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Interdigitative Coupling of Presumptive Hematopoietic Stem Cells to Macrophages in Endocloned Marrow Colonies

By Richard H. Lambertsen

Hematopoietic marrow colonies were studied ultrastructurally to investigate stromal cell–hematopoietic cell interactions during early hematopoiesis. An elaborate interdigitative coupling of mature marrow macrophages to morphologically undifferentiated colony cells is described. This coupling was found in undifferentiated and granulocytic colonies and established a physical linkage of the macrophage and undifferentiated cell. The spaces between the coupled cells contained an electron-dense material and appeared to be modified by the uptake or release of cytoplasmic vesicles of the macrophage. Based on available evidence that hematopoietic stem cell proliferation is controlled at a local, or stromal, level, and the finding that this interaction occurs in very early hematopoietic colonies, it is suggested that interdigitative couplings represent a mechanism of stem cell regulation. In addition, the observations indicate macrophages to be actively involved with the lodgement of morphologically undifferentiated hematopoietic cells in the marrow space.

ALTHOUGH IT IS GENERALLY AGREED that the later stages of differentiation and proliferation of hematopoietic cells are controlled largely by systemic factors,[1] much evidence suggests that the pluripotent hematopoietic stem cell pools are regulated at a local or "microenvironmental" level.[2-7] If isolated from peripheral influence, a local control of stem cell populations would provide a mechanism to prevent exhaustion of central stem cell reserves in the face of transient pathologic fluctuations in the blood. It has been suggested to operate in (1) the lodgement of pluripotent stem cells in the extravascular hematopoietic space from the circulation,[6] (2) the proliferation (self-replication) of stem cells in the hematopoietic space,[4,8] and (3) in the commitment (loss of pluripotency) of stem cells to differentiation.[5] Convincing evidence for a local, stromal control of stem cell proliferation has been presented by Croizat et al.[2] and Gidali and Lajtha[4]; in experiments on partial body irradiated mice, both groups showed that local stimulation of pluripotent stem cell (CFU-S) proliferation by local irradiation does not result in parallel, systemic changes in stem cell activity.

Accordingly, several workers have suggested that direct cell-to-cell interactions may be involved in the control and maintenance of hematopoietic stem cell pools.[8-10] In this study, I employed a hematopoietic colony-forming technique in an attempt to identify such interactions. Having previously found that endocloned hematopoietic colony formation is characterized by an initial prevalance of colonies of morphologically undifferentiated cells, and thereafter by a gradual increase in differentiated colony types,[11] I used the endocloning technique to investigate the ultrastructure of early hematopoiesis. As part of an analysis of the types of interactions and associations seen in early marrow colonies, this article reports the finding of an interdigitative coupling of presumptive hematopoietic stem cells to mature marrow macrophages.

MATERIALS AND METHODS

Thirty-two 1–2-mo-old C576J/Bl female mice (Jackson Laboratory, Bar Harbor, ME) were used; these were maintained on standard laboratory rodent feed (RMH-3000, Agway) ad libitum. Drinking water was acidified after irradiation to suppress intestinal Pseudomonas infection.

An alternate fraction whole body x-irradiation (850 R; 1.5 mm Cu half-value layer; 97.5 R/min) was used to produce hematopoietic marrow colonies.[11] Restrained mice were administered 850 R of x-rays by collimated beam to the entire body, except for the left hind leg, which was held outside the primary beam and shielded by a 4-mm thick lead collar fabricated from rolled 2-mm lead sheet. Ten hours later, mice were again restrained and administered 850 R by collimated beam to just the previously shielded left leg, with a 4-mm thick lead shield placed over the body. Using this technique, colony-forming units-marrow (CFU-M) emigrate from the initially shielded leg during the 10-hr interirradiation interval to seed into the radioablated marrow of the rest of the body. There they produce small, but detectable, colonies by 3 days postirradiation (PI).[11]

