Microencapsulated Human Bone Marrow Cultures: A Potential Culture System for the Clonal Outgrowth of Hematopoietic Progenitor Cells

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Currently the most successful methods for culturing human hematopoietic cells employ some form of perfused bioreactor system. However, these systems do not permit the clonal outgrowth of single progenitor cells. Therefore, we have investigated the use of alginate–poly-L-lysine microencapsulation of human bone marrow, combined with rapid medium exchange, as a system that may overcome this limitation for the purpose of studying the kinetics of progenitor cell growth. We report that a 12 to 24-fold multilineage expansion of adult human bone marrow cells was achieved in about 16 to 19 days with this system and that visually identifiable colonies within the capsules were responsible for the increase in cell number. The colonies that represented the majority of cell growth originated from cells that appeared to be present in a frequency of about 1 in 4000 in the encapsulated cell population. These colonies were predominantly granulocytic and contained greater than 40,000 cells each. Large erythroid colonies were also present in the capsules, and they often contained over 10,000 cells each. Time profiles of the erythroid progenitor cell density over time were obtained. Burst-forming units erythroid (BFU-E) peaked around day 5, and the number of morphologically identifiable erythroid cells (erythroblasts through reticulocytes) peaked on day 12. We also report the existence of a critical inoculum density and how growth was improved with the use of conditioned medium derived from a microcapsule culture initiated above the critical inoculum density. Taken together, these results suggest that microencapsulation of human hematopoietic cells allows for outgrowth of progenitor, and possibly preprogenitor, cells and could serve as a novel culture system for monitoring the growth and differentiation kinetics of these cells.
Key words: bone marrow cultures • hematopoietic progenitor cells • microencapsulation

INTRODUCTION

The eight mature blood cell types are believed to be derived from rare pluripotent stem cells that have the capacity for either self-renewal or differentiation along all hematopoietic lineages (e.g., refs. 2, 12). With differentiation and proliferation, stem cells give rise to single and multilineage committed progenitor cells, which in turn eventually produce a large number of mature blood cells. The initial long-term bone marrow cell cultures (LTBMCs) for human cells employed a static culture environment.[3] However, these cultures have rarely achieved the productivity or longevity found in cultures of other species.[1,4,11,13]

Human LTBMC productivity and efficiency was found to be significantly enhanced by increasing the medium perfusion rate to mimic the in vivo condition.[16,17] Subsequently, perfusion-based bioreactor systems have been developed to expand human hematopoietic cells from adult human bone marrow[7,15] as well as umbilical cord blood.[6] These cultures are not clonal; thus the kinetics of proliferation and differentiation from progenitor, or preprogenitor, cells cannot be monitored in these systems. Scientifically, such information is of great interest.

Microencapsulation, a technology that has been used for the growth of hybridoma[5,8] and islet cells,[8–10,14,18] has the potential to support clonal outgrowth of progenitor and perhaps preprogenitor cells. A combination of perfusion conditions and microencapsulation may provide a basis for the development of a culture system in which the full outgrowth of the mature progeny from a single cell can be monitored. Therefore, we have taken the first steps toward the development of a culture system that combines the benefits of rapidly perfused culture conditions with the potential clonal outgrowth of microencapsulated bone marrow cells. This article describes the chief characteristics of such a system, including total cell and progenitor cell production, cell and colony morphology, and the effects of inoculum density.

MATERIALS AND METHODS

Cells

Human bone marrow cells were obtained either from heparinized aspirates from the iliac crest of informed and consenting individuals or from mesh screens used during bone marrow harvests. The bone marrow was separated by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density

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gradient centrifugation, and the low-density mononuclear cells were collected and washed with Iscove's modified Dulbecco's medium (IMDM, Gibco Laboratories, Grand Island, NY) containing 2% fetal calf serum (Gibco) as described previously.[16]

Culture Medium and Hematopoietic Growth Factors

Cells were cultured in IMDM containing 10% fetal calf serum (Gibco), 10% horse serum (Gibco), 1% penicillin/streptomycin (Sigma Chemical Company, St. Louis, MO; 10,000 U/mL penicillin G and 10 mg/mL streptomycin), 0.036 *M* sodium bicarbonate (Sigma), and 10 μM hydrocortisone (17-hydroxycortiscosterone, Sigma). The media was supplemented with interleukin-3, granulocyte-macrophage colony stimulating factor, erythropoietin, and stem cell factor as described previously.[15]

