by centrifugation at 550 g for 10 min. to obtain a final volume of 5 ml. As demonstrated by reactivity for factor VIII antigen, the majority of endothelial cells were observed in fractions 3 and 4, with respectively 48.6+ 11.5 and 51.2 $\pm$ 13.2 percent factor VIII-positive cells.

For electron microscopy, a drop of cell suspension was mounted on a round coverslip pretreated with poly-L-lysine and fixed in a modified Karnovsky fixative in cacodylate buffer (0.114 M, pH 7.3) for 30 min. at 4°C. The coverslip was then rinsed and postfixed with 2% cold, similarly buffered OsO_4. After dehydration, the cells were critical point dried for scanning electron microscopy (SEM) with liquid CO_2. They were then sputter coated with gold-palladium and examined. For transmission electron microscopy (TEM), dehydrated cells were embedded in Spurr resin, thin-sectioned, stained with uranyl acetate and lead citrate, and examined.

Preparation of marrow endothelium in situ

Through a midline abdominal incision, the abdominal aorta was cannulated and the tip of the cannula was placed in the right femoral artery. The perfusate was retrieved from the inferior vena cava. To prevent clotting, 500 units of heparin were injected into the vein immediately before the cannulation.

The perfusion was done at 4°C. The perfusion was started with PBS containing 0.1% BSA at the flow rate of 4 ml/min. to wash out any circulating blood cells. At least 5 min. of perfusion was necessary to obtain clear perfusate from the inferior vena cava. Perfusion was then continued with ice-cold Karnovsky modified solution containing 0.7% glutaraldehyde, 0.1% paraformaldehyde in 0.113 M cacodylate buffer (pH 7.3, 330mOsm). The adequacy of fixation was estimated by the rigidity of leg muscles. Usually the infusion of 60 ml of fixative was necessary over a period of 15 min.

The tubular bones were then removed and the bony cortex was gently taken off using a bone cutter. The marrow tissue was placed in a petri dish containing fixative and was microdissected into small blocks. These blocks were further fixed with 2.4% glutaraldehyde and 1.0% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 h and then postfixed with similarly buffered 1% OsO_4 for 1 h.

For TEM, specimens were dehydrated in a graded alcohol series and embedded in Epon 812. Thin sections of gray-silver to silver range were obtained, stained with uranyl acetate and lead citrate, and observed in an electron microscope.

For SEM, fixed dehydrated specimens were subjected to freeze-cracking according to the method of Tokunaga et al. (21) in order to open vascular spaces and to make endothelial surfaces visible. The freeze-cracked specimens were then critical point dried, sputter coated as described above and processed for SEM.

Results

By SEM, in situ endothelial cells formed a pavement with numerous ridges and grooves (Fig. 1). Intercellular junctions were only occasionally well-perceived due to the coating. The surface of cells was generally smooth, although occasionally displayed some unevenness and contained numerous endocytic pits. Migrating cells were evident in all parts of endothelial surface and consisted of both erythroid and granulocytic cells. The formers showed smooth surfaces while the latters showed microprojections (Fig. 1).

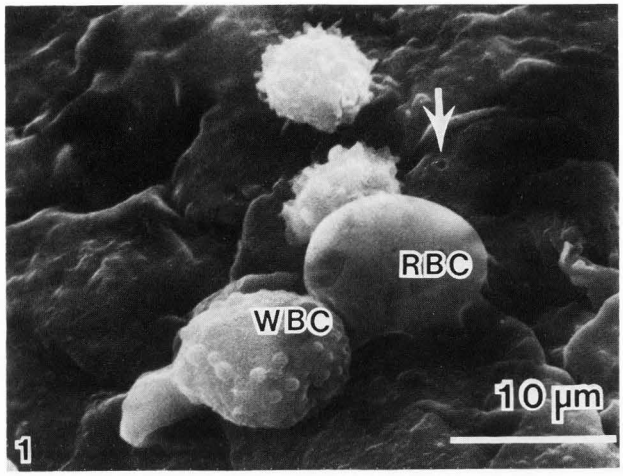
By TEM, in situ endothelium formed a continuous layer. It contained an elongated nucleus with considerable peripheral heterochromatin. Its thickness in the perinuclear region was up to 6-7 μm and this region contained most organelles including mitochrondria, ribosomes, rough endoplasmic reticulum and many vesicles (Figs. 2 and 3). The cytoplasm tapered off laterally, extending for a long distance. The thickness of the cell in this tapered region did not usually exceed 2 μm.

