Scanning Electron Microscopy

Volume 1986 | Number 2 Article 32

6-11-1986

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SCANNING ELECTRON MICROSCOPY/1986/II (Pages 621-629)
SEM Inc., AMF O'Hare (Chicago), IL 60666-0507 USA

0586-5581/86$1.00+05

ULTRASTRUCTURAL STUDIES OF INTERCELLULAR CONTACTS (JUNCTIONS) IN BONE MARROW. A REVIEW

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(Received for publication March 03, 1986: revised paper received June 11, 1986)

Abstract

This paper reviews ultrastructural studies of the intercellular contacts or junctions between cells of the bone marrow. Studies using tannic acid and glutaraldehyde as a fixative have shown pentalaminar complexes between many types of cells in marrow of mice and chicks. These intercellular contacts occur between adjacent stromal cells, between stromal cells and developing blood cells and, in marrow of mice, between migrating blood cells and cells of the sinusoidal wall. Because of their location and widespread occurrence, it is believed these contacts may represent a type of adherent junction helping to maintain an orderly arrangement of blood cells and stromal cells in the marrow. Migrating blood cells may use these contacts as anchoring sites during locomotion toward the sinusoids and in crossing the sinusoidal wall. On the other hand, since these junctions resemble gap junctions of other tissues, one should not exclude the possibility that they are involved in cellular communication. Freeze-fracture and lanthanum impregnation studies have failed to demonstrate these junctions in marrow. Studies using ruthenium red have shown apparent sites of attachment between cells of the marrow, but it is not known whether these sites correspond to the intercellular contacts seen in tannic acid preparations.

Key Words Bone marrow, intercellular contacts, intercellular junctions, ultrastructure, stromal cells, cellular migration.

Introduction

Hematopoiesis in bone marrow is a persistent process of cellular proliferation and differentiation in which cellular associations and interactions appear to play important roles (Wolf and Trentin, 1968; Tavassoli, 1975; Cline and Golde, 1979; Bentley, 1981, 1982; Dexter, 1982). In bone marrow of mammals the developing blood cells lie among stromal cells of the extravascular compartment. These stromal cells have numerous thin processes, shown particularly well by scanning electron microscopy (Weiss and Chen, 1975; Weiss, 1976), that extend among the immature blood cells. On the basis of fine structure (Berman, 1967; Weiss, 1976) and ultrastructural histochemistry (Weston and Bainton, 1979), the stromal cells appear to consist of at least two types, macrophages and reticular cells. Macrophages have close associations with cells of the erythroid series forming the erythroblastic islands described in the literature (Bessis, 1958; Berman, 1967). The reticular cells may also lie close to erythroid cells, but characteristically form close associations with cells of the granulocytic series (Weiss, 1976; Weston and Bainton, 1979). Recently, dark branched stromal cells have been shown to lie in close juxtaposition to developing blood cells in marrow during accelerated eosinophilopoiesis (Sakai et al., 1981), and during increased erythropoiesis (Brookoff et al., 1982). The reticular cells, in addition to forming an integral part of the stroma, partly cover the abluminal surface of endothelial cells of the sinusoidal wall, where they are referred to as adventitial cells (Weiss, 1965; Weiss, 1970). The adventitial covering of the endothelial cells is decreased during increased blood cell delivery to the sinusoids suggesting that adventitial cells play a role in modulating this process (Weiss, 1965; Weiss, 1970; Chamberlain et al., 1975; Leblond et al., 1975). As the blood cells mature, they migrate toward the sinusoids, come into contact with cells of the sinusoidal wall, and pass through temporary openings in the endothelial cells (DeBruyn et al., 1971; Campbell, 1972; Leblond

et al., 1975). This transcellular route of migration, thought to involve interaction between migrating blood cells and endothelial cells, may be of importance in determining which cells shall enter the sinusoids (DeBruyn et al, 1971; Campbell, 1972).

