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Proliferation, Migration and Platelet Release by Megakaryocytes in Long-term Bone Marrow Culture in Collagen Gel

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ABSTRACT. **The proliferation of megakaryocytes, their migration, and their platelet release processes were observed in long-term bone marrow culture in collagen gel. Megakaryocytes proliferated for more than 6 months, not only with myeloid cells but also with sinusoid-like capillaries. The megakaryocyte count decreased at 2 weeks of culture, increased to more than 400 at 6 weeks, and then decreased to about 100. Megakaryocyte colonies appeared after 2 weeks of culture; the number increased to more than 10 at 4 weeks of culture, and was maintained at that level.**

Morphologically, most fully mature megakaryocytes extended several long antennae-like processes, with periodic constrictions, demarcation membranes (DM), and platelet fields. Pro-platelets were released from these processes primarily by a "pinching off" mechanism, but some megakaryocytes released pro-platelets by the dissociation of DM, without extending long processes. Mature megakaryocytes migrated like ameba in collagen gel, and a few migrated to the abluminal side of the capillary or into the capillary; in both cases releasing pro-platelets into the capillary lumina. These observations were very similar to those noted *in vivo*.

Megakaryocytes are the homopoietic constituents of the bone marrow. They originate from hematopoietic stem cells, mature in the bone marrow, and then release platelets in their final maturation stage. However, where the platelets are released is still unclear.

Morphological studies of bone marrow have shown that megakaryocytes are located adjacent to bone marrow sinusoids; then extend their pseudopods, or projections, into sinusoids through endothelial fenestrae (3, 6, 16, 21, 26). These findings suggest that platelets are released from megakaryocyte processes by a "pinching-off" mechanism.

Several investigators have reported the presence of mature magakaryocytes and megakaryocyte nuclei in the pulmonary vessels (2, 10, 12, 13, 22, 25), where an increase in platelet count in the pulmonary circulation was also observed. This suggests that platelets were released in the pulmonary circulation from these megakaryocytes.

The growth and differentiation of megakaryocytes have also been studied *in vitro* with the megakaryocyte colony assay (CFU-Meg) and in systems involving freshly isolated megakaryocytes. CFU-Meg studies have identified several growth factors that are necessary for the growth of megakaryocyte colonies (9, 23). However, these megakaryocytes usually did not release platelets (17, 18). Freshly isolated megakaryocytes from bone marrow were therefore used to study platelet release (7, 15). In these systems, however, the stromal cell-megakaryocyte interaction cannot be studied, since there are no stromal cells in the culture. In Dexter-type long-term bone marrow culture (LTBMC), hemopoietic cells proliferate together with a layers of stromal cells (1, 5). However, in the LTBMC, megakaryocytepoiesis is very rarely seen, and if it does occur, it continues for only 5 weeks (30); further, the stromal cells do not form three-dimensional structures, such as capillary lumens.

We have recently succeeded in establishing long-term bone marrow cultures in collagen gel (LTBMC-G) (20). In this system, many megakaryocytes proliferate for up to 6 months and they release platelets, while myeloid cells also proliferated and sinusoid-like capillaries are formed. In this study, we examined the proliferation of megakaryocytes and observed stromal cell-megakaryocyte interaction, and the migration of, and platelet release by, megakaryocytes in LTBMC-G.

MATERIALS AND METHODS

Cells. Bone marrow was obtained from mice femora (C3H/He, 6–8 weeks old) by flushing with Fisher's medium using a syringe fitted with a 23-gauge needle. The marrow was

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dispersed carefully by several passages through a Pasteur pipet.

Collagen gel culture. Collagen gel was prepared as reported previously (19). Briefly, acidic collagen solution (Cellmatrix Ia, Nitta Gelatin Co., Osaka) was mixed with reconstitution buffer (2.2 g $NaHCO_3$ in 100 ml of 0.05 N NaOH and 200 mM HEPES) at 4°C. This was then mixed with 10× Fisher's medium (Gibco, NY) to adjust the osmotic pressure, and was added to an equal volume of marrow suspension. This mixture was plated onto 35-mm Petri dishes (0.4 ml per well; Corning, NY), and allowed to gel for 15 min at 37°C. Each dish contained approximately 2×10^6 bone marrow cells. The dishes were then overlaid with 2 ml of Fisher's medium, containing 20% horse serum and 1×10^{-6} M hydrocortisone 21-hemisuccinate, after which they were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C. Half of the medium in the dishes was changed weekly.

Observations. Cultures were observed under phase contrast microscopy for up to 25 weeks. Two gels were fixed every week with 4% paraformaldehyde, and were then stained histochemically for acetylcholinesterase (11) and counterstained with 0.5% toluidine blue. Megakaryocytes and megakaryocyte colonies were counted and the colony size measured. Megakaryocytes were identified as cells staining for acetylcholinesterase or having large nuclei (nuclear diameter > 16 microns). Immature megakaryocytes were distinguished from the mature cells by their low or undetectable staining for acetylcholinesterase and a high nuclear: cytoplasmic ratio. Small and large megakaryocyte colonies, consisting of more than 4 or more than 20 cells, respectively, were counted. Colony size was estimated from the length of the long and short axes of the colony.

