Endosteal Marrow: A Rich Source of Hematopoietic Stem Cells

Abstract. *Hematopoietic cells isolated from the endosteal bone surface, that is, the endosteal marrow cells, were found to consist mainly (60 to 80 percent) of lymphoid and late-stage normoblast-like cells. Unlike the cells they resemble, the endosteal marrow cells showed an affinity for Sudan black, demonstrable nucleoli (Feulgen reaction), and an absence of hemoglobin. Assays showed that over one-half of the endosteal marrow cell population may be the colony-forming units, the CFU-S of Till and McCulloch. Thus, high concentrations of stem cells could be obtained from the endosteal bone surface by means of the present isolation technique.*

The pluripotential hematopoietic stem cell is thought to be (i) morphologically similar to a small lymphocyte (*1*); (ii) identifiable by the colony-forming unit (CFU) technique (*2*); (iii) proliferated at the endosteal surface of bone (*3*); (iv) capable of development along the erythroid, myeloid, and thrombocytic lines (*4*); and (v) responsive to hematopoietic stresses (*5*). Most of the stem cells are thought to be normally in the G_0 state of the cell cycle, but under stress the fraction synthesizing DNA could be considerably higher than 10 percent (*6*). The rate of cell division was thought to be controlled by population feedback mechanisms (*7*). In this report, hematopoietic cells on the endosteal surface, that is, the endosteal marrow (EM) cells, were separated from those located more centrally in the axis of the bone marrow [the red marrow (RM) cells] and studied.

The bulk of the RM of the femoral diaphyses from young adult female Sprague-Dawley rats was forcibly expelled with air (by means of a 10-ml syringe) and the bone was split open. The remaining loosely adherent RM was washed away by gently waving the bone in Hanks basic salt solution (BSS). The thin, delicate, pale-brown film (the EM) visible on the bone was wetted with serum, scraped off with a rubber policeman into a round bottom plastic cup, and dispersed with a glass rod. The suspension was spread on glass slides, or diluted (Hanks BSS) for CFU assays.

Incompletely rinsed and scraped endosteal bone under scanning electron microscopy (SEM) (Fig. 1A) (*8*) revealed three distinctive areas of cells: one with large clumps of cells (probably RM) loosely attached to the bone surface; a second, consisting of a layer of individual cells and cells in small aggregates (probably EM) adhering flatly on the bone; and the third, a mosaic of osteoblasts embedded in the endosteum. A closer SEM view (Fig. 1B) showed that only a few erythrocytes were present among the EM cells, the latter with diameters of one to three times that of the former. The scanty erythrocytes would explain a general lack of redness of the EM, a characteristic found useful for distinguishing it from the RM.

Approximately 60 to 80 percent of the EM cells (Wright-Giemsa preparation) morphologically resembled lymphocytes (Fig. 1D) and late-stage normoblasts (Fig. 1H). The myeloblast (Fig. 1, G and H), at 10 to 20 percent, represented the next most numerous cell type. Cells in the 5 percent range included the reticulum cell, hemocytoblast of Bessis (*9*), (Fig. 1H), and proerythroblasts (Fig. 1, G, M2, and N2). Other cells present, generally less than 1 percent, were the immature or blastic forms of mast cell (Fig. 1I), lymphocyte (Fig. 1J), plasmacyte (Fig. 1K), megakaryocyte (Fig. 1L), osteoblast (Fig. 1A), and a variety of unusual forms (to be reported elsewhere). Notable by their general absence were the cells of the granulocytic series.

Detailed studies showed that lymphocytes and late normoblasts were rarely present in the EM. For example, Feulgen-reacted (*4*) EM cells resembling the above (Fig. 1, P2 and P3) demonstrated nucleoli, while the circulating lymphocyte (buffy coat, Fig. 1, P1) did not. In addition, both cells exhibited cytoplasmic staining with Sudan black (Fig. 1, O2 and O3), whereas lymphocytes treated similarly remained unstained (Fig. 1, O1). Because lymphocytes and normoblasts do not have an affinity for Sudan black (*4*), and because they do not normally demonstrate nucleoli, the EM population must in general not include these two types of cells which, conversely, are present in abundance in the RM. The relative absence of polychromatic normoblasts in the presence of early erythroid precursors was unusual but could be verified by benzidine tests for hemoglobin (*4*). Comparison of benzidine-reacted smears before (Fig. 1, M1 and N1) and after (Fig. 1, M2 and N2) Wright-Giemsa counterstaining revealed that generally only the erythrocytes and cells identifiable as young erythroblasts in Wright-Giemsa preparations contained hemoglobin. Late-stage normoblast-like cells exhibiting hemoglobin were rarely observed.

