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Isolation and Short-term Culture of Mouse Splenic Erythroblastic Islands

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ABSTRACT. **We isolated and cultured erythroblastic islands (EI) from the spleens of phlebotomized mice using a combination of collagenase digestion, unit gravity sedimentation, and Percoll density gradients separation. The isolated EI were composed of surrounding erythroid cells and central stromal macrophages (Mϕ), which were identified by Forssman antigen. While 60% of the erythroblasts incorporated bromodeoxyuridine, the Mϕ did not. EI could be maintained on a plastic dish for a short period in the presence of erythropoietin. Two hours later, the central Mϕ spread well and bound to erythroblasts via cytoplasmic processes. One day later, erythropoietic activity on the Mϕ surface continued, although their processes had retracted. Some EI showed synchronized expansion of erythroblasts and others showed differentiation to reticulocytes. Two days later, about 50% of the EI still showed erythropoietic activity and most erythroblasts differentiated to the orthochromatic stage. On the other hand, the Mϕ secreted colony-stimulating activity during the culture. It was infrequently observed that erythroid and myeloid populations simultaneously expanded on a central Mϕ. These results indicate that this EI culture system is useful for studying interactions between the stomal Mϕ and hematopoietic cells.**

Hematopoiesis involves interactive events between hematopoietic cells and stromal cells (1). It has been postulated that the stromal cells participate in segregation of hematopoietic cells according to maturational stage and lineage by a variety of adhesive interactions with hematopoietic cells (8, 18). Bone marrow-fixed macrophages (stromal Mϕ) establish stroma by extending long cytoplasmic processes and attach developing erythroid and myeloid cells in a collagenase-resistant fashion (5). In long-term bone marrow culture by which hematopoietic stem cells are maintained, immature myeloid cells proliferate on the Mϕ defined by monoclonal antibody F4/80 (1). The addition of erythropoietin (EPO) to the culture alternatively induces erythropoietic activity on the Mϕ (7). These lines of evidence suggest that immature myeloid cells or erythropoietin-responsive erythroid precursors adhere to the Mϕ, where they proliferate and differentiate into mature cells under the control of the Mϕ which are capable of secreting hematopoietic stimulating or inhibiting factors. However, the difficulty in enriching a sufficient number of hematopoietic tissue-fixed Mϕ has hampered the study of the interactions between the Mϕ and hematopoietic cells.

Mouse splenic red pulp stroma has also been recognized to regulate hematopoietic stem cell differentiation (21). Using immunohistological methods, we recently showed that Forssman glycosphingolipid is a marker for stromal Mϕ in bone marrow and splenic hematopoietic foci (15, 16). Splenic Forssman antigen-bearing (Forssman$^+$) Mϕ were shown to be involved in developing erythroid colonies induced by erythropoietic stress and this action was accompanied by morphological changes. Our results suggested that Forssman$^+$ Mϕ is an important stromal environment for splenic erythropoiesis. In the present study, to establish an in vitro system to analyze the functional role of the Mϕ in hematopoisis, we isolated and cultured erythroblastic islands (EI) from splenic erythropoietic foci of phlebotomized mice. We were able to obtain a workable number of EI containing Forssman$^+$ Mϕ and showed that this EI culture system is useful for studying interactions between the stromal Mϕ and hematopoietic cells.

MATERIALS AND METHODS

Animals. Eight to 16-week-old C3H/HeN (H-2^k) mice were purchased from Charles River Japan, Inc. Ten to 16-week-old C57BL/6(H-2^b) mice were obtained from the Shizuoka Agricultural Cooperative Association for Laboratory Animals.