While it is apparent that cellular associations and interactions are important in normal bone marrow, few studies have presented evidence of cellular junctions or membrane modifications suggesting interaction or attachment between stromal cells and blood cells, or between blood cells and cells of the sinusoidal wall. This review will discuss studies on marrow of mammals and birds by several investigators, including the author, designed to demonstrate possible sites of cellular interaction or attachment between developing blood cells and stromal cells. Studies showing possible sites of attachment or interaction between migrating blood cells and cells of the sinusoidal wall will also be reviewed and discussed. Previously unpublished ultrastructural observations by the author using ruthenium red to study bone marrow of mice, and tannic acid fixation to study bone marrow of chickens, are reported and discussed.

In the following sections material will be presented according to the electron microscopic techniques used. Conclusions based on re-examination of published studies and new material presented here will follow.

Lanthanum and Freeze-Fracture

Since its introduction by Revel and Karnovsky (1967), lanthanum has been a useful tracer for studying intercellular spaces, since it readily penetrates these spaces, is usually excluded from cells, and is electron dense. Lanthanum impregnation of tissues is also useful in identifying various types of intercellular junctions (Goodenough and Revel, 1970; Friend and Gilula, 1972). Techniques employing lanthanum as a tracer would therefore seem well suited for the study of cellular associations in bone marrow.

Freeze-fracture, another widely used technique for studying cellular junctions, involves the fracture of frozen cells and subsequent examination by electron microscopy of replicas of the fractured surfaces. This technique has also been used to demonstrate different types of junctions in many tissues (e.g., Friend and Gilula, 1972; Simionescu et al., 1975). Although bone marrow would appear to be difficult to study with freeze-fracture because of the large number of cell types present and their complex relationships, it has promise for the identification of cellular junctions or other membrane modifications between cells of bone marrow.

Tavassoli and Shaklai (1979) and Shaklai and Tavassoli (1979) have used lanthanum impregnation and freeze-fracture to examine bone marrow of rats following fixation by various procedures. Using a modified procedure for precipitating lanthanum in the intercellular space, Shaklai and Tavassoli (1977) have found this technique to be especially good for identifying thin processes of stromal cells that could be overlooked in standard electron microscopic studies. They occasionally saw small desmosome-like junctions between stromal cells and immature blood cells, but no tight, gap or septate junctions were identified. They did not report on blood cells in transit across the sinusoidal wall. By freeze-fracture they also demonstrated small desmosome-like junctions, possibly zonulae or fasciae adherentes, but again failed to find tight, gap or septate junctions. Freeze-fracture micrographs of bone marrow have also been published by Weiss (1976). These show such features of marrow as the basal lamina, stromal cell processes and the close association of various cell types, but the report mentions no findings of possible intercellular junctions. To date then, lanthanum impregnation and freeze-fracture have shown a few small desmosome-like junctions but have not presented evidence for other types of junctions between the cells of the bone marrow.

Tannic Acid

Fixation with glutaraldehyde in the presence of tannic acid enhances the staining properties of plasmalemmas and clearly reveals their trilaminar structure (Rodewald and Karnovsky, 1975; Wagner, 1976). Tannic acid is a mordant for heavy metal stains that gives increased contrast to membranes and certain extracellular materials (Simionescu and Simionescu, 1976; Sannes et al., 1978). Tannic acid enhances staining of adherent junctions but is excluded from occludent junctions. Tannic acid penetrates gap junctions binding to the membranes and filling the "gap" so that subsequent staining shows a characteristic pentalaminar complex (Van Deurs, 1975). For these reasons, tannic acid has been used to study intercellular junctions in several tissues (Van Deurs, 1975; Kalderon et al., 1977; Gilula et al., 1978).

With many of the considerations described above in mind, the author examined bone marrow of mice fixed with glutaraldehyde and tannic acid (see Campbell, 1980). After perfusion in situ with fixative diluted 1:1 with buffer, marrow was removed, cut into blocks, and fixed an additional hour in full strength fixative (4% glutaraldehyde, 4% tannic acid, 0.1 M phosphate). After fixation the marrow blocks were processed and embedded by standard methods. The ultrastructural features of marrow are largely unchanged by the added tannic acid so that identification of various marrow cells was not difficult. However, the cellular membranes are intensely stained. When the marrow cells are examined, regions are seen where the membranes of contiguous cells come together and occlude the intercellular space (arrows, fig. 1). At high magnification the plasmalemmas in these regions can be seen to fuse to form a pentalaminar complex consisting