The cytoplasm was quite rarefied and showed little electron density. Its salient feature was the presence of numerous endocytic pits and vesicles, some bristle-coated but most uncoated. These vesicles gave the cell a sponge-like appearance. When two cells came into contact, they usually overlapped for a variable distance. Small patchy, submembranous densities were occasionally seen in these overlapping areas, but tight junctions, as defined by pentalaminar membranous structures, were not seen and the membranes of the two cells retained their usual intercellular distance.

In SEM, isolated endothelial cells were round, about 10 μm in diameter. They displayed smooth surfaces with some ridges and grooves. The salient feature, again was the presence of numerous invaginations which probably corresponded to the endocytic pits seen in TEM (Fig. 4). By TEM the

Fig. 4. SEM of an isolated marrow endothelial cell. The cell is round, displaying smooth surface with some ridges and grooves. Note the presence of numerous invaginations (arrows) that may correspond to endocytic pits.

Fig. 5. TEM of several isolated marrow endothelial cells (E). Note the rarefied cytoplasm and the nuclei with peripheral chromatin condensation usually having a nucleolus. The salient feature is numerous cytoplasmic vesicles giving the cell a sponge-like appearance. This feature corresponds to endocytic vesicles seen in situ.



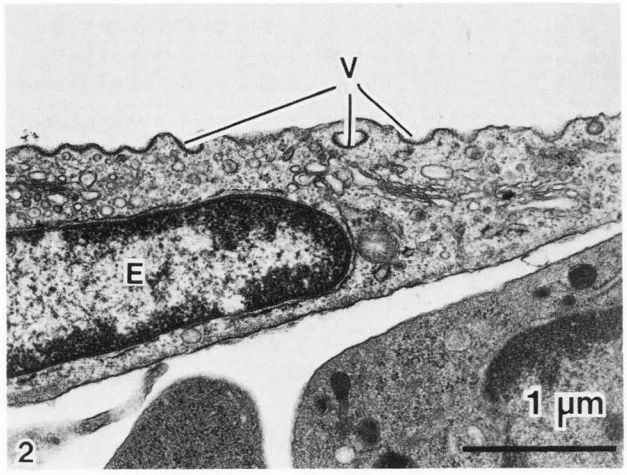
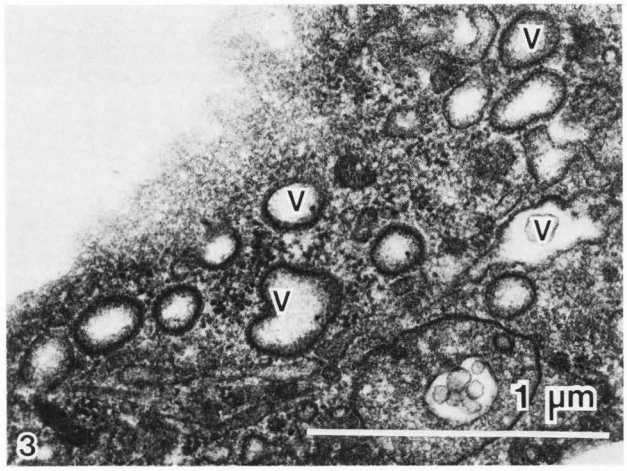
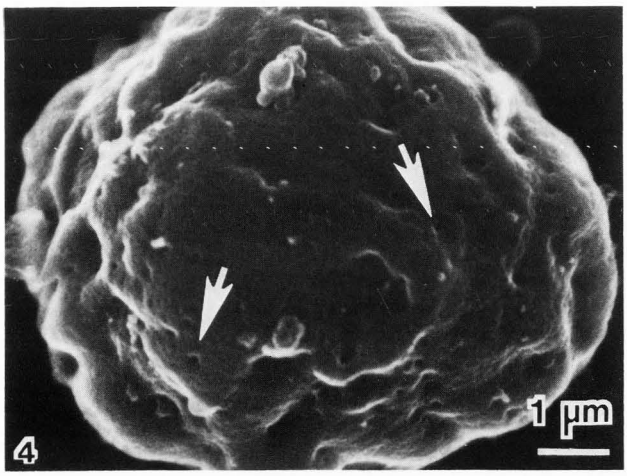