Other gels were fixed each week with 2.5% glutaraldehyde for 2 hr, stained with 0.5% toluidine blue, and then examined by light microscopy. Hemopoietic foci containing megakaryocytes were excised from toluidine blue-stained gels and processed for electron microscopy. They were post-fixed with 1% OsO_4 for 1 hr, dehydrated through a graded ethanol series, and routinely embedded in Epon 812. Ultra-thin sections, cut on a Reichert Ultracut E ultramicrotome, were stained with uranyl acetate and lead citrate and observed under a JEM 100CX electron microscope.

Ploidy distribution of megakaryocytes. For the calculation of megakaryocyte ploidy, gels were digested with 0.25% collagenase, type I (Sigma), at 37°C for 20 min and washed twice with culture medium; cyto-centrifuge preparations were then made. These samples were fixed in 70% ethanol for 10 min, digested with ribonuclease (Boehringer Mannheim, Germany) at 37°C for 30 min, and stained with 100 μg/ml propidium iodide (Sigma) for 10 min (4). The DNA content of each megakaryocyte was calculated by measuring the fluorescence intensity of propidium iodide with a microscope photometer MSP-65 (Zeiss).

RESULTS

As we reported previously (20) megakaryocytes were successfully maintained in mixed culture with myeloid

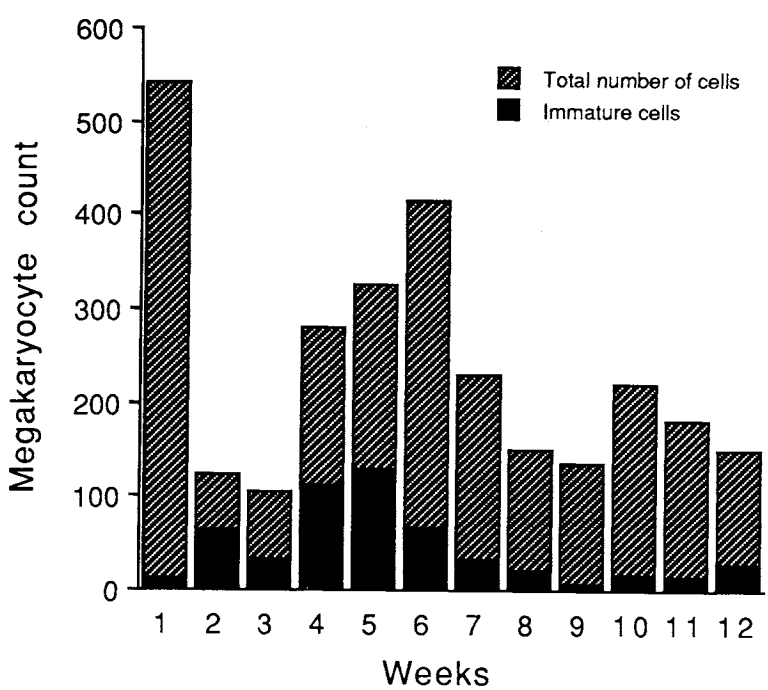

Fig. 1. Changes in megakaryocyte counts in LTBMC-G. The graph shows one series of data in duplicate experiments. Each point represents the mean for 2 culture dishes.

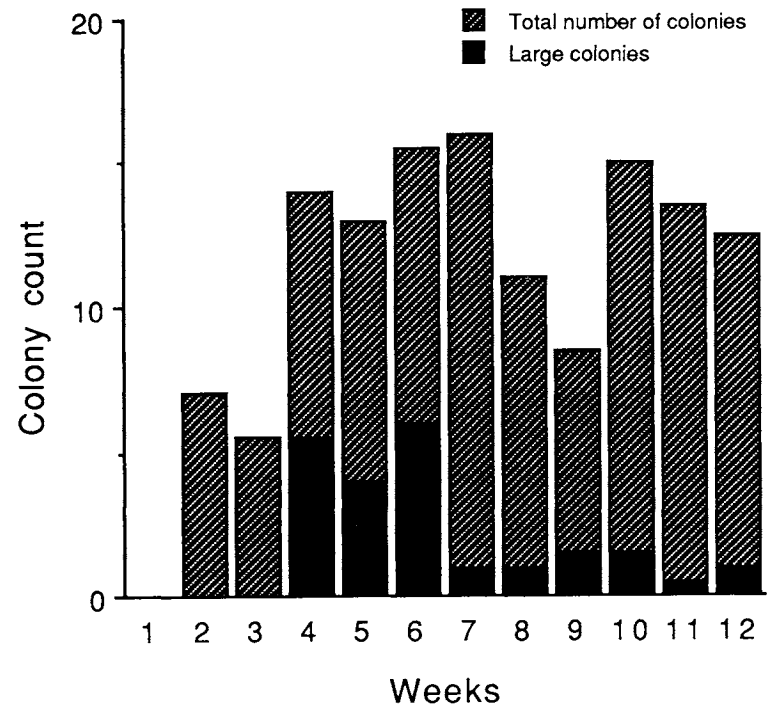

Fig. 2. Changes in megakaryocyte colony counts in LTBMC-G. The graph shows one series of data in duplicate experiments. Each point represents the mean for 2 dishes.