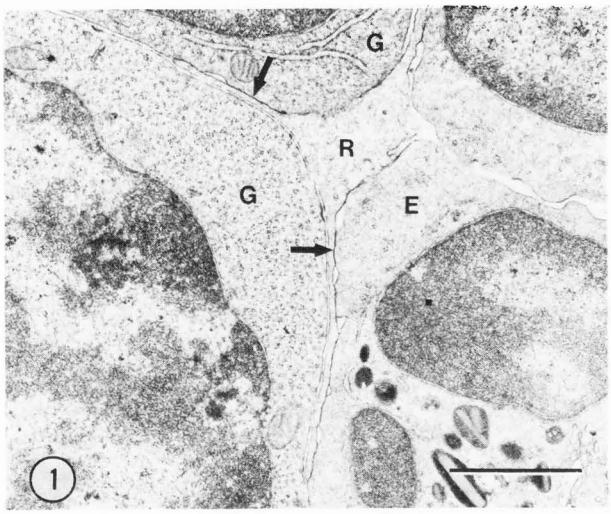


Figure 1. A process of a reticular cell (R) lies among several immature granulocytes (G), one of which is an eosinophil (E). Intercellular contacts between reticular cell and blood cells are shown at arrows. Mouse bone marrow fixed with tannic acid and glutaraldehyde. Stained with uranium and lead. Bar equals 1 µm

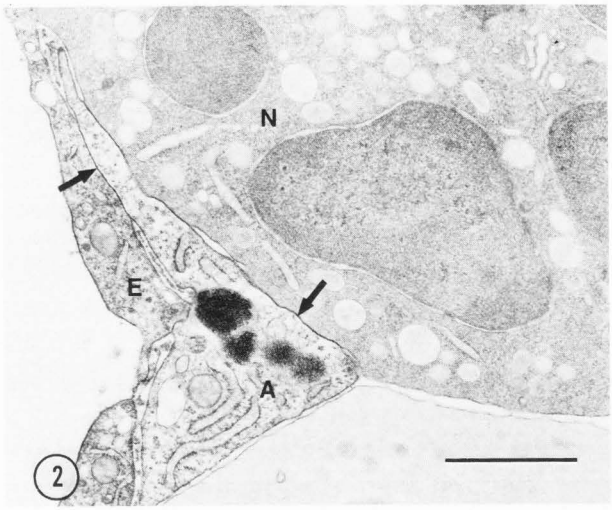


Figure 2. A neutrophil (N) lies adjacent to an adventitial cell (A) and endothelial cell (E) of the sinusoidal wall. The adventitial cell has intercellular contacts with the endothelial cell and neutrophil (arrows). Mouse bone marrow. Fixation and staining as in figure 1. Bar equals 1 µm.

of two dense inner leaflets, two intermediate electron lucent regions and a single thick layer continuous with both outer leaflets. For details of membranes at these sites see figures 3 and 6 (insets). These structures resemble gap junctions seen in other tissues fixed with glutaraldehyde and tannic acid (Van Deurs, 1975), but here they will be referred to as intercellular contacts, because corroborative evidence that they are gap junctions is lacking.

Macrophages typically have many intercellular contacts with erythroid cells, reticular cells less frequently. However, it is sometimes difficult to identify a thin process as belonging to a macrophage or reticular cell. Reticular cells, as illustrated in figure 1, frequently have intercellular contacts with granulocytes, but so do macrophages. In the author's experience processes of macrophages are more commonly associated with granulocytic cells than would be expected from the studies of Weston and Bainton (1979). Numerous intercellular contacts are observed between processes of adjacent reticular cells, between processes of reticular cells and macrophages (see Campbell, 1980), and between adventitial cells and endothelial cells of the sinusoidal wall (fig. 2).