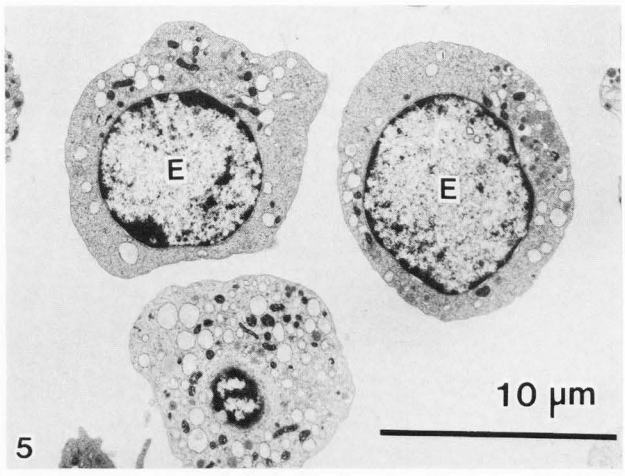
Fig. 1. SEM of in situ endothelium of bone marrow sinus viewed from the lumen. Endothelial cells form a pavement with numerous ridges and grooves. The cell surface is generally smooth, and contains invaginations (arrow). One red cell (RBC) and two other cells (WBC) in the process of migration are seen.

Fig. 2. TEM of in situ endothelium (E) of bone marrow sinus. The cell forms a continuous layer and contains an elongated nucleus with considerable peripheral heterochromatin. The cytoplasm is relatively thick in the perinuclear region which contains most cellular organelles. It tapers off laterally. The most salient organelle is endocytic pits and vesicles (V).

Fig. 3. TEM of tangentially sectioned marrow endothelium in situ. Numerous vesicles (V), mostly coated, are again the most prominent feature.







cells usually displayed rarefied cytoplasm and a relatively high cytoplasmic-nuclear ratio. The nucleus showed considerable peripheral chromatin condensation and usually a single nucleolus. Again, the salient feature was the presence of numerous cytoplasmic vacuolations, giving the cell a sponge-like appearance (Fig. 5).

Discussion

The method described herein yields relatively high purity preparations of bone marrow endothelium. These preparations can be used to study the function of marrow endothelial cells in vitro.

Since collagenase is used in the preparation of these isolated cells, the cell surface may be altered by this treatment and in studying the surface characteristics of isolated cells, this point should be kept in mind. Similar surface alterations by collagenase have been documented in isolated hepatocytes (11).

Structurally, isolated endothelial cells are round while in situ, they are elongated. This is, of course, expected since the elongated shape of endothelial cells in situ is not inherent to the cell but the result of their topography in the tissue. Similar rounding is also seen in liver endothelium which is otherwise elongated in situ (16,22). In considering the isolated form of endothelium versus the endothelium in situ, it is necessary to point out that in situ endothelium is polarized with the luminal surface having different characteristics compared to the abluminal surface (15). It is probable that this polarity is lost in the isolated form of endothelium.

Apart from the difference in shape, structural features of the cell, both in TEM and SEM, are remarkably similar in situ and in isolated forms. In SEM, both cells have smooth surfaces with invaginations that correspond to endocytic pits seen in TEM. In TEM both cells show a nucleus with dense peripheral heterochromatin, and high cytoplasmic-nuclear ratio. The cytoplasm in both situations is rarefied and displays a large number of vesicles, giving the cell a sponge-like appearance. In fact a similar sponge-like appearance has also been reported in capillary endothelial cells isolated from such other tissues as the liver (16,22).