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(*EI*). Mice were bled by heparinized capillary tubes from the retroorbital sinus four days (0.5 ml) before or four days plus one day (0.4 ml + 0.25 ml) before killing. After the mice were killed by cervical dislocation, the spleens were excised, washed in Eagle's minimum essential medium (MEM) (GIBCO, Grand Island, NY), and minced in the medium using scissors. The fragments of two spleens were incubated in 15 ml 0.075% type 4 collagenase from *Clostridium histolyticum* (Sigma, St Louis, Mo) and 0.004% deoxyribonuclease 1 (DNase) (Sigma) in MEM for 30 min in a 37°C water bath with constant shaking. After passing the suspension through a syringe with an 18 gauge needle several times, a small amount of undigested white tissue was discarded. The suspension was washed, and resuspended in 7 ml MEM containing 0.004% DNase. This suspension was gently layered on 35 ml MEM containing 30% horse serum (GIBCO) in a 50 ml plastic tube, Forty-five minutes later, aggregates sedimented on the bottom were collected, suspended in 20 ml MEM containing 50% Percoll (prior adjusted to 300 mOs/kg, Pharmacia Fine Chemicals, Uppsala, Sweden), and gently layered on 10 ml of 100% Percoll in a glass centifuge tube. The column was spun at an average of 400 g for 20 min. Red aggregates (EI fraction) enriched between the 50% Percoll column (density: 1064 g /ml) and the 100% Percoll column (density: 1122 g/ml) were harvested by Pasteur pipette, suspended in MEM, and washed by centrifuging at 100 g for 5 min. This enriched EI fraction was used for bromodeoxyuridine (BrdU) incorporation. After suspending the EI fraction in MEM containing 10% fetal bovine serum (Hyclone Lab, Logan, UT, lot 111826), 150 μl of the suspension ($1.0–3.0 \times 10^7$ cells/ml) was placed on a 35-mm plastic dish (no. 3001, Falcon, Oxnard, CA) or glass coverslip in 12-well dishes (Sumitomo Bakelite, Osaka). They were then incubated at 37°C in an atmosphere of 5% CO_2 in air. Ninety minutes later, the medium was decanted and loosely bound cells were removed by direct flushing with MEM. This treatment revealed EI on the surface. The EI were cultivated with alpha MEM containing 30% FBS, 5×10^{-5} mol/L mercaptoethanol (Sigma), and indicated concentration of recombinant erythropoietin (EPO) (EPOCH®, Chugai Phamacuitical Ltd., Tokyo). Morphological and immunocytochemical studies were performed 1, 2 and 3 days after beginning of the culture.

Scoring of EI. The large and well-spread macrophages attaching 4 or more erythroblasts which showed a positive benzidine reaction were scored as EI.

Antibodies. Anti-Forssman glycosphingolipid (GSL) serum was obtained by the immunization of a white rabbits as previously described (15). Rat Ig G monoclonal antibody F4/80 (2) was obtained from Dr. S. Gordon, University of Oxford, U.K. Rat IgG monoclonal antibody against Mac-1 antigen (M1/70) (17) and rat IgG monoclonal antibody against Ia antigens ($I\text{-}A^{b,d,q}I\text{-}E^{d,k}$) (M5/114) (3) were purchased from Hybritec Incorporated (San Diego, CA). Mouse monoclonal antibody against bromodeoxyuridine (BrdU) (9) was obtained from Becton Dikinson (Mountain View, CA).

Immunoperoxidase staining. Cytocentrifuge and cell-culture preparations were air-dried and fixed in acetone for 5 min at room temperature or in 2% paraformaldehyde for 60 min. Preparations were then stained with anti-Forssman serum using an indirect immunoperoxidase method and with F4/80, M1/70, M5/114 or anti-BrdU monoclonal antibody using an avidin-biotin-peroxidase complex (ABC) method (15).

Electron microscopy (EM). The culture samples were fixed with 2% paraformaldehyde and 1% glutaraldehyde in 0.1 mol/L phosphate buffer for 60 min at room temperature. For transmission EM studies, samples were post-fixed with 1% osmium tetroxide, dehydrated in ethanol, and embedded in epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEOL JEM-100CX. For scanning EM studies, the fixed samples were dehydrated, critical-point dried, coated with panadium-platinum, and examind with a HITACHI scanning EM S-570.