Nearly mature blood cells lying close to the sinusoidal wall frequently have intercellular contacts with adventitial cells (fig. 2, also see Campbell 1982); some of these cells have profiles that suggest they were fixed while migrating towards the endothelium. Some blood cells close to the endothelium have intercellular contacts with the endothelial cells and a few have processes that invaginate endothelial cells (fig. 3). Intercellular contacts may occur at several locations between these cells and adventitial or endothelial cells including the point of invagination (fig. 3) On occasion, blood cells are fixed while migrating through a pore in the endothelium. These cells typically have intercellular contacts with the endothelial cell at sites near the pore (fig. 4) and around at least part of the margin of the pore (Campbell, 1982). Blood cells of the sinusoidal lumen were largely removed by the fixation but of those remaining, none were noted to have intercellular contacts with endothelial cells.

Bone marrow of birds differs from that of mammals in that the erythroid cells lie within the sinusoidal lumina while the immature granulocytic cells lie in the extravascular compartment (Venzlaff, 1911; Jordan and Johnson, 1935). Electron microscopic studies (Campbell, 1967) have shown that the immature erythroid cells abut on the sinusoidal endothelium suggesting that they are attached to the endothelial cells. Also, granulocytic cells migrating across the endothelium to enter the sinusoids tightly fill the pores they pass through. Sorrell and Weiss (1982) studied the ultrastructure of bone marrow of the embryonic chicken following fixation with tannic acid and glutaraldehyde. They found pentalaminar intercellular junctions in the marrow that appear to be identical to those observed in the bone marrow of mice. In the intravascular compartment intercellular contacts, referred to as intercellular junctions by Sorrell and Weiss (1982), were seen between erythroblasts (including presumptive stem cells) and endothelial cells, and between adjacent

erythroblasts. In the extravascular compartment intercellular contacts were noted between contiguous reticular cells, and between reticular cells, presumptive stem cells and mast cells. No evidence was presented for intercellular contacts connecting immature or mature granulocytic cells and other cell types.

The author has also examined bone marrow of chickens following preservation with tannic acid and glutaraldehyde. While Sorrell and Weiss (1982) studied bone marrow from 15-day chick embryos, I examined marrow from 2-to 3-week-old chicks. My findings agree with those of Sorrell and Weiss (1982) concerning intercellular contacts between erythroid cells and between erythroid cells and endothelial cells (arrow, fig. 5). However, I have also seen many intercellular contacts between granulocytic cells and endothelial cells (fig. 6), between adjacent granulocytic cells, and between granulocytic cells and macrophages of the extravascular space. Intercellular contacts were also seen between contiguous stromal cells of the extravascular space as reported by Sorrell and Weiss (1982). Unfortunately, from the point of view of the present review, granulocytic cells in the process of penetrating or migrating across the endothelium have not been encountered.

Ruthenium Red

Ruthenium red has been widely used in animal tissues since Luft (1971) showed that this substance penetrates the intercellular space and binds to the surface of cells and to many intercellular materials. Ruthenium red is believed to bind to cell surface acid polysaccharides (Luft, 1971; Diericks, 1979) and when treated with osmium tetroxide forms an electron dense layer on the surface of cells. Luft demonstrated (1971) that ruthenium red readily stains desmosomes but is prevented from entering occludent junctions. Problems with ruthenium red are that it cannot be perfused through the vascular system and it penetrates tissue blocks only superficially (Luft, 1971). Nonetheless, it can be a useful stain of cell surface materials.

Sorrell and Weiss (1980) used ruthenium red staining in their ultrastructural studies of the bone marrow of chick embryos. They showed apparent attachments of erythroblasts to endothelial cells, of granulocytes to each other, and of granulocytic cells to processes of reticular cells. They observed a thinning of the surface material stained by ruthenium red at these sites.