Microencapsulation of Cells

(All concentrations are weight/volume.) The encapsulation procedure used in our laboratory was a modification of the technique originally developed by Lim and Sun.[8] Purified myeloid cells were resuspended in a sterile filtered 1.5% alginate (Keltone LV, Kelco, Chicago, IL) solution in 0.9% NaCl (Sigma, pH 7.4), at a concentration of 1.7×10^6 cells/mL alginate. Droplets were formed from the suspension by syringe-pump-driven extrusion at a rate of 0.6 mL/min through a 22-gauge stainless steel needle surrounded by a custom-made air jacket. Gel beads (0.7 to 0.8 mm in diameter) were obtained by collecting the droplets in a solution of 4°C, 1.5% calcium chloride (Sigma) for a maximum period of 25 min. All remaining microencapsulation steps were performed at room temperature. After washing twice with saline (0.9% NaCl, pH 7.4), the gel beads were coated for 6 min with 0.1% poly-L-lysine (MW 40 to 60 kD, Sigma) in saline. After a wash in 0.1% CHES [2-(*N*-cyclohexylamino)ethane-sulfonic acid, Sigma] buffer, pH 7.4, and a further wash in saline, the beads were finally coated with 0.15% sodium alginate in normal saline for 4 min. Following three washes in saline, the gel capsules were suspended in 1.5% trisodium citrate buffer, pH 7.4, for 8 min. After three more washes in saline, the capsules were washed and brought up to a total volume of 50 mL with culture medium (see above). The final total volume of capsules made was typically 80% to 90% of the initial alginate volume used. The controls for the following experiments used 12 to 13 mL of capsules per flask.

Culture Conditions

The capsules were grown in 50-mL glass spinner flasks (Belco Glass, Vineland, NJ) at an agitation speed of 70 rpm in a humidified 5% CO_2/95% air, 37°C incubator. Ten milliliters of the culture medium was exchanged daily. Cell counts were performed by removing a small volume of the well-agitated culture and placing it in a small, sterile dish. The total volume was brought up to 1 mL and the number of capsules was counted. The capsules were then gently broken by drawing the mixture into a 1-mL syringe and extruding it through a 23-gauge needle. Cell counts were performed in a hemocytometer. Dividing the total cell number per milliliter by the capsule number results in an average cell number per capsule.

Methylcellulose and Morphologic Assays

Clonogenic cells from the inoculum and harvested cells were enumerated as described previously.[15] Aliquots of harvested cells were centrifuged onto glass slides ("cytocentrifuged") and stained with Wright-Giemsa reagents before differential counts were performed. Cell viability was determined using trypan blue dye exclusion.

RESULTS

Culture Growth

Cell growth was found to be reproducible, as shown by the growth curves of three separate experiments (Fig. 1). These data show that the cultures had an average of 12- to 24-fold growth relative to the initial cell number. The brief exponential growth period had a doubling time of 3 to 4 days. Maximal cell density was reached around day 17.

Growth was monitored visually by examining the capsules under a microscope over time (Fig. 2). Initially the cells were evenly distributed throughout the capsules, and very few cell clumps were present (Fig. 2A). The capsule diameter was approximately 0.75 mm, and initially each capsule contained around 400 cells. The majority (>98%) of these cells were viable, as assessed by trypan blue dye exclusion. By the third day, clusters of varying sizes had developed in essentially all capsules (Fig. 2B). However, by