The high degree of vesiculation, seen in these capillary endothelia, can be attributed to the fact that these endothelia are specialized in large-volume exchange of solutes (13). This exchange takes place via different vesicular transport systems, most of which are still unexplored. It is the presence of these vesicles that gives the cell a sponge-like appearance both in isolated form and in situ.

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Discussion with Reviewers

W.H. Knospe: What was the maximum concentration of endothelial cells after elutriation?
Authors: This was 51.2+ 13.2% by fluorescent staining and 89.7% by EM criteria. This occurred in fraction 4.

W.H. Knospe: Could the authors comment on the intensity of factor VIII antigen staining in these cells? Is the intensity of the reaction as heavy as those cells from the liver or the aorta? Do all cells show factor VIII antigen?
Authors: The intensity of staining in these cells is much lighter than those of the aorta, and even slightly lighter than liver endothelium. Sinal endothelia generally produce factor VIII antigen but they do not store them. This is evident from the fact that this type of endothelium lacks Weibel-Palade bodies that are thought to be storage sites of factor VIII. Consequent to low intensity of staining, there is some variation in staining in different cells. The discrepancy between the proportions of endothelial cells as judged by fluorescent staining and EM suggests that some of the cells may not stain by fluorescent staining.

T.M. Seed: The apparent lack of discrete Weibel-Palade bodies in factor VIII positive marrow endothelial cells is interesting. What type of immunofluorescent pattern was pattern was observed in these cells? Ultrastructurally, is any evidence for alternate, non WB factor VIII containing granules?
Authors: The pattern of immunofluorescence was intracellular diffuse, or finely granular pattern. We did not encounter any alternate WB bodies. The sinal endothelia are generally thought not to store factor VIII as endothelia of larger vessels do.

F. Campbell: I have difficulty seeing that the vesicles of the in situ endothelial cells and isolated endothelial cells are very much alike, especially since those of the isolated cells seem much larger. Are bristle-coated vesicles present in the isolated endothelial cells?
Authors: We have not seen them; but isolated endothelium may undergo some modifications as a result of isolation procedures. This may lead to morphological alterations. Not all features of in situ endothelium are expected to be present in isolated endothelium.

F. Campbell: If fractions 3 and 4, containing about 50% endothelium, were used to study colony-stimulating factors or other aspects of hematopoiesis, it would be important to know what other cell types are present. Are reticular cells or macrophages contained in these fractions?
Authors: They are generally mononuclear immature cells, some with lymphoid features. Their nature is not known at this time, but they are morphologically distinguishable from endothelial cells.

Reviewer V: What is the evidence that the structure shown in Figure 4 is an endothelial cell? Cannot other bone marrow cell types show invaginations or use endocytotic pits for internalization of materials?
Authors: Positive identification of cells in all these purification steps were made, not on the basis of electron microscopy, but on the basis of progressive increase in the proportion of positivity for factor VIII antigen using immunofluorescent technique, during various purification steps. As mentioned in the text this proportion rose gradually from less than 1% to 51.2%. Concordantly, the proportion of cells with this type of morphology rose from 3.9% to 89.7%. Clearly, if we increase the proportion of one parameter concordantly with the others, the two parameters must have certain relationship to each others, particularly since this morphology has been ascribed to other capillary endothelia.

Of course, many types of cells in the bone marrow show invaginations and endocytosis. Any cell that has contact with the outside does internalize materials. But the features described here, i.e. smooth surface with invagination in SEM and sponge-like appearance in TEM for the cell of this size has only been described for endothelial cells of capillary type in several other tissues and there is no reason to believe that marrow capillary endothelium should be any different.

Reviewer V: What features of the cells shown in Figure 5 at low magnification identify these positively as endothelial cells? It is not uncommon for cells to show vacuoles in suspension.
Authors: The feature that positively identifies all these cells as endothelial cells is their factor VIII positivity. Cells commonly show vacuoles in suspensions or otherwise. But only certain cell types show positivity for factor VIII antigen.