I have used ruthenium red to study bone marrow of mice following fixation by vascular perfusion. Ruthenium red stains the surface of marrow cells well near the block edge, but seems to penetrate best into regions where the intercellular space is rather wide, probably due to shrinkage during fixation. In these regions (fig. 7), numerous sites can be seen where the stained surfaces of adjacent cells are in contact (arrows). Interestingly, ruthenium red has penetrated the stromal cells and stained them darkly. In many sites the stained surface of stromal cells and the surface of blood cells are in contact with each other. Ruthenium red penetrates marrow blocks more deeply through the sinusoids when they are empty as a result of perfusion. In this case it is possible to examine regions of endothelium where ruthenium red has been presented to the luminal surface. When one is fortunate enough to encounter a blood cell migrating across the endothelium in such a location, it is apparent that the ruthenium red has stained the luminal portion of the migrating cell intensely (fig. 8) but it has been largely excluded from the region of close membrane apposition between the migrating blood cell and the endothelial cell, suggesting the presence of an intercellular junction around the edge of the pore.

Figure 3. A neutrophil (N) has a process (P) that invaginates the endothelial cell (E). An intercellular contact is shown at the arrow. Fixation and staining as in figure 1. Bar equals 1 μm. Inset shows detail of the membranes at the intercellular contact. Bar equals 0.1 μm.

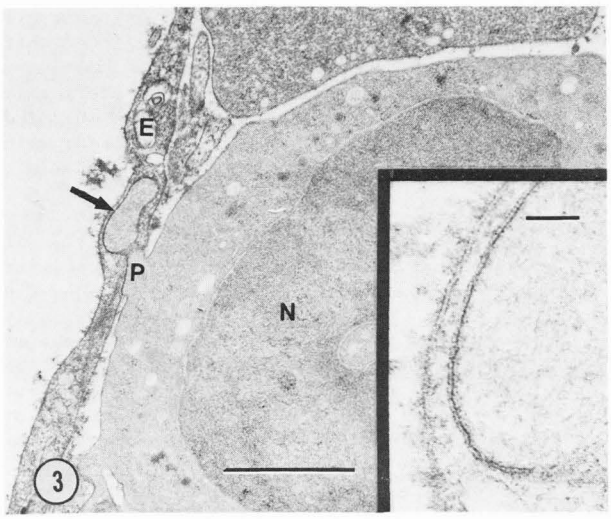
Figure 4. A blood cell, probably a monocyte, is passing through an opening in the endothelium (E) to enter the lumen of a sinusoid (S). Note intercellular contacts between the blood cell and endothelial cell (arrows). Mouse bone marrow. Fixation and staining as in figure 1. Bar equal 1 μm.

Figure 5. An endothelial cell (E) lies between an erythroblast (ER) of the sinusoidal lumen and granulocytes (G) lying in the extravascular space. An intercellular contact between the erythroblast and endothelial cell is shown at the arrow. Chicken bone marrow fixed in 4% glutaraldehyde, 4% tannic acid, 0.1 M phosphate. Stained with uranium and lead. Bar equals 1 μm.

Figure 6. A granulocyte (G) lies adjacent to an endothelial cell (E) of the sinusoidal wall. An intercellular contact is shown at the arrow. Chicken bone marrow. Fixation and staining as in figure 5. Bar equals 0.1 μm.

Figure 7. Bone marrow of mouse impregnated with ruthenium red according to the procedure of Luft (1971). A stromal cell (S), partially filled with ruthenium red, and several blood cells (B) are labeled. Arrows show sites of possible attachment between cells. Bar equals 1 μm.