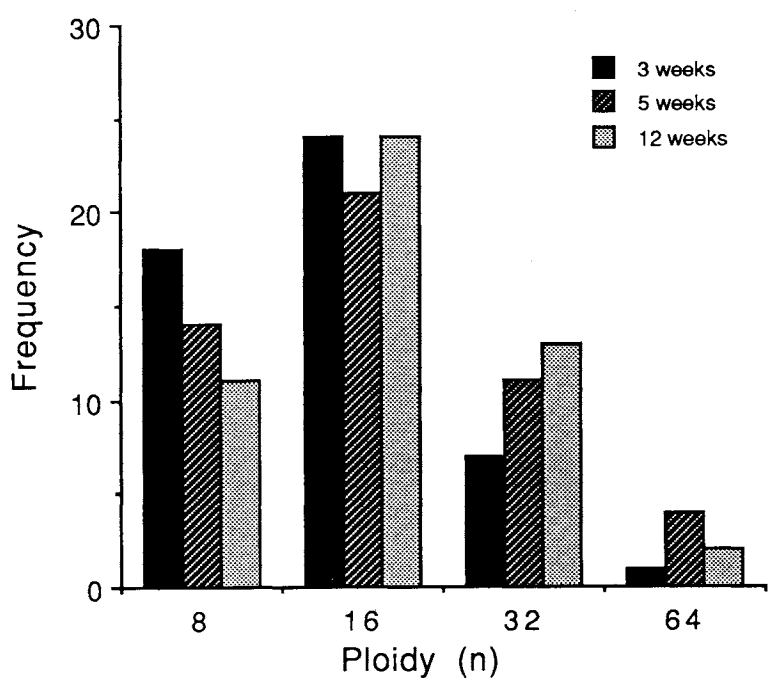

Fig. 3. Ploidy distribution of megakaryocytes after 3, 5, and 12 weeks of culture. The graph shows one series of data in duplicate experiments. Fifty megakaryocytes were calculated at each point.

and stromal cells, in which sinusoid-like capillaries formed, for more than 6 months. Figs. 1 and 2, respectively, show the megakaryocyte count and megakaryocyte colony count for the first 12 weeks. After 1 week of culture, more than 500 megakaryocytes were observed although there was no colony formation. Almost all were mature cells and showed pro-platelet formation (Fig. 4a, b). After 2 weeks of culture, the megakaryocyte count decreased markedly and many clusters of pro-platelets (with acetylcholinesterase-positive granules) were observed. At the same time, the number of immature megakaryocytes increased and megakaryocyte colonies appeared. These findings suggest that the megakaryocytes observed after 1 week in culture were derived from the bone marrow and not formed *in vitro*. The megakaryocyte count then gradually increased, to more than 400, after 6 weeks of culture, and thereafter decreased to about 100–200. The proportion of immature cells peaked at week 5, then decreased. Megakaryocyte colonies appeared after 2 weeks of culture, and their number increased to more than 10 after 4 weeks (Fig. 2). Large colonies, consisting of more than 20 megakaryocytes, appeared at week 4, at which point they comprised about 30% of the total number of colonies. From week 7, the number of large colonies decreased to a low level. The average megakaryocyte count per colony was 7.5 cells at 2 weeks; this increased to 36.2 cells at 4 weeks, and then decreased. The size of the colonies also increased up to week 6, at which time the major axis of the largest colony was 3.2 mm and the colony contained more than 60 megakaryocytes. The lifespan of a megakaryocyte colony was about 3–4 weeks. Gener-

