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Human Bone Marrow Contains Fibroblast Colony Forming Cells

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In a liquid culture of human bone marrow, the development of fibroblast colonies begins to take place on day 4. Twenty per cent fetal calf serum is used as the stimulus for fibroblast colony growth. Human bone marrow contains 47 ± 4 fibroblasts colonies per 2×10^5 nucleated cells plated. Bone marrow fibroblast cultures using agar or methylcellulose restrict colony formation. This fibroblast culture method may be useful for pathognomonic and clinical studies in hematological disorders.

(Key Words: Fibroblast colony, Human bone marrow, Liquid culture)

INTRODUCTION

Several studies have reported culture methods in which fibroblast colonies can be grown from a single cell sourse (3 4, 8, 9). There are several techniques and materials which have been used to initiate and sustain colony growth in these systems. Friedenstain et al performed fibroblast colony formation from the bone marrow of guinea pigs, rabbits and humans, and from spleen cells of guinea pigs and rabbits using a liquid culture (3, 4). Metcalf did it from mouse pleural cells using an agar culture (8). Wilson et al did it from mouse bone marrow using a methylcellulose culture (9). Attempts to use these systems for growing human bone marrow fibroblast colonies have rarely been studied.

The present work was undertaken to devise a simplified method of fibroblast colony formation from human bone marrow. The fibroblast colony growth of human bone marrow was obtained using a liquid culture medium containing fetal calf serum (FCS). This culture method gave a higher frequency of fibroblast colonies than in the previous report (4).

MATERIALS AND METHODS

Preparation of bone marrow. Bone marrow was drawn from 10 volunteers who were 20 to 40 years old and had not hematological disorders. All bone marrow for the cultures was obtained from the sternum. The specimens for cultures were collected by aspirating 0.5 to 1 ml of bone marrow into a syringe rinsed with 1:1000 heparin just prior to use. The aspirate was then transferred to a test tube. Bone marrow cells were separated by dextran

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sedimentation at room temperature for 30 min and washed three times in RPMI 1640 medium.

Culture method. For all experiments 35 × 10 mm plastic dishes (Falcon Plastics) were used. Ten thousand to two million cells in 2 ml of medium were placed in each dish. The culture medium consisted of RPMI 1640 and 5 to 50% heat-inactivated FCS. Fifty units each of penicillin and streptomycin were added to each 1 ml of medium. At weekly intervals, half, of the alliquots were replaced with fresh medium. Agar and methylcellulose culture systems were used for some cultures, incorporating 0.05 to 0.3% agar or 0.1 to 1% methylcellulose in RPMI 1640 with 20% FCS. Cultures were incubated for 28 days in 5% CO_2 in a fully humidified incubator.

Colony counting. Colonies developing at the bottom of the dish were counted by staining with Giemsa stain using a binocular dissecting microscope (×40).

Microscopic study. Cover slides were fixed at intervals up to 28 days in ethanol and stained with Giemsa stain, by McJunkin's method for peroxidase, by the PAS method for polysaccharides, and by Li's method (6) for esterase. To examine phagocytic activity of the fibroblasts, Gardner's method (5) was used. Polystylene latex particles (Dow Chemical Co.) in a physiological saline solution were added to the culture dish. The dish was then placed in a reciprocating-shaker bath at 82 rpm at 37°C. After a 30-min incubation, the cover slides were fixed in formalin vapor and stained with Giemsa stain. The slides were then placed in 100% xylene for 3 h with occasional shaking and observed microscopically.

RESULTS

Development of fibroblast colonies in monolayer cultures of bone marrow cells. In the early days of cultivation neutrophils or round cells with small nuclei and dark cytoplasm were predominant. On the second day there was a small number of large stretched cells with pale cytoplasm and ovoid nuclei containing several nucleoli among the cells. Individual fibroblasts formed small aggregates of cells. From the 4th day the number of these aggregates increased as did the size of individual aggregates (Figs. 1 and 2). On the 6th day separate foci of six to 40 large pale fibroblasts appeared among the histiocytes. All leukocytes had practically disappeared from the surface. A detailed size analysis was made on day 7 of incubation of all aggregates containing fibroblasts in cultures of 2×10^5 bone marrow cells. The result is shown in the histogram in Fig. 3. Fibroblast colonies varied widely in size from 6 to 100 cells, and the fibroblasts in the colonies were spread loosely, containing each other. On the 7th to 10th days the fibroblasts had formed large discrete colonies each of which contained 20 to 500 cells (Fig. 4). The number of histiocytes decreased sharply. On the 9th day the fibroblast colonies began to fuse. A morphological study of the fibroblast colonies showed that all cells had prominent nucleoli and cytoplasmic vacuoles. When peroxidase was tested, no enzymatic activity was demonstrated. PAS and α-naphthyl butylate esterase staining were positive. By testing phagocytic activity using polysyrene latex particles, it was observed that the fibroblasts had no phagocytic activity. The fibroblasts dif-

fered distinctly from the histiocytes.