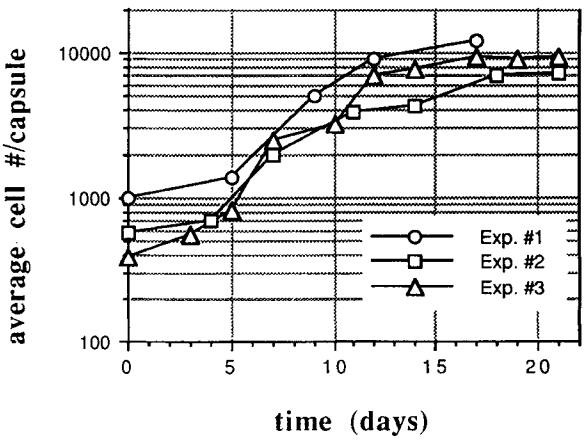

Figure 1. Growth curves of three separate experiments. Other results from experiment 3 are shown in Figures 2, 3, and 4, and results from experiment 2 are shown in Figure 5. These growth curves reflect an average number of cells per capsule, calculated as described in the Materials and Methods section.

day 7 each capsule contained only one or two significant colonies (Fig. 2C). The variation in colony morphology was quite clear by day 14 (Fig. 2D), and maximum colony size was achieved by days 17 to 19 (Fig. 2E).

Two clearly distinct colony morphologies were apparent. One type consisted of a single large, dense ball of cells, whereas the other consisted of multiple spherical clumps that often became brilliant red in color. The latter colonies resembled the burst-forming units erythroid (BFU-E) colonies that form in the methylcellulose assay. Examination of the culture after 10 to 12 days showed highly uneven colony distribution among the capsules. Some capsules contained one or more very large colonies, whereas others contained only small colonies or no cells at all.

A differential morphological count of stained cytocentrifuged cells from the culture at day 21 was performed (Fig. 2F). Approximately 72% of the cells were granulocytic, 22% were macrophagic, and 6% were erythroid. Around one-fifth of the macrophages (or 4% of the total cell population) were many times the size of other cells and contained dark, angular masses. The other macrophagic cells were much smaller and had highly vaculated cytoplasm. Therefore, the culture as a whole contained a multilineage cell population.

Colony Morphology

As described above, the colonies exhibited distinct morphology by day 14. Therefore, to determine what cell types these colonies contained, representative day 21 capsules were selected, isolated, photographed, and broken, and the cells were recovered. Cell counts and differential counts were then performed.

The capsule shown in Figure 3A is representative of the most packed capsules in the culture. About 9% of the capsules contained colonies within this size range and with this very dense, centralized morphology. This capsule contained 42,000 cells and was 98% granulocytic based on differential counts of cytospins (Fig. 3B).

The dominant colony in the capsule shown in Figure 3C is representative of the burst-like colonies described above. In this particular case, a smaller white cell colony (light brown to yellow in color) also is present at the bottom edge of the capsule. This capsule contained 11,000 cells. Based on morphological analysis (Fig. 3D), approximately 78% of these cells were late-stage erythroid cells, and 22% were macrophages.

Lineage Development

The number of progenitor cells present in the culture was monitored over time using methylcellulose assays performed on cells that were harvested from the microcapsules. The total number and density of erythroid progenitors (BFU-E's) peaked around day 5. Subsequently, the number and density of BFU-E's declined exponentially. This result is highly reproducible and can be seen in Figure 4. The number of granulocyte-macrophage progenitors (CFU-GM's) was also monitored during multiple experiments, and although the total number of these progenitors increased throughout the culture period, a consistent trend in production (such as timing or height of peaks) was not observed (results not shown).

The number of morphologically identifiable erythroid cells in the culture was also assessed over time using differential counts. Figure 4 shows that the percentage of morphologically identifiable erythroid cells (erythroblasts through to reticulocytes) peaked around day 12 and then steadily decreased for the rest of the culture period. Comparing both the erythroid progenitor data and the morphological data, one can observe the rate of erythroid lineage development within this culture system.

Inoculum Cell Density Effect

To determine how culture growth is a function of inoculum density, two experiments were performed. In the first, 42×10^6 fresh marrow cells were encapsulated in 25 mL of alginate. Twelve milliliters (*A*), 6 mL (*B*), and 3 mL (*C*) of the capsules were placed in separate flasks, so that the number of cells per capsule was the same initially but the number of cells per flask varied. The total volume of all flasks was brought up to 50 mL with medium, and the cultures were grown and sampled as described (Materials and Methods). Monitoring cell number per capsule over time showed that culture *A* grew well but the other cultures did not (results not shown). Examination of the capsules themselves showed that by day 17, 54% of the capsules from culture *A* contained fairly large colonies, whereas only 8% of the capsules from culture *B* contained colonies, and only 1% of the culture *C* capsules contained colonies.