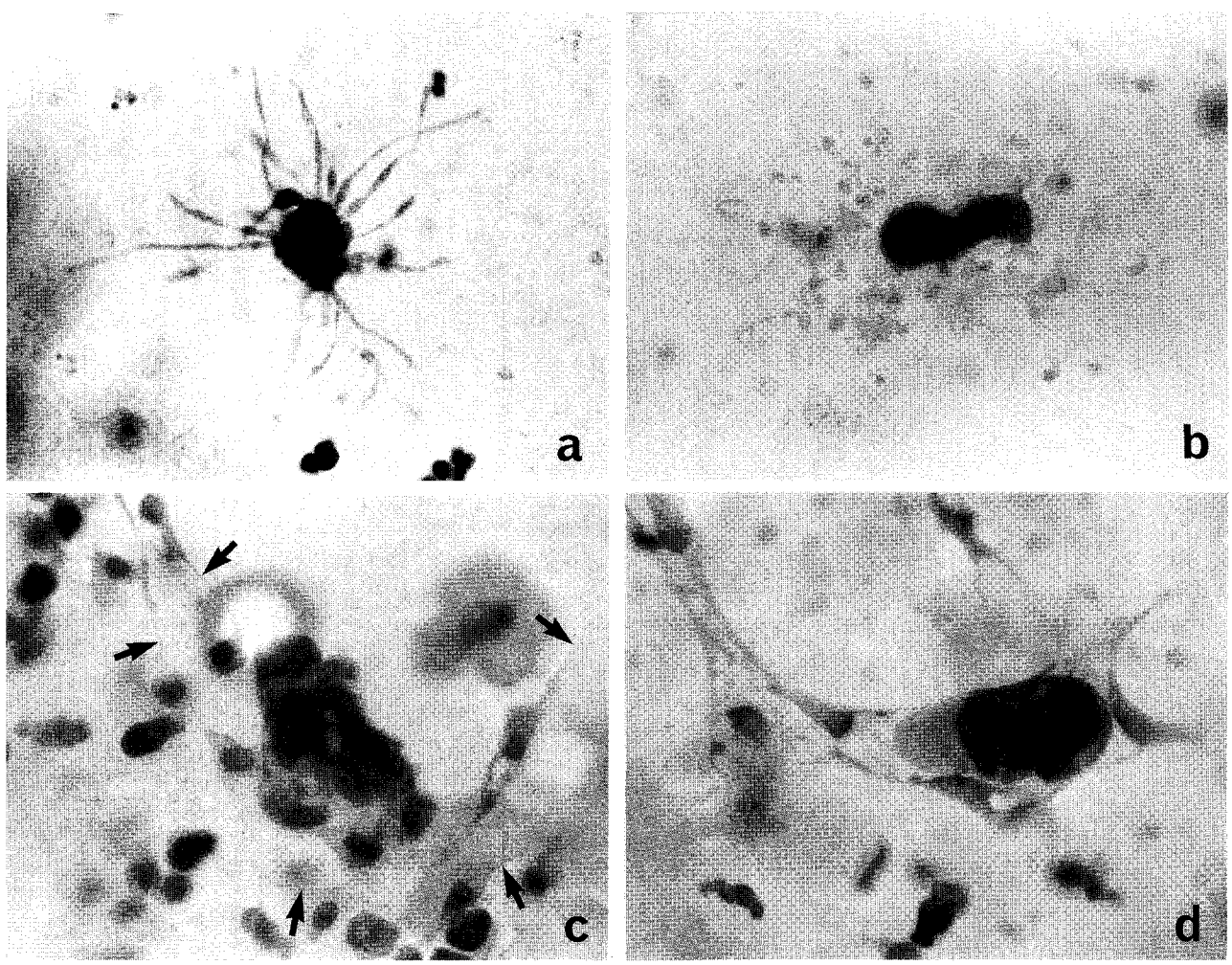

Fig. 4. Light microscopic pictures of megakaryocytes (toluidine blue stain). a, mature megakaryocyte showing more than ten long processes with periodic constrictions (×380). (Some of the processes are out of focus); b, megakaryocyte that has already released pro-platelet fragments, several of which have thin tails (×760).; c, megakaryocyte situated just outside a capillary wall (arrows, ×900); d, megakaryocyte in a capillary lumen (×900).

ally, the distance between the immature megakaryocytes was small, while the distance between mature cells was large. Several megakaryocytes were found to be further than 1 mm from neighboring megakaryocytes, and we could not estimate from which colony they had migrated. Fig. 3 shows the ploidy of megakaryocytes at 3, 5, and 12 weeks of culture. Almost all of the megakaryocytes showed 8–32 n ploidy. The number of megakaryocytes showing 8 n ploidy at 3 and 5 weeks was greater than that at 12 weeks. This finding is compatible with the higher ratio of immature megakaryocytes seen at 3 and 5 weeks compared with that at 12 weeks (Fig. 1).

Morphological observations revealed that some megakaryocytes had an ameba-like shape. Serial observations by phase-contrast microscopy demonstrated megakaryocyte migration. Several megakaryocytes migrated more than 50 microns in a day, but the majority migrated only a short distance. The majority of fully mature megakaryocytes extended long antenna-like processes, with periodic constrictions (Fig. 4a), the number of these processes ranging from one to more than ten. However, a few fully mature megakaryocytes had pyknotic nuclei and granular cytoplasm without any long processes. Fig. 4b shows a megakaryocyte with several discharged pro-platelet fragments around it. The nucleus of this megakaryocyte was lobulated, pyknotic, and almost bare. The size of the pro-platelet fragments varied, and some had tadpole-like tails, which suggested that they were formed by fragmentation of the long processes. A few megakaryocytes were located just outside the capillary wall (Fig. 4c). It was not clear whether these cells extended their processes into the capillary lumen. Fig. 4d shows a megakaryocyte that had migrated into a capillary lumen, but this was very rarely seen (it occurred with around 1% of megakaryocytes).