Cytochemistry. Cytocentrifuge preparations or cell-culture preparations were stained for acid phosphatase (ACPase) with naphthol AS-BI phosphate as a substrate, for nonspecific esterase with alpha-naphthyl butyrate, and for granulocyte esterase with naphthol AS-D chloroacetate.

Simultaneous detection of BrdU incorporation and ACPase activity. The isolated EI were pulsed for 30 min at 37°C with 10 μg/mL BrdU. After washing in MEM, clusters were suspended in MEM containing 10% FBS and cytocentrifuged. They were then fixed in acetone and ACPase activity was detected as described previously. After washing with distilled water, they were treated with hydrochloric acid, neutralized with sodium tetraborate, and stained for incorporated BrdU using the ABC method.

Preparation of conditioned-medium (CM). The medium conditioned by spleen cells stimulated by pokeweed mitogen (PWM-SPCM) was prepared according to the previous report (10). The Mϕ CM was prepared as follows. After the EI fraction isolated from two spleens was atttached on two 35 mm

Table I. YIELD OF EI FROM A BLED C3H/HE MOUSE SPLEEN[a].

Spleen weight (mg)	244±30
Total nucleated cells[b] ($\times 10^8$)	4.5±0.9
EI: single cell ratio	1 : 520
Enriched EI fraction	
Nucleated cells ($\times 10^7$)	8.2±2.9
EI ($\times 10^6$)	1.2±0.4
EI: single cell ratio	1 : 27
Differential count of EI fraction (%)[c]	
Mϕ	3.4±0.8
Erythroid cells	90.1±1.5
myeloid cells	1.8±0.3
Other cells	4.4±0.9

[a] Data is shown as mean or mean±SD (range) of 5 independent experiments with 10 C3H/He mice. [b] This fraction was obtained after collagenase treatment and contained $2.0 \sim 3.8 \times 10^8$ erythrocytes. [c] Data with May-Giemsa stain.

plastic dishes (Falcon), the overlaying hematopoietic cells were stripped from the Mϕ with Ca^{2+},Mg^{2+}-free Hanks' solution. This treatment yielded about 6 to 8×10^4 cells/dish stromal Mϕ of 85% purity. After each of the dishes received 10 Gy irradiation, they were cultured with alpha MEM containing 10% FBS for a day. The supernatant was collected, freed of cells by centrifugation, and then tested for colony stimulating activity against mouse bone marrow cells with appropriate controls of the additive alone.

Detection of colony stimulating activity (CSA). CSA in the conditioned medium from stromal macrophage culture was examined. Briefly, 1.0 ml of bone marrow cell suspension (5.0×10^5 per ml) from which adherent cells had been removed, was mixed with 2.0 ml of 2.2% methylcellulose (Muromachi Kagaku, Tokyo) in alpha MEM medium, 1.0 ml of FBS, and 1.0 ml of Mo CM. A control culture was performed in the presence of PWM-SPCM. One ml aliquots of the mixture were plated into 35 mm plastic dishes and incubated in a CO_2 incubator at 37°C in an atmosphere of 5% CO_2. Seven days later, colonies composed of more than 50 cells were expressed as CSA.

RESULTS

Enrichment of erythroblastic islands (EI). EI were isolated by mild collagenase treatment and following unit gravity sedimentation. Most single cells including erythrocytes were removed and the sediments contained