In short, the 60 to 80 percent of the EM cells that resemble the lymphocyte and polychromatic normoblasts are, in fact, a class of cells that is different from any of the cells normally found in the RM. Because of their uncertain identities they could be termed the "unidentified endosteal marrow cells" (UEMC) for the present. In general, they have round to oblong nuclei that exhibit densely packed chromatin, and show thin rims of deeply basophilic to slightly eosinophilic cytoplasm. In addition, these cells, which are 6 to 20 μm in diameter, are

Table 1. Colony-forming units on mouse spleen compared with the number of rat EM cells injected, and the determination for the "f" factor; S.E., standard error.

Cells injected per mouse	CFU's per spleen		CFU's per cell injected
	Mean ± S.E.	*N*	
	Experiment 1		
100	28.3 ± 3.1	10	0.28
50	15.8 ± 2.1	10	0.32
25	7.5 ± 1.1	13	0.35
	Experiment 2		
60	15.3 ± 3.7	6	0.26
40	8.8 ± 1.4	9	0.22
30	8.5 ± 1.7	12	0.28
20	6.3 ± 0.6	12	0.32
0	0.3 ± 0.1	11	
	Experiment 3		
57	12.5 ± 2.1	23	0.22
36	8.3 ± 1.9	14	0.23
25	5.5 ± 1.6	18	0.22
"f"*	12.7 ± 2.8	13	
0	0.2 ± 0.1	11	

*Two primary recipient mice were each injected with 2520 EM cells within 1 hour after whole-body x-irradiation of 900 R. They were killed 3 hours later, their spleens were forced through a 200-mesh stainless steel wire screen, and an 8.0-ml suspension was made with Hanks BSS. Each secondary recipient was then injected intravenously with 0.25 ml of the cell suspension (or 1/16 of the splenic content), approximately 4 hours after exposure to 900 R. The fraction of CFU's per cell injected in experiment 3 averaged 0.223. Thus, the CFU's per primary recipient spleen was 562 (0.223 × 2520) and the number injected in the secondary recipient was 35.1 (562/16) CFU's each. The "f" factor was then 12.7/35.1, or 0.36. Earlier workers reported "f" factors of approximately 0.20 (*15*).

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stained with Sudan black and exhibit multiple nucleoli.

In a study of the uptake of tritiated thymidine (*10*) with five control and five rats made anemic by bleeding, there was an anemia-induced elevation of the labeling indices (LI, percentage of cells labeled): from 16.6 ± 3.2 (mean ± 1 standard error of the mean) to 26.7 ± 2.5 for the EM population; from 11.9 ± 2.3 to 14.9 ± 3.0 for the large-sized (12 to 20 μm) (Fig. 1, Q2 and Q3) UEMC; and from 2.0 ± 0.2 to 9.9 ± 0.5 for the small-sized (6 to 11 μm) (Fig. 1, Q1) UEMC within 3 hours of anemia induction (*11*). The magnitude of the LI's of the large- and small-sized UEMC in stressed and unstressed animals were generally similar to the stem cell LI's reported previously (*12*). The marked and rapid response suggests a direct relation between the anemic stress and the cells of the EM.

Three separate CFU assays were made with injections of EM cells (*13*) into supralethally irradiated male ICR/Swiss mice (20 to 100 cells per mouse) (*14*). Table 1 shows the results and Fig.