Femoral marrow tissue for electron microscopy of hematopoietic colonies was collected at 4, 5, 6, and 8 days PI from the initially unshielded right leg. Eight animals were killed at each PI interval by cervical dislocation following ether anesthesia. The right femur was immediately removed, immersed in ice-cold paraformaldehyde-glutaraldehyde fixative,[12] and split lengthwise with a razor blade to enhance fixative penetration. After 1 hr of cold fixation, the femur with marrow was cut into 1 cu mm pieces and fixed for an additional 2 hr at room temperature. These were washed in 0.1 *M* cacodylate buffer (pH 7.4) and decalcified overnight in 10% EDTA. After a second wash in buffer, the tissue was postfixed in 1.1% aqueous osmium tetroxide, dehydrated through graded acetone, and embedded in Epon-araldite (Polysciences, Warrington, PA). Three to eight tissue blocks were examined from each animal. One-micron thick

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sections, stained with 1.5% aqueous toluidine blue, permitted the light microscopic localization of hematopoietic colonies. Blocks were trimmed accordingly, and thin sections were made with a diamond knife on an MT-2 Porter-Blum ultramicrotome. Sections were mounted on copper grids and stained with saturated aqueous uranyl acetate and lead citrate[13] for examination at 80 Kvp under a Seimens Elmskop 1A electron microscope. Using the same methods, ultrastructural study of the aplastic uncolonized regions was also carried out.

RESULTS

"Coupling" of hematopoietic cells to macrophages, as used here, describes an active cellular association that physically links these two cell types. Its defining characteristic was an interdigitation of hematopoietic cell and macrophage cytoplasmic processes. Such coupling was observed only between morphologically undifferentiated colony cells and mature marcophages within undifferentiated and granulocytic colonies. Although a close association between macrophages and hematopoietic cells was also characteristic of erythrocytic colonies, interdigitative coupling of erythrocytic colony cells to macrophages was not observed. In undifferentiated and granulocytic colonies, such couplings occurred infrequently.

The hematopoietic cells found coupled to macrophages were small, approximately 5 μ in diameter. They had an irregularly round nucleus that was euchromatic centrally and heterochromatic adjacent to the nucleolemma. Nuclear pores were conspicuous. The cytoplasm was scant and unspecialized, although centrioles and a poorly developed Golgi apparatus were sometimes evident. The plasmalemma had a variable configuration (see below) and, at times, was evaginated from the cell surface into narrow microvilli. I have termed these cells "presumptive hematopoietic stem cells" (PSC), based on their lack of cytoplasmic differentiation and the context in which they occurred (see Discussion).

Macrophages participating in coupling junctions were mature. They typically were of elongate form and made contact with stromal reticular cells. Cytoplasmic inclusions in these cells used for their identification included numerous phagosomes, vesicles, and heterolysosomes. The plasmalemma was variably developed into filopodia. Similar macrophages occurred throughout the uncolonized marrow and appeared to be active in the repair of the hematopoietic tissue space. The question of the origin of macrophages in the endocloned marrow is considered elsewhere.[11]

The simplest cell association considered to be interdigitative coupling consisted of a slender cytoplasmic process of a PSC invaginating the cytoplasm of a macrophage (Fig. 1). There were similarities in the appearance of the macrophage–PSC interface of this association with that of more elaborate interdigitations (compare Fig. 1B with Fig. 2B). As shown in Fig. 1B, small membrane-bound vesicles containing a granular electron-dense material occurred in the macrophage cytoplasm near the region of the association, which showed an area of plasmalemmal separation between the cells. In more elaborate couplings, as exemplified by Fig. 2, a large segment of the perimeter of the PSC was apposed by cytoplasmic processes of a macrophage. Microvillous processes of the PSC invaginated cytoplasmic pockets within the macrophage and/or intertwined with loops or folds of the macrophage surface. Membrane-bound vesicles containing a granular electron-dense material lay within the macrophage cytoplasm along these invaginations; in certain instances, these appeared to be releasing their contents into, or forming from, the intercellular space (Fig. 2, B and C).

The PSC processes within a macrophage were separated from the macrophage's plasmalemma in many areas by a distinct, regular, electron-lucent space approximately 30 nm wide. This space usually contained a fine, filamentous material perpendicularly aligned between the parallel plasmalemmae of the macrophage and hematopoietic cell, suggestive of an orientation of matter in an electric field. In other areas, the plasmalemma of neither the PSC nor macrophage was distinct.

Interdigitative coupling of more than one PSC to a single macrophage was not observed.