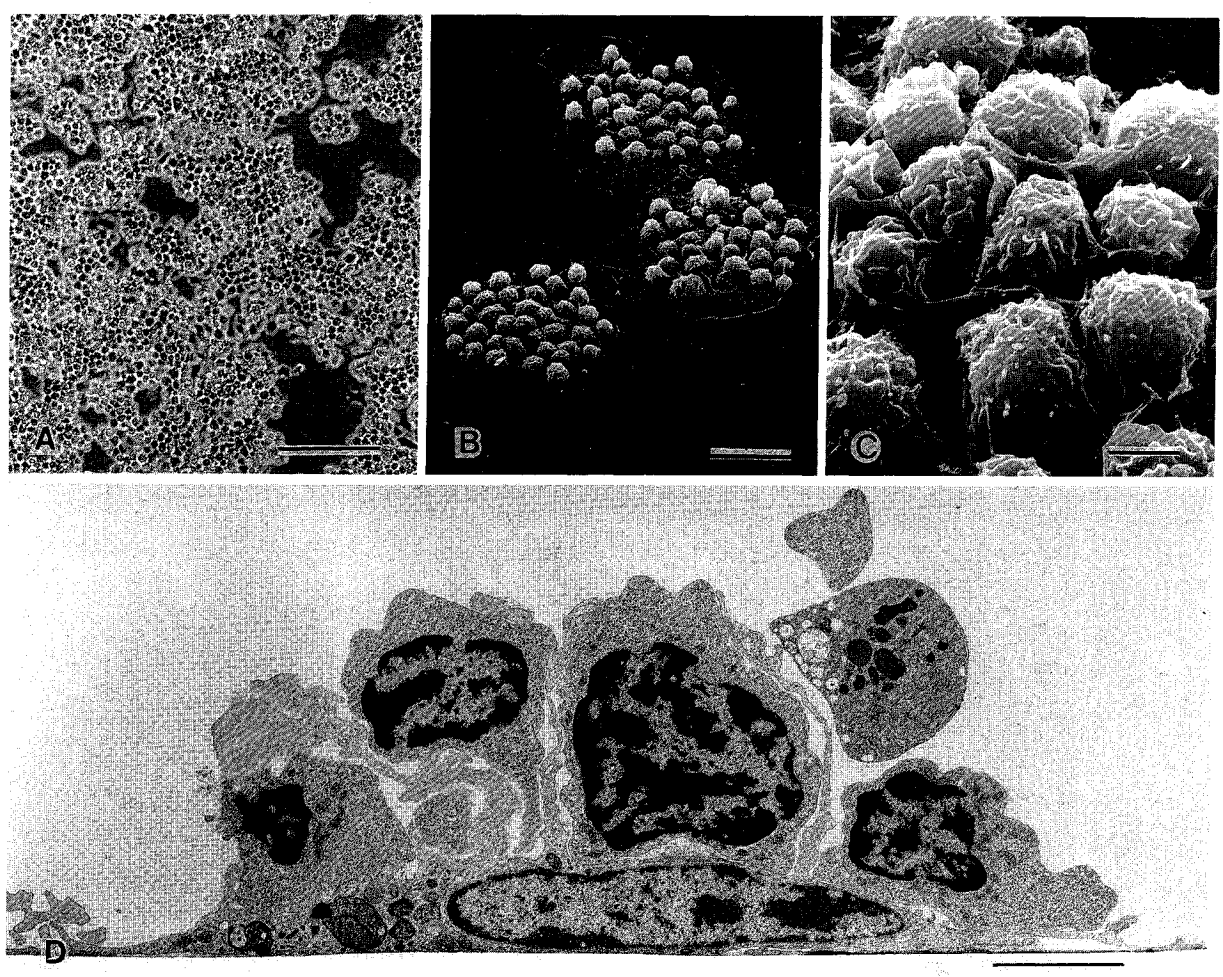

Fig. 1. Morphology of isolated erythroblastic islands (EI).
A. Phase contrast microscopy two hours after inoculation of EI fraction. Bar, 100 μm.
B. Scanning electron microscopy (SEM) of EI two hours after inoculation. Three EI individually composed of a central Mϕ and many overlaying erythroblasts. Bar, 20 μm.
C. Higher magnification of SEM. The membranous processes of the Mϕ embrace erythroblasts with irregular and folded surfaces. Bar, 3 μm.
D. Transmission electron microscopy. The long processes of well-spread Mϕ extend between the erythroblasts. Bar, 3 μm.