A repeat of this experiment was subsequently performed with 55×10^6 fresh marrow cells encapsulated in 33 mL of alginate. In this case, 13 mL (*A*), 6.5 mL (*B*), and 3.3 mL (*C*) of the capsules were placed in separate flasks, respectively, and treated as before. A fourth culture (*S*), also containing only 3.3 mL of capsules, was supplemented with conditioned medium in the following manner. From day 1 through day 3, 20 mL of medium was exchanged with 10 mL fresh medium and 10 mL conditioned medium from the control culture (*A*). From day 4 on, 30 mL of medium was exchanged with 10 mL fresh medium, 10 mL conditioned medium, and 10 mL frozen conditioned medium from the previous experiment's control culture.

The growth curves of all four cultures are presented in Figure 5. As before, the control culture (*A*) grew best, and culture *C* did not grow well. However, in this case culture *B* grew better than during the previous experiment, although not as well as the control culture (*A*). These results were confirmed by observation of the capsules themselves. Culture *A* had one or more colonies per capsule, with the usual morphology. Culture *B* also had a number of colonies, but they tended to be different morphologically from the control culture. These colonies often had a feathered appearance

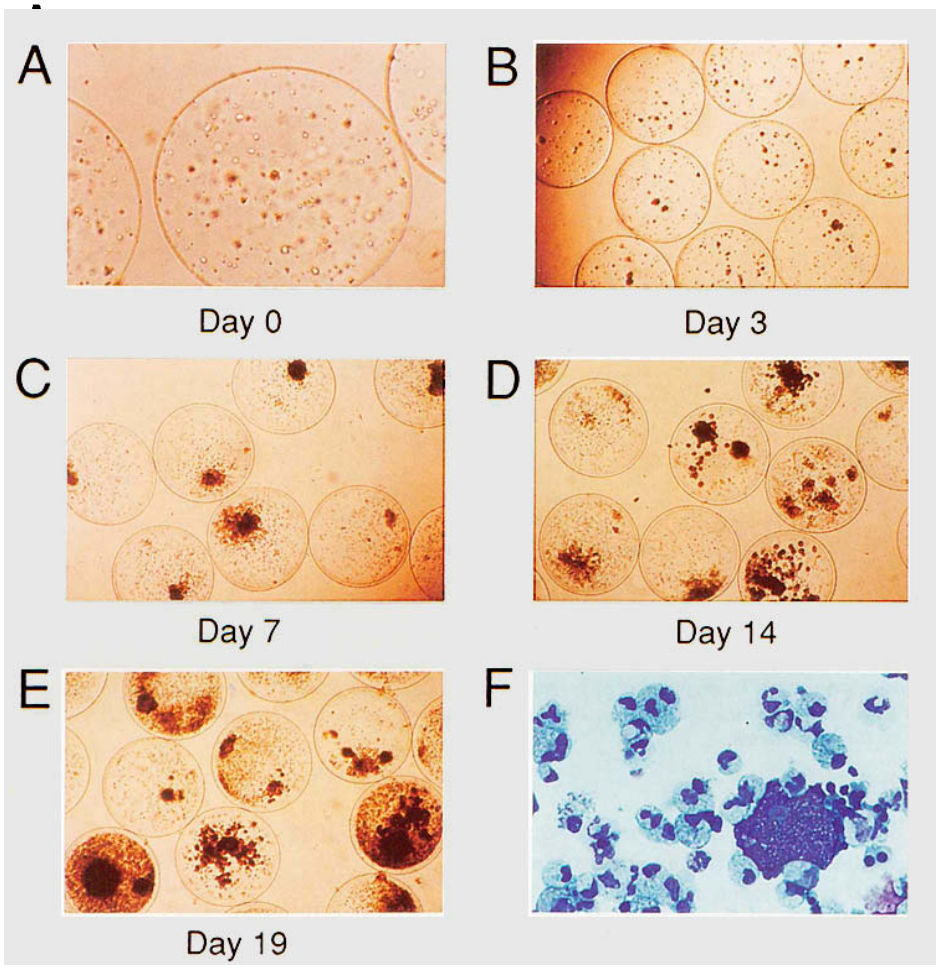

Figure 2. Photomicrographs of marrow containing microcapsules (from experiment 3, Fig. 1) at various times during the culture period (A–E) and of Wright–Giemsa stained cells from day 21 capsules (F).