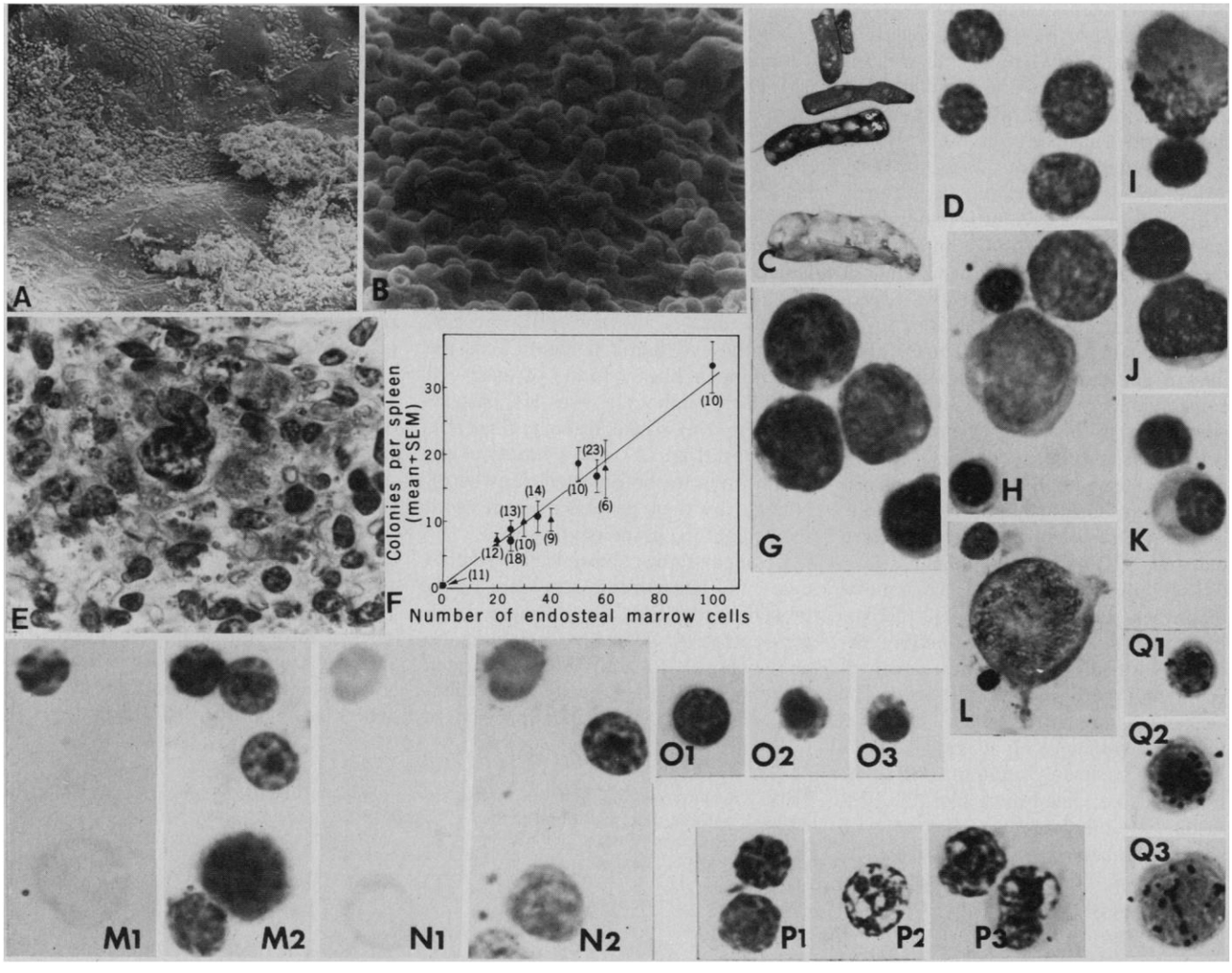

Fig. 1. Endosteal marrow cells. Unless otherwise indicated, the figures are magnified ×3000 and the cells were stained with Wright-Giemsa (WG) stain. (A) Scanning electron micrographs (SEM, ×150) of three distinctive endosteal surface areas: right bottom, loosely attached red marrow cells which could be removed by gentle rinsing; left middle, endosteal marrow cells which resisted gentle rinsing but could be scraped away; and center top, osteoblasts in a mosaic pattern visible after scraping with a rubber policeman. (B) A closer view (SEM, ×600) of the endosteal marrow area. Scattered erythrocytes are seen among nonosseous cells closely adherent to the endosteum. (C) The large spleen with confluent colonies is from a mouse injected with 100 EM cells, and the remainder are from mice injected with 25 cells each (×2). (D) Lymphoid cells exhibiting nucleoli. (E) A section of a splenic nodule showing a megakaryocytic nucleus in the process of endomitotic activity. Barr bodies (peripherally located mass of sex chromatin) seen in many of the nuclei of the large immature cells indicate that the male mouse spleen had been repopulated with hematopoietic cells derived from a female donor (×1000, hematoxylin and eosin). (F) A composite curve of three CFU studies showing a linear relation between the EM cells injected and CFU's observed. (G) Myeloblasts and young erythroblast. (H) Myeloblast, hemocytoblast, and two cells resembling late-stage normoblasts. (I) Young mast cell. (J) Immature lymphocyte. (K) Immature plasmacyte. (L) Megakaryoblast (×1840). (M) and (N) Benzidine-stained smears: (M1) before WG counterstaining, showing stained hemoglobin of a crenated erythrocyte (top upper) and an early erythroblast; (M2) after WG, showing three nonhemoglobin-bearing cells, two of which resemble late-stage normoblasts; (N1) before WG, two cells with hemoglobin, a mature erythrocyte (upper) and a younger nucleated form; (N2) after WG, one of the hemoglobin-free cells bears a marked resemblance to a polychromatic normoblast. These findings suggest that many cells resembling normoblasts in the EM are not nucleated red cells. (O) Sudan black (SB) staining: (O1) circulating lymphocyte