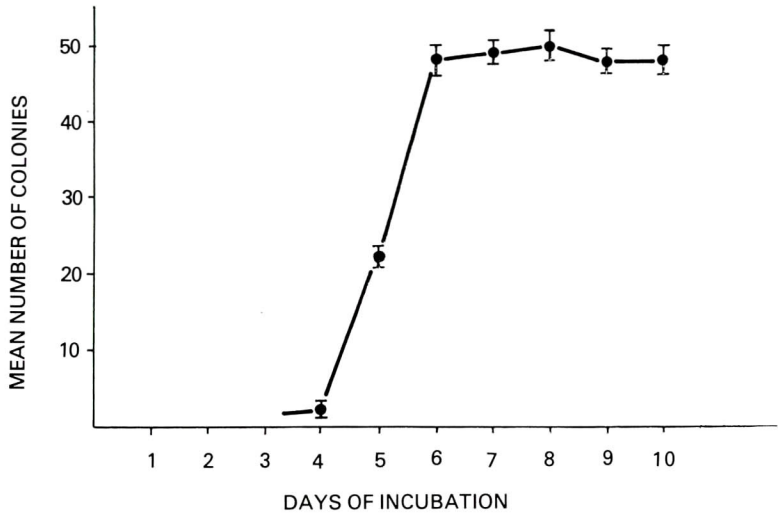

Fig. 1 Increase in mean number of fibroblast colonies with continued incubation. Each culture contains 2×10^5 bone marrow cells. Vertical bars are standard deviations of mean values from four cultured dishes.

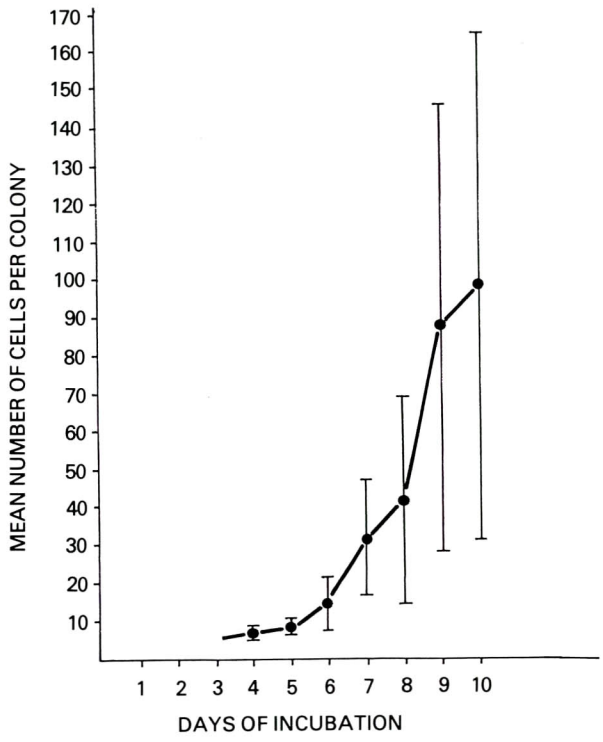

Fig. 2 Increase in mean size of fibroblast colonies with continued incubation. Each culture contains 2×10^5 bone marrow cells. Vertical bars are standard deviations of mean values from colonies of four cultured dishes.