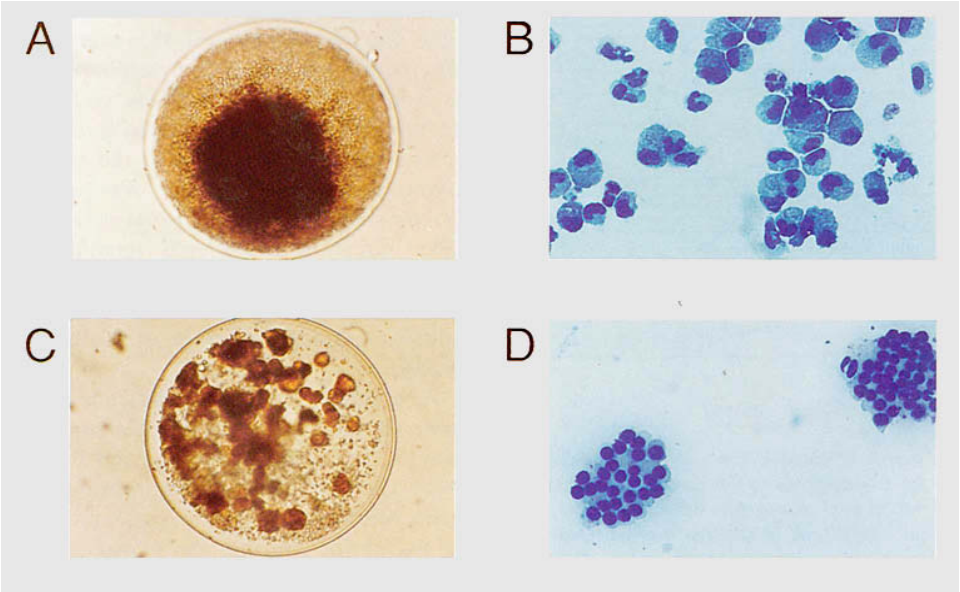

Figure 3. Photomicrographs of representative colony containing microcapsules (from experiment 3, Fig. 1) on day 21 (A and C) and their corresponding cytospins (B and D).

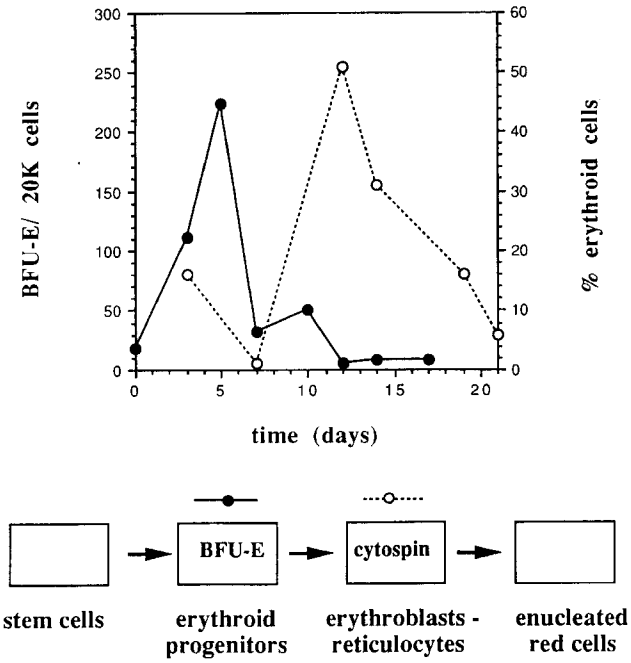

Figure 4. Graph and diagram depicting the progression of erythroid lineage development within the capsules over time. The number of erythroid progenitors (BFU-E) (●) was determined using the methylcellulose assay, and the percentage of erythroid cells (○) was determined by differential counts of cytospins. (Data from experiment 3, Fig. 1).

and, in some cases, were extremely dark in color. Again, only 1% of the capsules from culture *C* contained colonies. However, the addition of conditioned media did improve the growth of culture *S*. The morphology of these colonies was similar to those seen in culture *B*, but the growth was eight times better than in the culture without the addition of conditioned media, culture *C*.