showing a lack of SB staining; (O2 and O3) small mononucleate cells with cytoplasmic staining which indicates that they are neither lymphocytes nor late normoblasts since the latter cells are known to be SB negative (*4*). (P) Feulgen reaction: (P1) lymphocyte (buffy coat) demonstrating no unstained (Feulgen negative) nucleolar spaces; (P2 and P3) lymphoid elements of the EM exhibiting distinctive nucleolar areas indicating that these lymphocyte-like cells are not lymphocytes. The presence of nucleoli indicates that they are blastic cells. (Q) Autoradiographs: (Q1 and Q2) cells resembling late-stage normoblasts and showing radioactive granules that indicate that they are not late erythroid precursors because late normoblasts do not enter mitosis; (Q3) a lymphoid cell after the incorporation of [^{3}H]thymidine.

1C shows examples of splenic colonies. Strikingly similar linear relations between the numbers of EM cells injected and the CFU's observed were noted, Fig. F. In the third study, the "f" factor (*15*) was found to be 0.36 and the CFU per EM cell injected was 0.223, suggesting that the majority [(0.233/0.36) = 0.62] of the EM cells must be CFU's. Since only the UEMC were sufficiently numerous (up to 80 percent) to account for the number of colonies observed and because their function has not been established, they must be considered the prime candidate stem cells.

Tissue sections of splenic colonies showed many foci of developing megakaryocytes and immature cells, Fig. 1E. The presence of Barr bodies observed in the nuclei of a large fraction of the latter cells indicate that the CFU's were derived from cells of female animals (*16*). Since the recipient mice were all males, this observation would be tantamount to direct evidence that these colonies were developed from exogenous stem cells obtained from the donor animal, a female rat.

Thus, although the exact form or morphology of the hematopoietic stem cell remains to be described, it is possible that the majority of the EM cells, that is, the UEMC, are CFU's. If true, the present technique for the separation of the EM and RM may prove useful in the study of the hematopoietic stem cell under normal as well as pathological conditions.