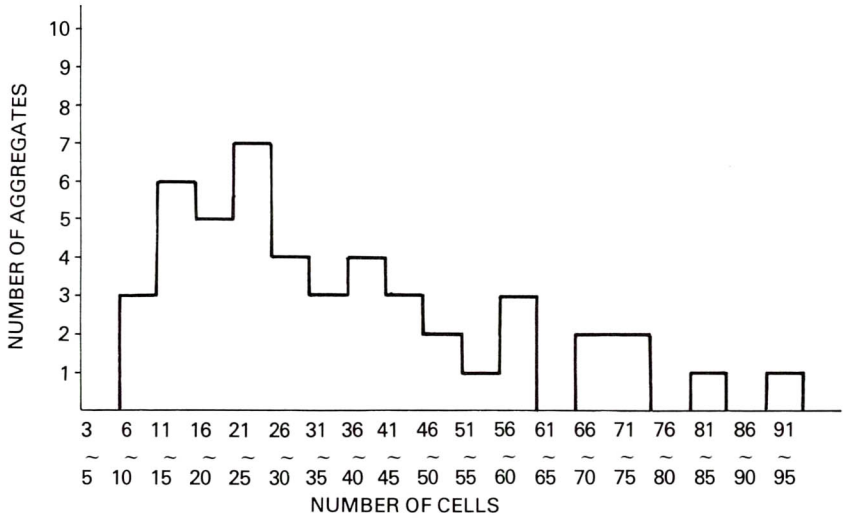

Fig. 3 Histogram of size distribution of cell aggregates containing fibroblasts, six or more cells in size, on day 7 cultures of bone marrow cells. Pooled data from three cultures containing 2×10^5 bone marrow cells.

Fig. 4 Fibroblast colony in a 9 day culture of bone marrow cells. Giemsa stain × 60 magnification.

Incidence of fibroblast colonies in monolayer cultures. Bone marrow cells suspensions at concentrations of 1×10^4 to 1×10^6 per 2ml of culture medium were incubated in the presence of 20% FCS. Titration of the number of bone marrow cells indicated a linear relationship between the

number of bone marrow cells cultured and the number of fibroblast colonies that developed (Fig. 5). This suggested that the fibroblast colony was of a single cell origin. At the lower extreme, eg., less than 1×10^5, the number of colonies was very few, and the higher extreme, eg., greater than 5×10^5, there was marked fusing between the colonies. Optimal cell growth on days 6 and 9 was noted when approximately 1 to 5×10^5 cells were plated. At this time, the optimal number of colonies containing more than six cells was approximately $25/10^5$ seeded cells.

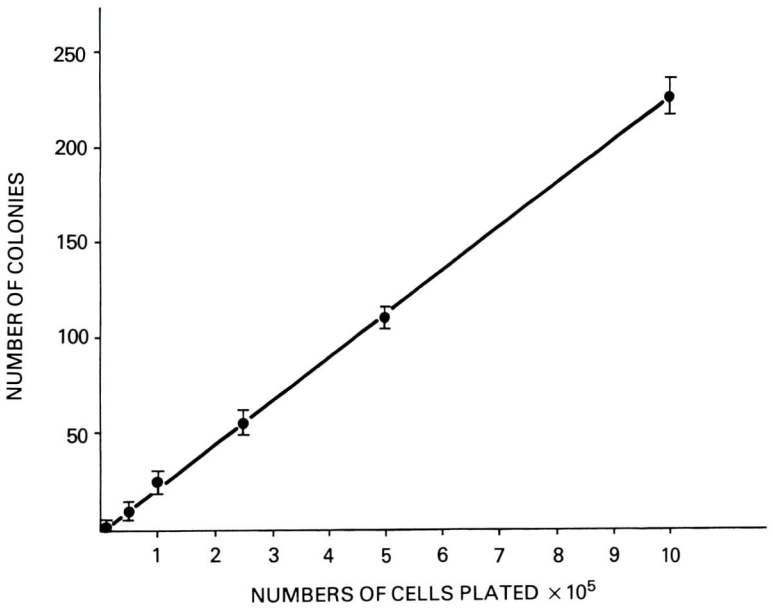

Fig. 5 Fibroblast colony formation with explantation of different numbers of bone marrow cells. Vertical bars are standard deviations of mean values from five normal subjects.

Optimal FCS concentration. The development of fibroblast colonies appeared to be a reasonably specific consequence of mixing bone marrow cells with FCS. Concentrations of FCS from 5 to 50% v/v produced colony growth. However, concentrations of FCS greater than 25% and lesser than 10% were suppressant (Table 1), and no colony was observed without adding FCS to the medium. The largest number of colonies was noted when a final concentration of 20% FCS was chosen. In addition, although colony growth was achieved at FCS concentrations of 5, 10, 15, 20, 25, 30, 40 and 50%, a significant number of colonies of greater than 30 cells in size was noted at 10 to 30% (Table 1). Thus, growth of larger colonies appeared to be more selective.

Comparison of agar and methylcellulose cultures with liquid cultures. The growth of the fibroblast colonies was tested in 0.3 to 0.05% agar and 1 to 0.1% methylcellulose. Growth was equal in 0.05% agar and 0.1% methylcellulose to that in liquid cultures and severely restricted in more than 0.1% agar and 0.25% methylcellulose (Table 2).