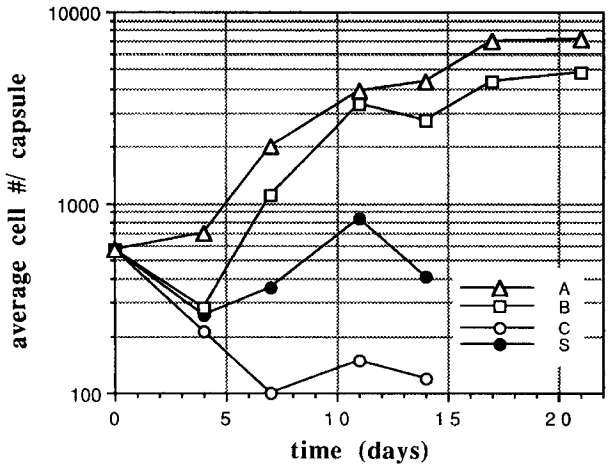

Figure 5. Cell growth curves of cultures with varying inoculum cell densities (*A*, *B*, *C*, and *S*): *A* corresponds to the normal control culture of 13 mL of capsules/50 mL of total culture, *B* to 6.5 mL of capsules, *C* to 3.3 mL of capsules, and *S* to 3.3 mL of capsules supplemented with conditioned medium. Also, *A* corresponds to experiment 2 in Figure 1. These growth curves represent the average cell number per capsule, calculated as described in the Materials and Methods section.

DISCUSSION

Multilineage hematopoietic growth is achievable in alginate poly-L-lysine capsules with rapid medium exchange. Twelve- to 24-fold overall expansion in total cell number occurred in approximately 17 days, a result that is comparable to other reported perfused human hematopoietic systems.[6,7,15] By the end of the culture period, about 9% of the capsules contained very large granulocytic colonies. Because these colonies are so much larger than all other colonies, the cells that initiated the formation of these colonies (approximately 1 in 4000 cells) must be responsible for the majority of the overall cell growth. This observation, confirmed by cytospin results from the culture as a whole, indicates that the system is predominantly granulocytic, although erythroid growth is also significant around day 12. This peak in the number of identifiable erythroid cells in the culture was preceded by a peak in the number of erythroid progenitor cells around day 5. Since sampling is a simple, reproducible procedure, our preliminary work with erythropoiesis suggests the use of microencapsulated bone marrow may be a useful way to follow the kinetics of lineage development in a perfusion system.

As alluded to above, the presence of distinct colonies in some capsules and none in others suggests that the colony growth in these capsules originates from single clonogenic cells. Therefore, with further refinement of this system in terms of cell number per capsule, microencapsulation may be useful for studying the clonal outgrowth of perfused human hematopoietic cells. In order for this refinement to be realized, capsule size will need to be reduced and/or

a lower inoculum density will need to be used. Recently, improved microencapsulation technologies have been described which allow for capsule diameter reduction.[10,18] These methods may prove to be viable alternatives to the droplet generator used in our studies. Also, if a lower inoculum density needs to be used, the limitations associated with a minimum inoculum density will need to be overcome. Since the critical inoculum density effect described by our results is due to variation in cell number per flask, but not cell number per capsule, it seems likely that a soluble growth factor is being produced and is responsible for the minimum inoculum size. Therefore, working with lower inoculum densities may involve either identifying this growth factor and supplying it exogenously or using conditioned medium in conjunction with fresh medium.

In conclusion, the hematopoietic culture system that we describe here combines the benefits of rapid medium exchange with the potential clonality provided for by microencapsulation. This system thus holds the potential for being a useful method for the study of the kinetics of growth, lineage development and differentiation of hematopoietic progenitor, and possibly preprogenitor, cells. Further studies are needed to develop this approach and definitively ascertain its use as a clonal culture system.

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