Electron microscopy showed that the majority of fully mature megakaryocytes had long processes (Fig. 5), with several constrictions, platelet fields, and DM areas. Fig. 6 shows a megakaryocyte releasing pro-platelet fragments. The nucleus of this cell became pyknotic,

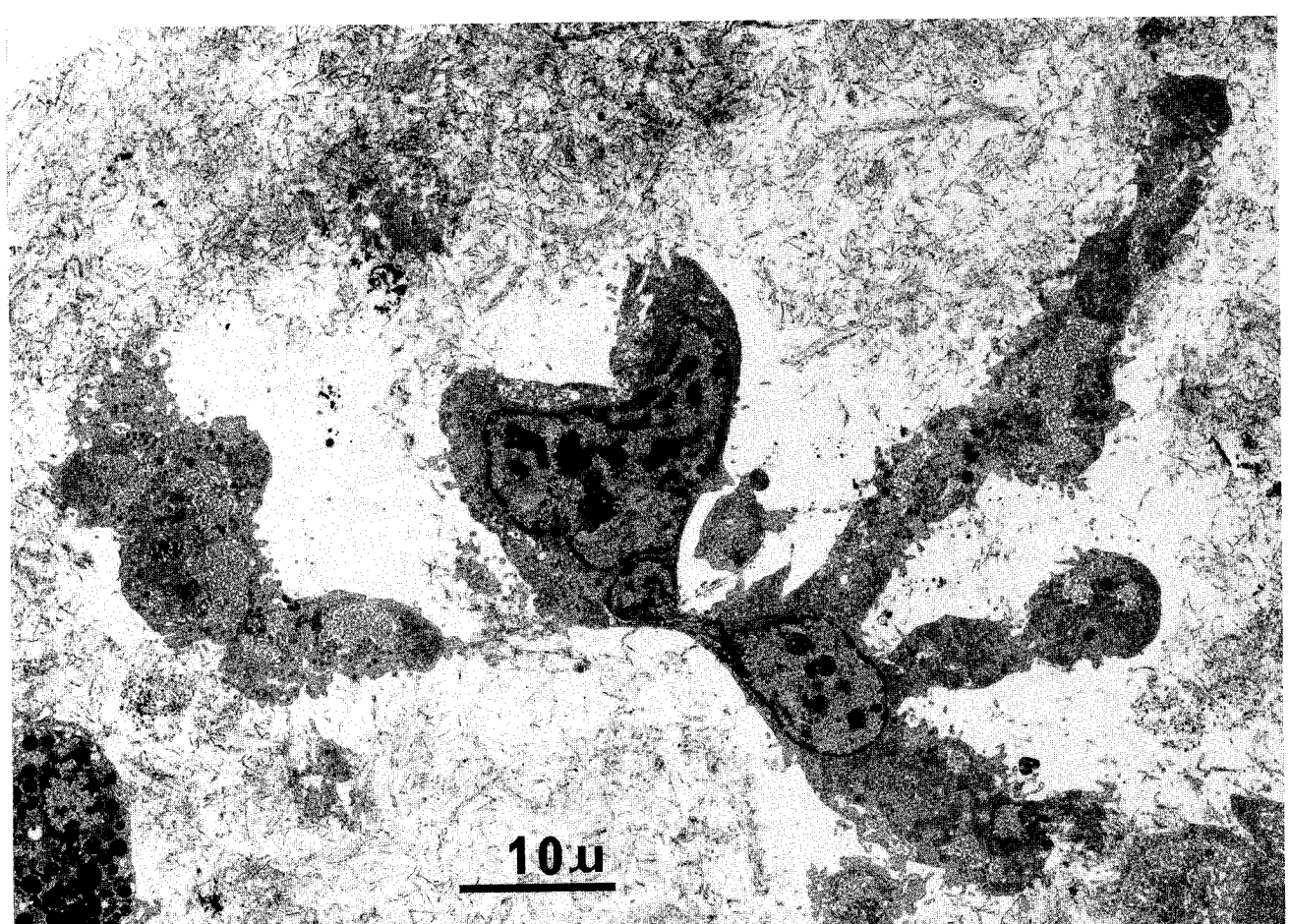

Fig. 5. A fully mature megakaryocyte exhibiting long processes with constrictions. Platelet fields and DM areas appear alternately in these processes. (×2,000)

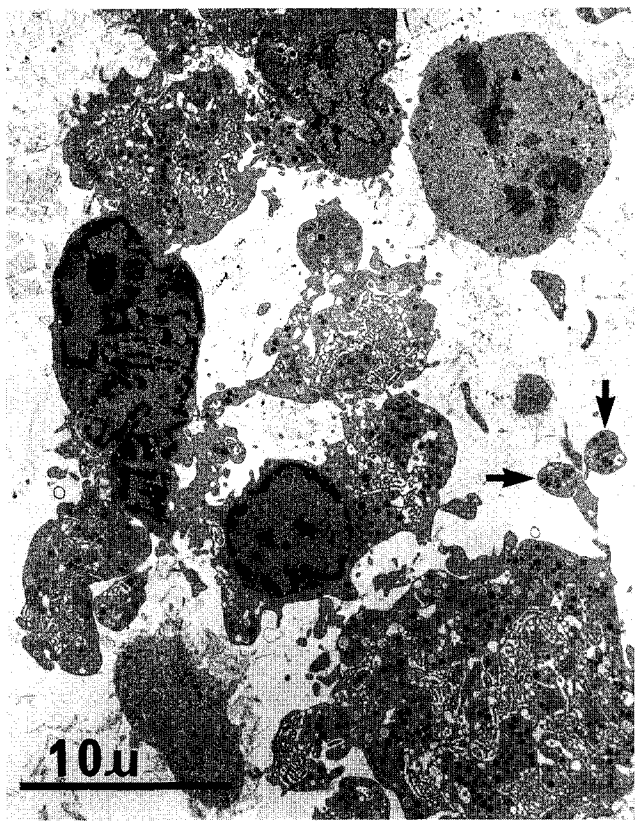

Fig. 6. A megakaryocyte discharging pro-platelets of various sizes; the small pro-platelets (arrows) are similar in size to platelets. (×2,800)