Table 1 Fibroblast colony formation and mean size of fibroblast colonies in the presence of FCS from 5 to 50%. Optimal colony formation and the largest colony formation were achieved at a concentration of 20%. Mean number with standard deviation from five normal subjects. Each culture contains 2×10^5 cells.

Percentage of FCS	No. of colonies	No. of cells per colony
0	0	0
5	24 ± 2	10 ± 4
10	33 ± 3	26 ± 17
15	46 ± 3	29 ± 16
20	47 ± 3	31 ± 15
25	41 ± 3	25 ± 15
30	30 ± 2	18 ± 12
40	13 ± 2	11 ± 5
50	6 ± 2	7 ± 2

Table 2 The effect of increasing agar and methylcellulose concentration on the growth of fibroblast colonies. Each culture contains 2×10^5 cells. Each observation is the average from colonies of five normal subjects.

Concentration	No. of colonies on day 7 cultures
Agar	
0.3%	0
0.15%	9
0.1%	21
0.05%	44
Methylcellulose	
1%	0
0.5%	14
0.25%	27
0.1%	48
Liquid culture	47

DISCUSSION

It has been suggested that normal fibroblasts are distinguishable from transformed fibroblasts by their inability to form colonies in agar (7). However, the present experiments have demonstrated that fibroblast-like colonies can be grown regularly in primary cultures of human bone marrow cell populations if FCS is incorporated in the medium. This report has described a simple technique for obtaining human bone marrow fibroblast colony growth in a liquid culture. With this system it is possible to selectively clone fibroblasts. On the basis of morphology and the absence of peroxidase and phagocytic activity, the characteristics of the cells studied herein appeared to be fibroblasts. The colony forming unit may consist of a single cell. Each colony constitutes a cell clone. The chromosome analysis revealed that each of the ten fibroblast colonies in mixed cultures of male and female guinea pig donor cells appeared to contain dividing cells from one donor only, and that the number of colonies in mixed cultures did not

differ from those in cultures of cells from separate donors (3). A linear increase in the number of colonies with increasing numbers of explanted cells was observed. These results support the view that the fibroblast colonies are clones.

The results obtained using this system show similar colony size and morphology to guinea pig systems (3). The number of the fibroblast colonies in cultures of guinea pig bone marrow was approximately 2 per 10^5 marrow cells. However, the frequency of such cells of healthy human bone marrow was approximately 25 per 10^5 marrow cells. The reason why the ratio of fibroblast colony forming cells from human bone marrow is greater than that from guinea pigs is probably due to the difference in the counting stage and colony criteria. In our experiment the number of colonies was counted on day 7 and cell aggregates containing more than six cells were considered as a colony, while counting was performed on days 10 to 12, and more than 50 cell aggregates were considered as colonies in the guinea pig report (3). Colonies usually begin to fuse from day 9, and the colony number decreased after day 9.

The growth of the fibroblast colonies in liquid culture was the same in 0.05% agar and 0.1% methylcellulose, but it was severely restricted in more than 0.1% agar and 0.25% methylcellulose layers in proportion to their increasing concentrations. The frequency of agar colony forming cells from mouse bone marrow (8) was less than that of guinea pigs (3). The superiority of liquid cultures tends to suggest that fibroblast colony forming cells and their progeny must adhere to a substrate such as glass or the bottom of a dish, and that the supporting medium such as agar and methylcellulose may inhibit adhesion of fibroblasts following restriction of fibroblast colony formation.

The growth of colonies from human bone marrow cells depends on the use of FCS. The most appropriate concentration of FCS is 20 per cent. Fetal calf serum has been suggested as a source of fibroblast colony stimulating factor. The physicochemical properties of the fibroblast colony stimulating factor remain to be determined.

Studies by Dexter and his colleagues (1, 2) have shown that, if marrow cells are cocultured in liquid with underlayers of either thymic or marrow-derived stromal cells, there can be an exceptional degree of self-maintenance of stem cells involving extensive self duplication. Although the feeder cells are heterogenous, an analysis of population interactions is only possible if the individual cells can be cloned and examined for functional activity as pure populations. There is fragmentary evidence for the fact that fibroblasts might function as stromal cells. Friedenstain et al (4) have demonstrated that when marrow-derived fibroblast colonies were harvested and grafted under the kidney capsule, a bone trabecule developed, enclosing foci of proliferating hemopoietic cells. This experiment suggested that the fibroblast colonies were in fact hemopoietic stromal colonies. The use of fibroblast growth in vitro with selective cloning opens the possibility of studying fibroblast functions as microenvironmental or stromal cells. This system will be useful for investigation of disorders of the hemopoietic environment such as hypoplastic anemia.

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