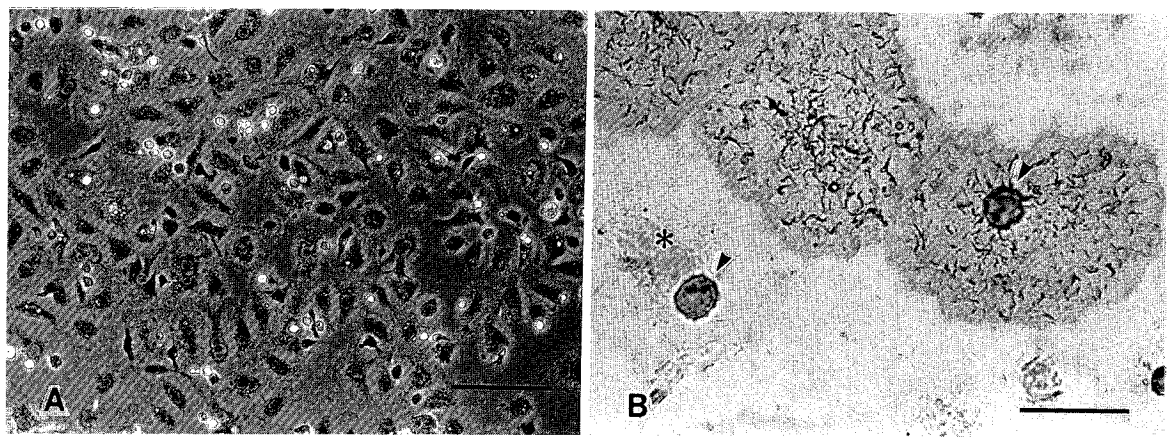

Fig. 2. Characteristics of central Mϕ of EI.
A. Phase contrast microscopy of underlaying Mϕ after stripping erythroblasts. Well-spread Mϕ are evident. Bar, 100 μm.
B. Immunoperoxidase staining of underlaying Mϕ with anti-Forssman antiserum. Membrane folds of the Mϕ are clearly stained. Arrows show non-stripped erythroblasts which bind Forssman$^+$ and Forssman$^-$ (*) Mϕ. Formalin-fixed sample: methylgreen counterstain. Bar, 20 μm.

many EI of various sizes. Percoll density gradients served to separate these EI from lower density cells, including myelomonocytic cells and lymphocytes. As shown in Table I, these procedures resulted in about a twentyfold enrichment of the EI content. The final EI fraction obtained from the spleen of a bled C3H mouse, contained 90% erythroid cells and 3.4% Mϕ as defined by May-Giemsa staining.

Characterization of EI. Simultaneous identification of ACPase activity and BrdU incorporation of isolated EI was performed to examine whether the stromal Mϕ were replicating. None of the Mϕ incorporated BrdU, whereas 60% of surrounding cells did (data not shown).

Under a phase contrast microscope after 2 hours culture of the EI fraction on the plastic surface, many EI were observed to comprise of underlaying large, flattened Mϕ and overlaying cells (Fig. 1A). Scanning electron microscopy of EI in culture revealed well-spread Mϕ embracing many erythroblasts within their membranous processes (Fig. 1B, 1C). Transmission electron microscopy clearly showed evidence of this close interaction between Mϕ processes and overlaying erythroblasts (Fig. 1D). Monocytes or immature granulocytes were sometimes attached to the Mϕ. However, Mϕ processes did not circumscribe these types of cells. The erythroblasts were released from the Mϕ in the Ca^{2+},Mg^{2+}-free Hanks' solution, so that Mϕ comprised 85% of adherent cells (Fig. 2A). The addition of 0.5 mM Ca^{2+} or 5 mM Mg^{2+} into the solution completely inhibited this release. The binding was also dissociated by 0.002% trypsin or 5 mM lidocain. Immunocytochemically, 92% of the Mϕ proved positive for Forssman antigen (Fig. 2B). Functionally, the Mϕ vividly phagocytized latex particles. Table II provides a summary of the characteristics of the central Mϕ of EI. The results indicated that the Mϕ of splenic EI have similar characteristics to those of bone marrow Mϕ obtained by collagenase digestion of the tissue (5, 6).