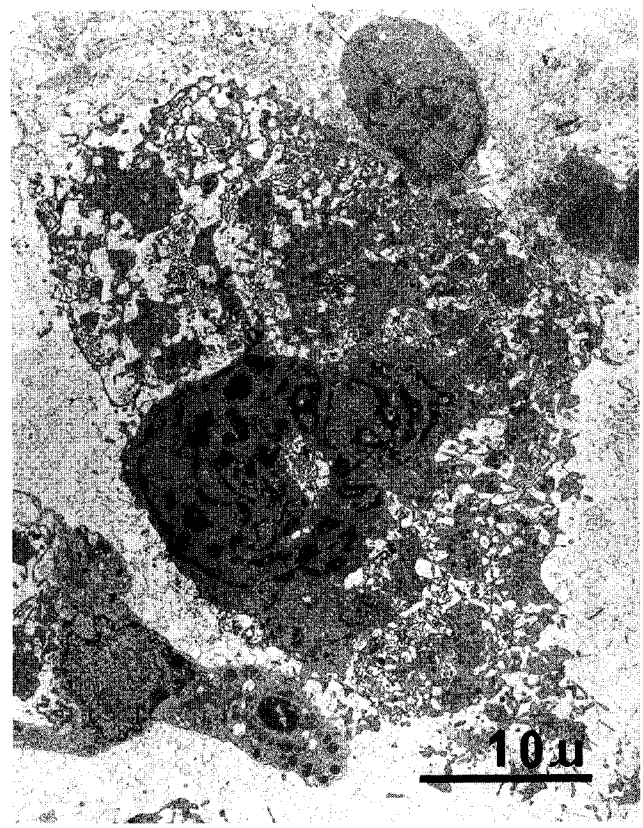

Fig. 7. A fully mature megakaryocyte. Platelet fields are dissociated by dilated DM. (×2,300)

and showed complicated invaginations, being covered with only thin cytoplasm, except at both sides, which were connected to cytoplasmic knobs. There were large fragments with several platelet fields and DM areas, and there were also several small fragments similar to mature platelets. This was the main type of platelet formation found in this culture. In contrast, several megakaryocytes showed another type of platelet formation (Fig. 7), in which the megakaryocytes had condensed and lobulated nuclei, but no cytoplasmic processes. Their peripheral zone was broken, and their DM had dissociated into the platelet field.

We also observed megakaryocyte migration and the release of platelets into the capillary lumens. Fig. 8 shows a megakaryocyte body located just outside a thin capillary wall, while pro-platelets were seen in the capillary lumen, suggesting that these pro-platelets were released from the megakaryocyte outside the capillary. Fig. 9 shows the same megakaryocyte as that shown in Fig. 4d. A fully mature megakaryocyte with a lobulated nucleus was seen in the capillary lumen. All of the megakaryocytes we observed in the capillary lumens were of the mature form, and some had released pro-platelets (Fig. 10).

We did not observe any interaction between the megakaryocytes and other stromal cells, such as fibroblastic cells and macrophages, which often interact with myeloid and erythroid cells.

DISCUSSION

In LTBMC-G, in contrast to conventional CFU-Meg and LTBMC, megakaryocytepoiesis was maintained for a long period and these megakaryocytes developed fully and released platelets. We have recently succeeded in maintaining long-term hemopoiesis with megakaryocytes on stromal cells isolated from mouse spleen (manuscript in preparation). In this culture system, megakaryocytes did not show platelet release, as was the case with LTBMC. McLeod *et al.* (17) observed platelet release from megakaryocytes in CFU-Meg in a plasma clot culture when the culture contained erythropoietin. However, the addition of erythropoietin did not induce platelet formation in the megakaryocytes found in long-term hemopoiesis on the spleen stromal cells mentioned above. Lanotte (14) found that megakaryocytes released platelets in CFU-Meg in collagen gel. Thus, it is