Figure 8. Bone marrow of mouse impregnated with ruthenium red as in figure 7. A monocyte has a process (P) passing through a pore in the endothelium (E). Ruthenium red (R) has stained the luminal surface of the blood cell and endothelial cell but is largely excluded from the pore. Bar equals 1 μm.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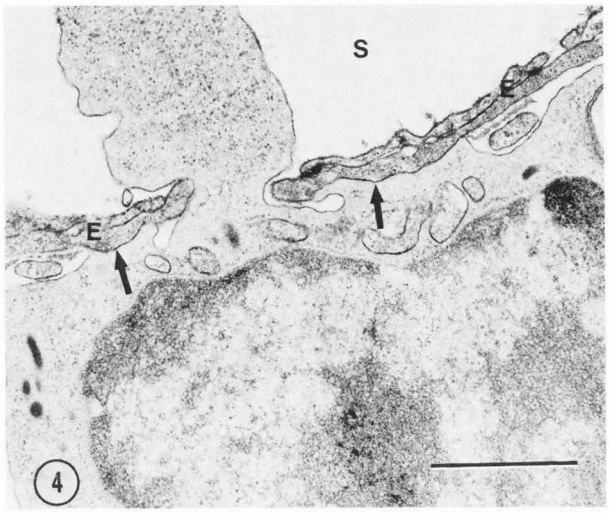
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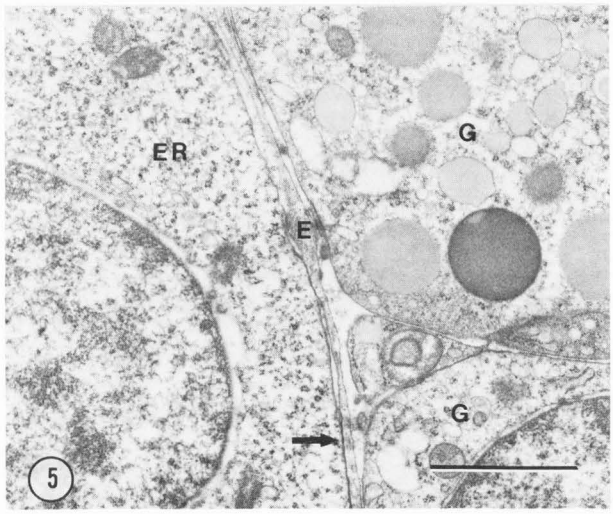
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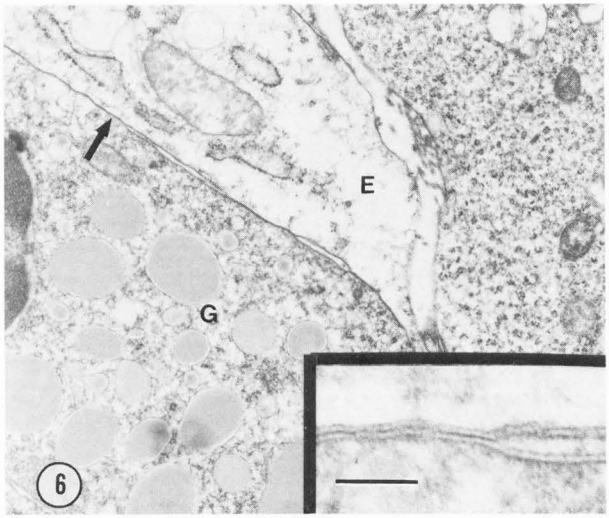
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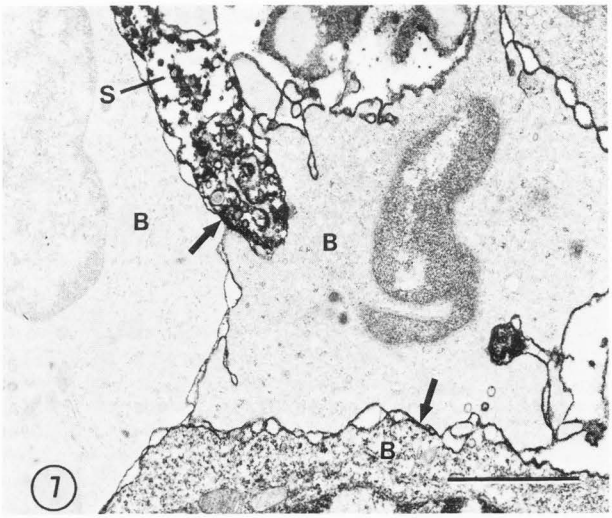
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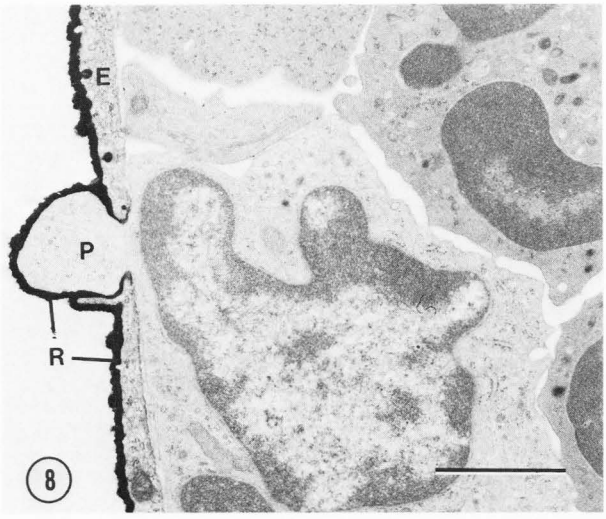
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The ruthenium red studies have shown many sites where marrow cells are in contact with each other, but it is not known whether the sites of apparent attachment seen with ruthenium red correspond to the intercellular contacts observed in the tannic acid studies.