Short-term culture of EI in the presence of erythropoietin (EPO). In this study, we used the EI isolated from the mice which were bled four days then one day before killing. When they were cultured in the presence of human recombinant EPO, the EI remained on the Mϕ surface for a short period although the Mϕ retracted their cytoplasmic processes (Fig. 3A). In the absence of EPO, erythropoietic activity completely disappeared within 24 hours (Fig. 3B). Table III shows

Table II. Characteristics of central Mϕ of splenic EI.

1. Morphology	Large and well-spread cells with long cytoplasmic projections and frequent binucleus
2. Surface antigens	
F4/80	+ (100%)
Forssman	+ (92%)
Mac-1	−
Ia	− ~ + (faintly positive, 20%)
3. Enzyme-cytochemistry	
Acid phosphatase	+ (100%)
Alpha-naphthyl-butyrate esterase	+ (100%)
Peroxidase	− ~ +
4. Function	Binding of erythroblasts in collagenase-resistant and Ca^{2+}, Mg^{2+}-dependent manner, Secretion of colony-stimulating factor

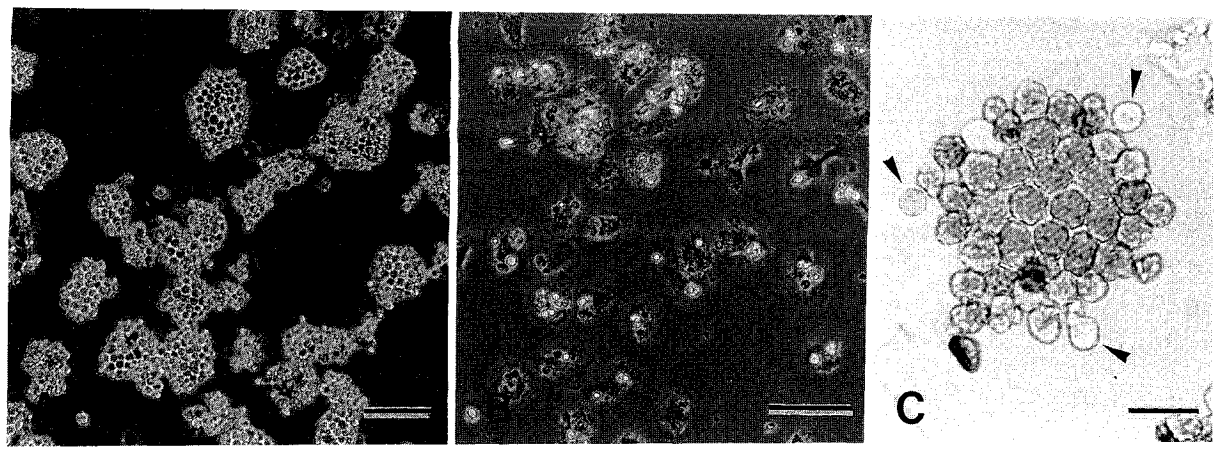

Fig. 3. Effects of EPO on EI culture.
A. Day 1. Phase contrast microscopy of EI that wee maintained in the presence of EPO. Bar, 50 μm.
B. Day 1. Phase contrast microscopy of EI cultured without EPO. Erythroblasts have disappeared and underlaying Mϕ are evident. Bar, 50 μm.
C. Day 2. Synchronous expansion of erythroblasts in the center of Mϕ surface. Arrow heads show extruded nuclei. Diaminobenzidine staining. Bar, 10 μm.

the relationship between EPO concentration and stimulation of the erythropoietic activity on the Mϕ surface. Considering these results, we performed this culture in 30% FBS and 2 unit/ml EPO for morphological studies.

One day later under these conditions, erythropoietic activity on the Mϕ persisted at different stages among EI. Some EI showed synchronous expansion of erythroblasts at the center of Mϕ surface. At the periphery, more differentiated erythroblasts and anucleated cells were seen. Others were composed of only differentiated erythroblasts and/or reticulocytes. Two days later, most erythroblasts on the Mϕ showed orthochromatic stage and some showed enucleation (Fig. 3C). Three days later, some Mϕ attached reticulocytes and extruded nuclei remained undigested.