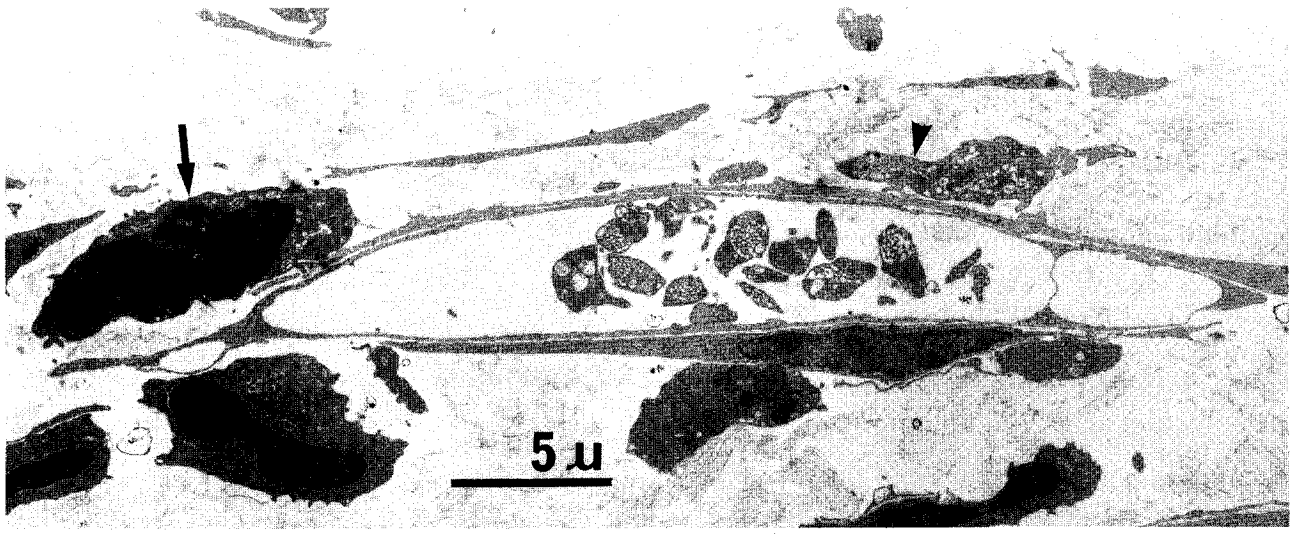

Fig. 8. A megakaryocyte releasing platelets into a capillary lumen. Its body (arrow) and process (arrowhead) remain outside the capillary. (×4,000)

conceivable that the collagen gel and well differentiated stromal cells in LTBMC-G may play an important role in the maturation and platelet release of megakaryocytes. These factors that promote platelet release are not yet clear, however, it has been reported by Williams et al. (30), that medium conditioned with LTBMC stimulated CFU-Meg formation on the addition of WEHI-3 conditioned medium. They also reported that megakaryocytes from CFU-Meg released platelets even in soft agar culture, on the addition of conditioned medium from LTBMC. Thus, humoral factors produced by stromal cells may also play an important role in the proliferation and differentiation of megakaryocytes in LTBMC-G.

Morphological observations of LTBMC-G showed that the majority of megakaryocytes had elongated processes and liberated platelets by a "pinching off" mechanism. This observation is similar to those made in isolated megakaryocytes (7, 15). To the best of our knowledge, our findings showed, for the first time, that

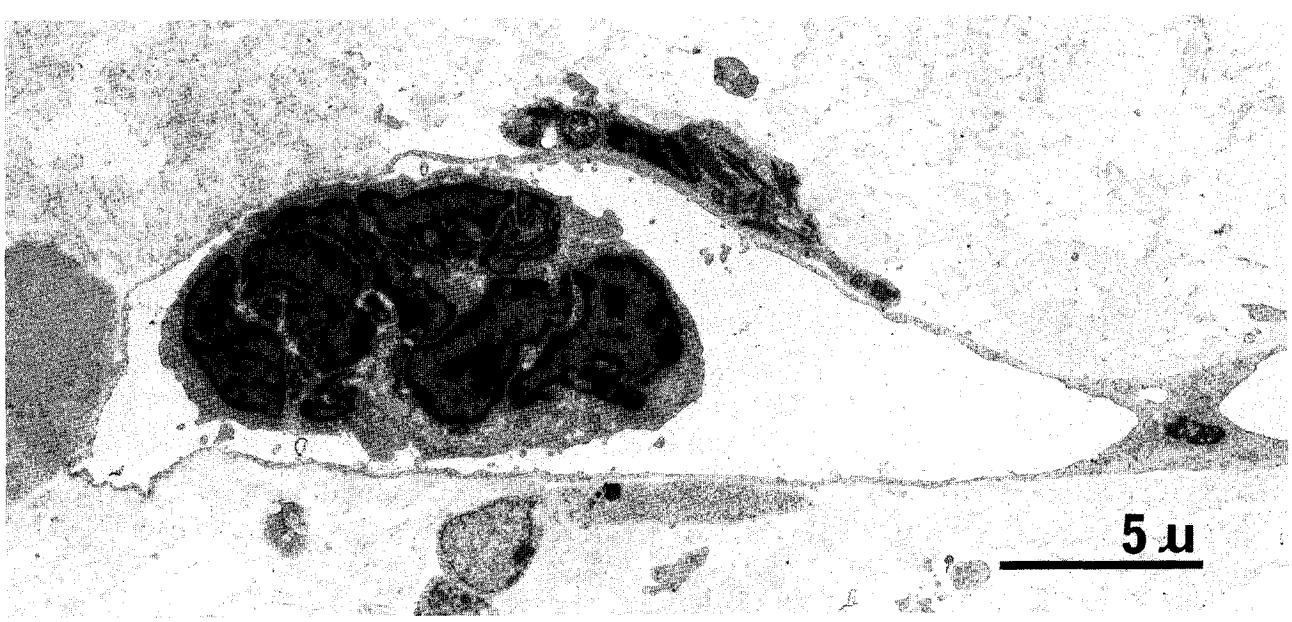

Fig. 9. A megakaryocyte in a capillary lumen (same cell as that shown in Fig. 1d). The nucleus is condensed and lobulated. (×5,000)