DISCUSSION

This study reports the discovery of interdigitative couplings between morphologically undifferentiated hematopoietic cells and mature marrow macrophages. The small mononuclear cells found coupled to macrophages have been identified as stem cells tentatively, in view of the well known difficulties in establishing the differentiative potential of early hematopoietic cells using morphologic techniques. However, the possibility that what I term PSC are, in fact, pluripotent cells is supported by their small mononuclear character and lack of cytoplasmic differentiation. Such attributes place these cells within a morphotypic class of hematopoietic cells, which includes the earliest and least differentiated cells contributing to marrow repopulation;[14,15] moreover, they indicate similarity to the cells identified by several workers as the most likely candidates for the pluripotent hematopoietic stem cell.[16–18]

Additional evidence for this interpretation comes from the context in which these cells occurred. As has been demonstrated by means of the spleen colony technique for both exocloning[19] and endocloning[20] procedures, pluripotent hematopoietic cells multiply rap-

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Fig. 1. (A) Simple interdigitative coupling of a mature marrow macrophage (M) to a morphologically undifferentiated hematopoietic cell (PSC). This occurred within a granulocytic colony at 6 days postirradiation. A mitochondrion occurs in the macrophage cytoplasm in the region of this association (×7,300). (B) High magnification (×60,750) of PSC process where it invaginates the macrophage cytoplasm. Membrane-bound vesicles containing a granular, electron-dense material occur in this region.

idly in the regenerating, colonized marrow during the 2–8-day posttransplantation (or postirradiation) period. Thus, marrow colonies, which constitute the regenerating hematopoietic population, do include pluripotent stem cells. By comparison, "committed" granulocyte/macrophage progenitors, as assayed by methylcellulose culture, reach a nadir at 3 days PI in lethally irradiated bone marrow recipients and show only low level signs of a regenerative increase at 4 days,[21] whereas erythropoiesis, as measured by ^{59}Fe uptake, is maximally depressed in endocolonized marrow 3–5 days PI.[22] Hence, the probability of finding pluripotent stem cells in the very early marrow colonies studied here should be high.

Certain functional attributes of this coupling can be inferred: (A) it is an active association, involving modifications of both the macrophage and PSC; (B) it establishes extensive areas of apposition of the macrophage and PSC plasmalemmae; (C) it creates confined spaces between these cells; and (D) these spaces may be modified by the uptake or release of small cytoplasmic vesicles of the macrophage. Further, the interdigitation of cytoplasmic processes clearly establishes a physical linkage between the interacting cells (Fig. 2),