Conclusions

There is a great deal of evidence, primarily from in vitro studies, that stromal cells provide the microenvironment necessary for normal hematopoiesis (see Dexter, 1982; Bentley, 1982). The stromal cells also produce substances that may play important regulatory roles as well (Shadduck et al., 1983; Gallagher et al., 1983; Zipori et al. 1985). The physical relations between stromal and developing blood cells are well-documented through numerous electron microscopic studies (see Introduction), but the mechanisms by which they interact are poorly understood.

In many tissues intercellular junctions, likely sites of cellular interaction, can be seen by standard electron microscopic techniques; their ultrastructure has been further elucidated by freeze-fracture and intercellular tracer studies (reviewed by Staehelin, 1974). In bone marrow, intercellular junctions have not been seen using standard electron microscopic techniques, and studies using other techniques have presented conflicting findings. Intercellular contacts or junctions, resembling gap junctions are seen when bone marrow is fixed in the presence of tannic acid, but other than a few small desmosomes, have not been observed when other procedures are used. A satisfactory explanation for these conflicting observations is not apparent.

Investigators who work with bone marrow soon realize how difficult it can be to obtain good preservation of marrow cells. The route of administration of fixative, the method of opening the bone, and many other factors can affect bone marrow ultrastructure. How much this contributes to conflicting reports is hard to tell, but should always be borne in mind.

It would seem unlikely that the intercellular contacts seen with tannic acid are artifacts due to the tannic acid. The presence of tannic acid during fixation preserves complex carbohydrates in cartilage (Takagi et al., 1983) and improves the preservation of some membrane proteins (Fujikawa, 1983). Also, the studies of Mizuhira et al. (1981) have shown that addition of tannic acid to glutaraldehyde fixatives precipitates and thus should improve *in situ* preservation of several neutral and basic proteins, and glycoproteins. All these studies indicate that tannic acid should improve preservation of membrane-associated proteins and glycoproteins and therefore should improve membrane ultrastructure. It seems likely then that the intercellular contacts observed in studies using tannic acid are not artifactual but represent structures that are not preserved with other techniques. Sorrell and Weiss (1982) have previously suggested that these intercellular contacts may be very labile, an idea consistent with the mobile nature of marrow blood cells where rigid intercellular junctions could interfere with normal hematopoiesis (Tavassoli and Shaklai, 1979).

Consideration of the location of the intercellular contacts in bone marrow of chickens and mice suggests a role in cellular adherence. In bone marrow of chickens, the intrasinusoidal erythroblasts are apparently anchored to each other and to the endothelium, thus preventing their premature entry into the circulation. In bone marrow of mice, adherent junctions may interconnect the processes of stromal cells, thus providing a stable meshwork to contain the blood cells. Likewise, attachment of developing blood cells to stromal cells could be important in assuring the orderly maturation of the blood cells. One of the strongest suggestions that these contacts are important in cell-to-cell adherence comes from their association with migrating blood cells of the marrow. It is generally accepted that blood cells require a substrate upon which to migrate. In the case of bone marrow, the stromal cells and cells of the sinusoidal wall are the likely substrate. Blood cells that appear to be migrating toward the endothelium often have intercellular contacts with adventitial cells. Intercellular contacts also occur between nearly mature blood cells and endothelial cells. Blood cells that are penetrating the endothelium may have intercellular contacts with endothelial cells at several sites, including the site of penetration. Intercellular contacts occur between blood cells that occupy pores in the endothelium and endothelial cells. All these observations are consistent with the intercellular contacts as points of attachment used by migrating blood cells. Similarly, the ruthenium red studies presented here show apparent attachment of migrating blood cells to surrounding endothelial cells. Evidence that identical contacts are involved in blood cells migration across endothelium elsewhere has been presented in studies of lymphocyte migration across high-endothelial venules of lymph nodes (Campbell, 1983).