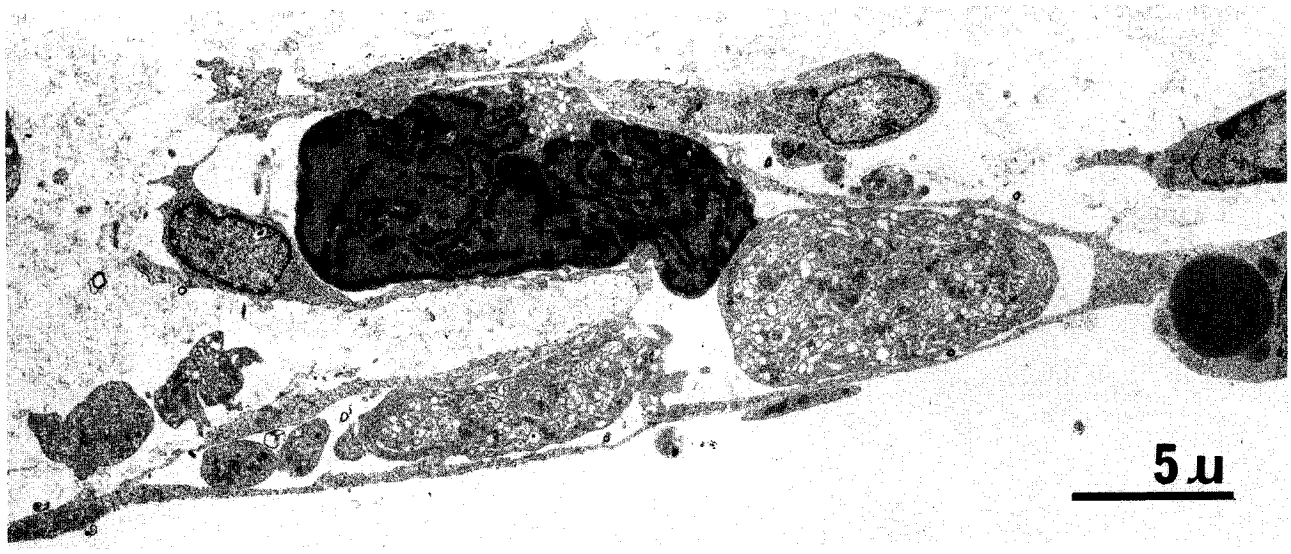

Fig. 10. A megakaryocyte in a capillary lumen. Its nucleus is covered with very thin cytoplasm; almost all of the cytoplasm is separated into small and large pro-platelet fragments. (×4,000)

megakaryocytes *in vitro* have the capacity to migrate; a few migrated to the abluminal side of the capillary and liberated platelets into the capillary lumen. These observations are very similar to *in vivo* findings. In bone marrow, megakaryocytes preferentially have para-sinusoidal locations and their long processes reach into sinusoids, penetrating the endothelial layer (3, 16, 21, 24). Platelets are formed by "pinching off" at intervals along these processes.

Although many investigators have reported megakaryocytes in the pulmonary circulation, the migration of these cells into marrow sinusoids has rarely been observed. Only Tavassoli & Aoki (27, 28) have reported the migration of entire megakaryocytes into bone marrow sinusoids. In this study, we demonstrated, for the first time, the migration of megakaryocytes into capillary lumens *in vitro*, thus confirming the migration of megakaryocytes into bone marrow sinusoids. We also showed pro-platelet release from megakaryocytes in the capillary lumen without the help of the bloodstream. *In vivo*, these megakaryocytes that migrated into sinusoids may reach the pulmonary vessels via the bloodstream. Platelets would then be liberated by the fragmentation of megakaryocyte cytoplasm (8, 13, 29).

These findings support the idea that platelets are formed both in the bone marrow and in circulating blood. These two processes may cooperate in platelet production.

As we have mentioned above, LTBMC-G not only showed the three-dimensional configuration of stromal cells but also maintained multilineal hemopoiesis. This culture more closely resembles bone marrow than conventional cultures, and its advantages are that morphological observations can be made of stromal cell-hemopoietic cell interactions. However, the rate of association between megakaryocytes and capillaries is very low in this culture system. This may be due, partly, to the low capillary density in LTBMC-G. This low density probably reflects the lack of chemotactic factor or cell-cell interaction that may play an important role in megakaryocyte-endothelial association *in vivo*. Thus, in this system, only those megakaryocytes that encounter capillaries by chance can associate with them. Those factors that regulate the differentiation and migration of megakaryocytes may be clarified in this system.

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