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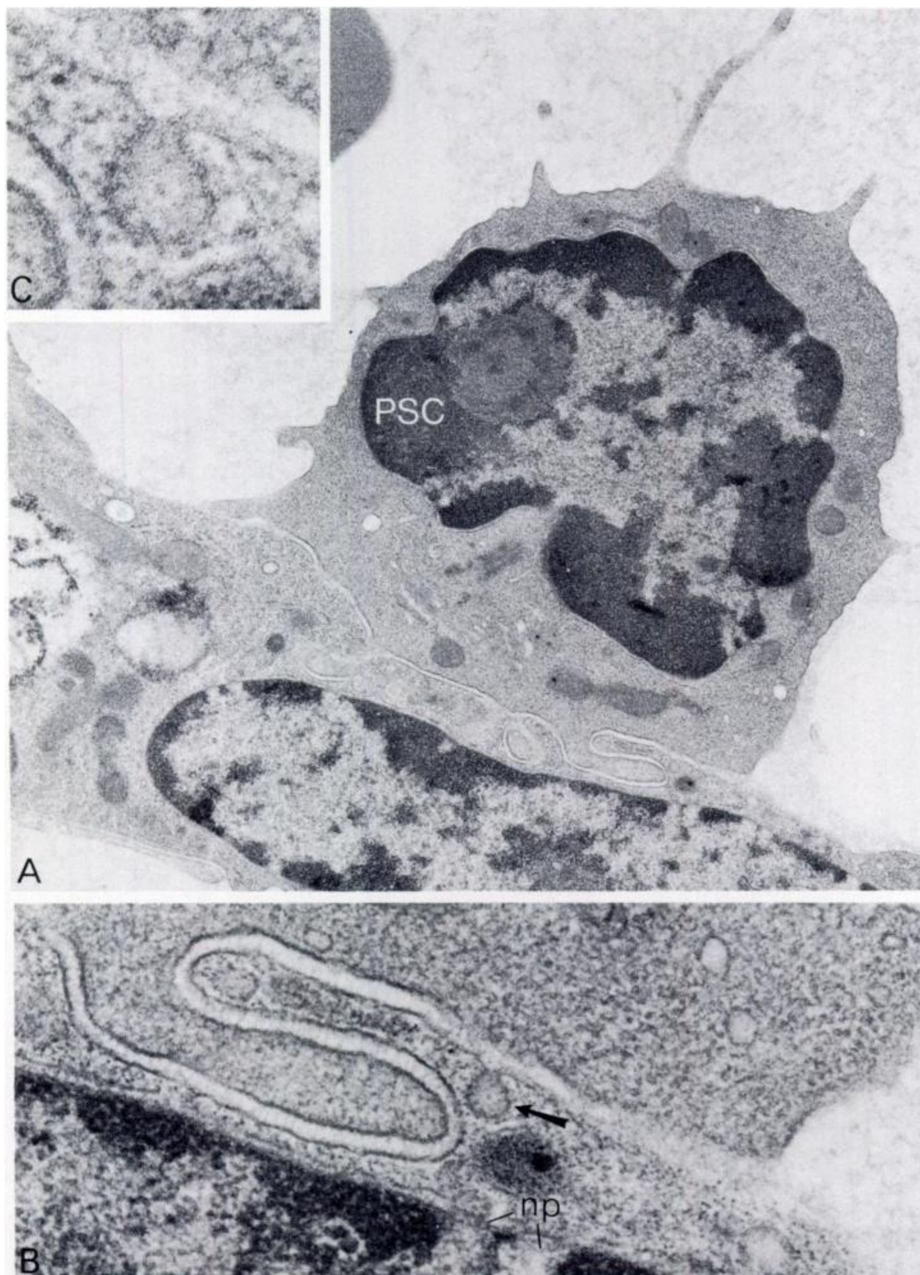

Fig. 2. (A) Interdigitative coupling of a presumptive hematopoietic stem cell (PSC) and macrophage at 4 days postirradiation. This occurred within a morphologically undifferentiated colony. Note the lack of cytoplasmic differentiation of the PSC, well developed centriole, and the slender microvilli along its free surface. Cytoplasmic processes of the PSC are invaginated into the perinuclear region of the macrophage below (×18,000). (B) High magnification (×45,000) of right side of coupling shown in (A). A well defined intercellular space is evident between the PSC and macrophage surface membranes in the area of interdigitation and contains a filamentous material aligned perpendicularly between the macrophage and PSC in this region. A membrane-bound vesicle containing an electron-dense granular material is evident within the macrophage cytoplasm (arrow) and appears to be releasing its contents into, or forming from, the intercellular space. A granular material is evident between the macrophage and PSC near this vesicle and toward the extracellular space. Macrophage nuclear pores (np) also occur near this region. (C) High magnification (×56,250) of macrophage vesicle described in (B).

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thus indicating that macrophages contribute to the lodgement, or holding, or undifferentiated hematopoietic cells in the marrow space.

Considering these points, interdigitative coupling of PSC to macrophages evidently does involve short-range intercellular communication, perhaps of a multifunctional nature. Also, there is support for the specific possibility that these interactions are involved with stem cell regulation. This comes from the probability that the cells termed PSC are, in fact, pluripotent hematopoietic stem cells, and the now strong evidence, provided by Wright and coworkers, that marrow macrophages are capable of controlling stem cell proliferation. These investigators have found that media conditioned by normal and regenerating marrow contain, respectively, an inhibitor and a stimulator of CFU-S replication and that both factors are produced by marrow macrophages.[23-25]

Thus, in light of available evidence, the present findings suggest that interdigitative coupling of stem cells to macrophages may represent the actual mechanism of local control of hematopoietic stem cell pools indicated by the work of Croizat et al.,[2] Gidali and Lajtha,[4] and others. They lead to the simple hypothesis

that the confined intercellular spaces created by interdigitative couplings provide a means to hold proliferative and/or differentiative hemokines in active concentrations at a stem cell's plasmalemma, without substantially altering the hemokines' systemic or regional levels. Definitive testing of this hypothesis awaits the development of specific markers for hematopoietic stem cells that can be applied at the ultrastructural level.

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