The view that these intercellular contacts are sites of cellular adhesion is supported by the studies of Skaer et al.(1979). In platelet aggregates fixed with glutaraldehyde and tannic acid, they observed intercellular contacts having a pentalaminar structure identical to that of those discussed here. Freeze-fracture studies of these platelet aggregates failed to demonstrate gap junctions, leading these authors to refer to these contacts as "gap contacts." In the first 5 to 15 seconds of platelet aggregation, these contacts are the only ones seen, although other types are noted later. These authors regard these "gap contacts" as sites of adhesion between adjacent platelets. The similarity to the contacts

associated with migrating blood cells and other cells of the marrow is obvious. Finally, if the intercellular contacts associated with migrating blood cells are involved in locomotion, they must assemble rapidly along the leading edge of migrating cells and disassemble rapidly as the cell progresses. The studies of Skaer et al. (1979) suggest that these contacts may form very rapidly.

On purely morphological grounds, the intercellular contacts described here would appear to be typical gap junctions, resembling as they do gap junctions described by others following fixation with tannic acid (Van Deurs, 1975; Gilula et al, 1978). The occurrence of gap junctions in bone marrow would not be surprising in view of their widespread occurrence between differentiating cells of other systems (Gabbiani et al., 1978; Kelley and Fallon, 1978; Yee and Revel, 1978). However, since freeze-fracture has not shown the aggregates of particles characteristic of other gap junctions, it seems unwarranted to call them gap junctions. Nicholson et al. (1983) have reported that gap junctions from different tissues have different proteins and suggest that gap junctions may be a heterogeneous group of junctions varying from tissue to tissue. Whether the intercellular contacts of bone marrow may then prove to represent a type of gap junction is still speculation. Neither the possibility that the intercellular junctions of bone marrow play a role in intercellular communication or in intercellular adherence, or both, can be excluded yet.

In summary, the location of the intercellular contacts in bone marrow suggests a role in maintaining appropriate cellular interrelationships for hematopoiesis and in forming attachment sites for locomotion of blood cells across the sinusoidal wall.

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Discussion with Reviewers

Reviewer I: Does the number of intercellular contacts change as the developing blood cells in the marrow become more mature?

Author: I don't know. Such an analysis would be very difficult because of differences in the quality of preservation throughout the marrow and because a nearly perpendicular plane of section is needed to resolve the membranes at an intercellular contact.

Reviewer I: Since the function of intercellular contacts between cells in the bone marrow appears to differ from that of contacts between endothelial cells and blood cells migrating through them, could the structural and functional differences of these intercellular contacts be related?

Author: I don't agree that a different function may necessarily be subserved by these contacts at different sites within the marrow. In both instances the contacts appear to be related to cellular adherence. However, the function(s) of these contacts is unknown.

S.A. Bentley: Recent work from our laboratory (Bentley and Tralka, Experimental Hematology, 11:129-138, 1983) indicates the presence of fibronectin at sites of interaction between stromal cells and granulocytic precursors in long term bone marrow culture. How might this type of interaction relate to the junctions you have described using tannic acid and glutaraldehyde fixation of intact bone marrow preparations?

Author: Fibronectin is, of course, a substance that may be present at the intercellular contacts, but to my knowledge no histochemical findings have shown its presence there. Chen and Singer (J. Cell Biol., 95:205-222, 1982) have demonstrated fibronectin associated with "close contacts" (30-50 nm) and "intercellular matrix contacts" (>100 nm) of cultured fibroblasts, but these contacts seem quite different from the intercellular contacts described here. The fibronectin labeled in your studies is not associated with a narrowing of the intercellular space and thus would appear to be related more closely to the "intercellular matrix contacts" described by Chen and Singer.