The percentage of Forssman$^+$ Mϕ of the Mϕ forming EI was 93% on day 1 and 87% on day 2. A few Mϕ binding erythroblasts were negative for Forssman antigen.

As described earlier, myeloid cells were found in some isolated EI. On day 2 of EI culture, immunolabeling for Forssman antigen also revealed a cluster of immature granuloid cells on Forssman$^+$ Mϕ (Fig. 4A). It was infrequently observed that erythroid and myeloid cells simultaneously expanded on a central Mϕ (Fig. 4B, 4C).

Detection of colony-stimulating activity (CSA). We examined CSA in the supernatant from 24 hours culture of enriched stromal Mϕ fraction which received 10 Gy or no radiation. Irradiation prevented the proliferation of a small number of contaminated cells. As shown in Table IV, both irradiated and non-irradiated stromal Mϕ secreted CSA. Most colonies consisted of Mϕ.

Table III. Effects of erythropoietin (EPO) concentration on erythropoietic activity of isolated EI in culture[a].

EPO concentration (unit/ml)	% Mϕ binding erythroblasts[b]	
	Day 1	Day 2
0	0.0±0.0	0.0±0.0
0.03	7.7±7.3	0.5±0.6
0.06	28.0±11.7	3.4±1.9
0.13	51.3±7.0	9.1±3.5
0.25	59.4±12.5	24.3±3.8
0.5	66.4±5.0	31.4±8.0
1.0	66.8±6.5	43.6±5.8
2.0	68.9±9.5	55.8±8.1

[a] Data is shown as the mean±SD (n=4). [b] The percentage of Mo binding for at least 4 erythroblasts was calculated.

DISCUSSION

In the present studying using EI culture, we clearly showed that erythroid and myeloid cells attach to and develop on splenic red pulp Forssman$^+$ Mϕ in the pres-

Table IV. Colony stimulating activity of culture supernatant of central Mϕ.

Source of CM	Colony count/10^5 bone marrow cells[a]
− (negative control)	0
PWM-SPCM (positive control)	175±2.1
Mϕ CM	43±9.6
Irradiated-Mϕ CM	58±5.9

[a] Data is shown as mean±SD (n=4).