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References and Notes

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8. For the scanning electron microscopy, endosteal marrow attached to bone was fixed in 6 percent glutaraldehyde solution for 24 hours before examination.
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10. Bone pieces with EM attached were exposed to [^{3}H]thymidine in saline solution for 15 minutes, rinsed four times, and further incubated (room temperature) in a fifth inert saline rinse for 45 minutes. The EM was scraped off the bones; slides were prepared, then coated with NBT2 (Kodak) emulsion. They were developed and counterstained with Giemsa diluted 1:30 with a phosphate buffer, *p*H 5.6, 2 to 6 days later [T. M. Fliedner, E. D. Thomas, L. M. Myer, E. P. Cronkite, *Ann. N.Y. Acad. Sci.* **114**, 510 (1964)].
11. Up to 50 percent of the calculated blood volume was removed from each rat by way of the jugular vein [J. K. Gong, C. A. Glomski, N. L. Frederiksen, A. J. Lawson, J. P. Daley, *Radiat. Res.* **65**, 83 (1976)].
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13. Hemocytometer counts of cell suspensions were verified in a Coulter Counter, then diluted (Hanks BBS) to yield the required cell concentrations per 0.25 ml for each injection. Mice were anesthetized with ether and injected via the jugular vein at approximately 2 hours after whole-body x-irradiation (250 kV peak) of 850 or 900 R. Control animals were each injected with 0.25 ml of Hanks BSS. Spleens were fixed in Bouin's fluid when the mice succumbed or when they were killed (6 to 10 days later) and examined 24 hours later.
14. The ICR/Swiss mice were obtained from Health Research Inc., West Seneca Laboratories.
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Myosin: Immunofluorescent Localization in Neuronal and Glial Cultures

Abstract. *The distribution of intracellular myosin was studied by the double antibody immunofluorescence method in primary organotypic neuronal cultures and two established neuronal and glial cell lines. An array of parallel filaments aligned with the major cellular axis and a three-dimensional subsurface network were shown to react with two different myosin antibodies. The presence of myosin-rich filaments in regions known to contain actin filaments suggests that these proteins interact to generate the motive force in nonmuscle contractile systems.*

Most theories concerning the mechanisms responsible for cellular and intracellular movement involve fibrous systems. At present, two major fibrous proteins, myosin and actin, have been implicated in cell motility. Discrete filaments of cytoplasmic actin have been isolated and shown to bind heavy meromyosin, and have been localized by immunofluorescence studies (*1*). Myosin from nonmuscle cells has been isolated and identified biochemically but its intracellular disposition and localization has not been determined (*2*). The role of these two contractile proteins in skeletal muscle has formed the basis for the general assumption that they participate in axoplasmic transport. In order to confirm and clarify the role of actin and myosin in neuronal tissues, we undertook the present study.

Since cultured neuronal tissue exhibits a high level of intracellular movement (axoplasmic flow) as well as considerable morphogenetic movement (neurite extension and radial glial migration), it serves as an excellent model for the study and localization of elements essential to motility (*3*). We studied the intracellular distribution of cytoplasmic myosin in primary cultures of chick sensory ganglia and established neuronal and glial cell lines.

Myosin from chick gizzard smooth muscle is biochemically similar to myosin isolated from embryonic chick brain (*4*). We therefore used an antibody prepared against highly purified chick gizzard myosin in a double antibody immunofluorescence study to localize myosin in cultured neuronal tissues. All experiments were repeated with an antibody prepared against human uterine smooth muscle myosin to assess further the intracellular distribution of myosin in neuronal and glial cells. Both antibodies, prepared in the laboratory of Gröschel-Stewart (*5*), have been shown to react solely with myosin and not with other contractile proteins (*6*).

Primary organotypic cultures of embryonic chick dorsal root ganglia and monolayers of murine neuroblastoma (S20) and rat Schwann cells (RN22) were grown on collagen-coated or uncoated glass cover slips either in closed Maximow chambers or in open petri dishes incubated in a humidified atmosphere of 5 percent CO_2 at 35°C (*7*). Cultures were rinsed free of media with three changes of phosphate-buffered saline (PBS), *p*H 7.6, fixed in 2 percent paraformaldehyde in PBS and air-dried after changes in graded acetone. In one series of experiments, cultures were treated with a detergent solution (0.1 percent Triton X-100 in PBS) prior to fixation to facilitate the penetration of the antibodies (*8*). Air-dried specimens were incubated for 1 hour at 37°C in moist chambers with the antibody against purified chick gizzard myosin or human uterine myosin. Control cultures were incubated with nonimmune rabbit serums and nonimmune

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