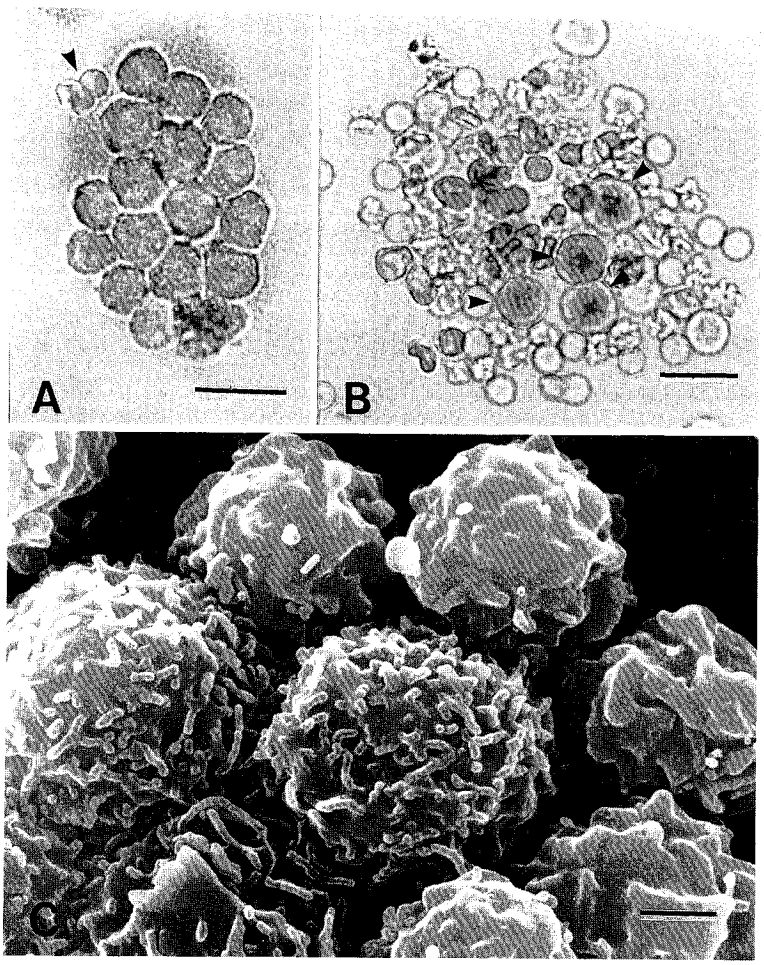

Fig. 4. Myelopoietic activity of cultured EI.

A. Day 2. Expansion of immature granulocytes on a Forssman$^+$ Mϕ. Arrow head shows erythroblast-enucleation at the periphery of the Mϕ surface. Bar, 10 μm.

B. Day 2. Four immature granulocytic cells identified by naphtol AS-D chloroacetate esterase staining (arrow heads) in EI which shows many erythroblast-enucleations. Bar, 10 μm.

C. Day 2. Scanning microscopy showing simultaneous expansion of erythrocytic and monocytic populations on a Mϕ. Erythroblasts showing many pits in deeply grooved surface and monocytic cells with characteristic surface villous projections. Bar, 1 μm.

ence of EPO.

Erythroblasts in EI were embraced by central Mϕ within their cytoplasmic processes on plastic surface immediately after isolation. At this point, they were not easily released from the Mϕ by vigorous pipetting. However, as EI were further cultured, the processes of the Mϕ were retracted and the binding could be dissociated by pipetting. Therefore, the Mϕ appear to reinforce the binding of erythroblasts by extending the processes between them.

As demonstrated by Bessis, *et al.* (4), erythroid cells of isolated EI showed different maturation stages and formed a gradient of maturation relative to distance from the center of Mo. In the culture with EPO, synchronous expansion of erythroblasts occurred in the center of Mϕ surface. The presence of EI possessing 8 to 32 synchronous orthochromatic erythroblasts at day 2 of culture, implies that some isolated EI contain EPO-sensitive late erythroid precursors, probably corresponding to CFU-E, and EPO play an important role in the maintenance of EI.

Rich, *et al.* (14) reported that mouse bone marrow and spleen Mϕ obtained by cultivating bone marrow or spleen cells in teflon bags, secrete EPO. Recently, they showed that a subpopulation of bone marrow-fixed Mϕ are capable of producing EPO using a combination of in situ hybridization and immunohistochemistry with F4/80 antibody (19). In the culture without EPO used here, however, the erythropoietic activity of EI disappeared within 24 hours. This indicated that Mϕ in

splenic erythropoietic foci did not secrete enough EPO to sustain the erythropoietic activity. To obtain clear evidence for this, another approach using immunocytochemical methods with specific antibody against EPO is necessary.

It is of interest that cluster-forming immature myeloid cells attach and proliferate on Forssman$^+$ Mϕ. Our previous experiments or short-term bone marrow culture without EPO, also showed that developing myeloid cells were attached to Forssman$^+$ Mϕ (13). The presence of bone marrow "granulocytic islands" described by Weiss (20) or "granulocytic clusters" obtained by collagenase digestion of the bone marrow (5) supports the idea that the attachment of immature myeloid cells to the Mϕ is not an artificial event based on their affinity to the Mϕ during the isolation but a reflection of in situ EI form.

Much data has been obtained concerning the secretion of CSA from Mϕ (11, 12). However, its significance in steady-state myelopoiesis is still unknown, since the Mϕ used in the studies is different from hematopoietic tissue-fixed Mϕ. The present result that Forssman$^+$ Mϕ secreted CSA, suggests that stromal Mϕ involved in splenic erythropoietic foci potentially contribute to myelopoiesis.

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