Single Cell Origin of Multilineage Colonies in Culture

Evidence That Differentiation of Multipotent Progenitors and Restriction of Proliferative Potential of Monopotent Progenitors Are Stochastic Processes

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Abstract. In this paper, we report analysis of differentiation in human hemopoietic colonies derived from a single cell. Cord blood mononulear cells and panned My-10 antigen-positive bone marrow and cord blood cells were plated in methylcellulose medium containing erythropoietin and conditioned medium. Initially, we performed mapping studies to identify candidate colony-forming cells. Subsequently, using a micromanipulator, we transferred single cells individually to 35-mm dishes for analysis of colony formation. Cellular composition of the colony was determined by identifying all of the cells in the May-Grunwald-Giemsa stained preparation. Of 150 single candidate cells replated, 63 produced colonies. The incidences of single lineage colonies included 19 erythroid, 17 monocyte-macrophage, and 9 eosinophil colonies. There were 18 mixed hemopoietic colonies consisting of cells in two, three, four, and five lineages in varying combinations. In some instances, we noted the predominance of one lineage and the presence of very small populations of cells in a second or third lineage. These results provide evidence for the single-cell origin of human multilineage hemopoietic colonies, and are consistent with the stochastic model of stem cell differentiation in man. They also indicate that restriction of the proliferative potential of committed progenitors is a stochastic process.

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Received for publication 3 August 1984.

The Journal of Clinical Investigation, Inc.
Volume 74, December 1984, 2193–2197

Introduction

We have recently provided evidence for the single-cell origin of mouse hemopoietic colonies expressing multiple lineages in variable combinations (1). While several types of human multilineage colonies have been described (2–5), the evidence for their clonal origin has been indirect and has involved the analysis of Y chromatin (4) or studies of glucose-6-phosphate-dehydrogenase mosaicism (6). To study the single-cell origin of human multilineage colonies, we carried out serial observations of colony formation from candidate progenitors to mono- and multilineage colonies in culture, and developed a method to predict candidate colony-forming cells with a reasonable degree of accuracy. Using the micromanipulation technique developed in the murine system (1), we isolated single hemopoietic progenitors, replated them in culture, and performed differential counts of all resulting hemopoietic colonies. We then cultured My-10 antigen-positive cells (7) that had been separated by a panning technique, and found a high incidence of mixed hemopoietic colonies. The results were similar to those of the murine multilineage colonies of single-cell origin, and documented the clonal nature of several types of bi-, tri-, tetra-, and pentalineage colonies revealing varying combinations of lineages. These observations are consistent with the stochastic principle of stem cell differentiation in man and provide additional support for the stochastic model of stem cell differentiation we have previously proposed (8). Differential counting of the total cells in an apparently homogeneous colony sometimes revealed predominance of one lineage and the presence of very small populations of cells in second or third lineages. These observations may indicate that restriction of proliferative potential of single lineage progenitors is a stochastic process and not a consequence of the maturation of single lineage progenitors.

Methods

Primary cell culture. Mononuclear cells were isolated from umbilical cord blood samples by use of Ficoll-Paque and were cultured at a concentration of 2×10^4 cells/ml by using a modification (9, 10) of the methylcellulose culture described by Iscove et al. (11). A marrow sample from a normal volunteer and an additional umbilical cord blood sample were enriched for progenitors by panning with monoclonal anti-My-10 antibody (7), and plated in culture at 2×10^3/ml and 1×10^3/ml cell concentrations, respectively. We used 2 U/ml of partially purified human urinary erythropoietin with a specific activity of 370 U/mg protein (kindly provided by Dr. Makoto Kawakita, Kumomoto University, Japan) and 2.5% (vol/vol) medium conditioned by phytohemagglutinin-stimulated leukocytes. Dishes were incubated at 37°C in a humidified atmosphere flushed with 5% CO_2 in air.

Indirect immune adherence with anti-My-10 antibody. My-10 is a 115-kD cell surface glycoprotein identified by a mouse monoclonal antibody raised against the KG-1a blast cell line. Details of the hybridoma production (12) and antibody specificity (7) are reported elsewhere. Because the My-10 antigen has been shown to be expressed preferentially on human colony-forming cells, we used My-10 antibodies to enrich the population. The panning technique of Engleman et al. (13) was used. Tris buffer, pH 9.2, containing 75 μg of affinity-purified goat anti-mouse IgG was added to 60-mm-diam nontissue culture dishes. After 3 h, the dishes were rinsed three times with Hanks' balanced salt solution (HBSS) and stored at 4°C in HBSS containing 0.2% bovine serum albumin (BSA). Nonadherent low-density marrow leukocytes were adjusted to 5×10^6/ml in RPMI 1640 (Flow Laboratories, Inc., McLean, VA) with 0.2% BSA and incubated (30 min, 22°C) with an equal volume of spent hybridoma supernate. Cells were then washed with HBSS containing 0.2% BSA. 5–10 million cells in 2 ml of the same cold medium were placed in a petri dish coated with goat anti-mouse IgG and kept at 4°C. 1 h later, the dish was rocked gently and incubation was continued for another hour. The nonadherent cells were harvested by rocking and gentle pipetting with three 2-ml volumes. The adherent cells were released by three rinses with vigorous pipetting.

Identification and culture of single hemopoietic progenitors. As we will discuss in the Results section, serial observations (mapping studies) of colony formation from small blast cell colonies to multilineage colonies provided a method for identifying hemopoietic progenitors with some degree of success. Accordingly, on days 2–5 of culture, each candidate progenitor cell was lifted with a fine capillary tube with a diameter of ~30 μm attached to a micromanipulator (Hacker Instruments, Inc., Fairfield, NJ) with motorized adjustments. To ensure that only single cells were transferred from the primary culture, the contents of the pipette were blown into a second dish containing complete methylcellulose medium under direct microscopic visualization. After thorough washing of the pipette with sterile water followed by sterile α-medium, the candidate progenitor cell was again aspirated using the micromanipulation technique and plated into a third dish containing complete culture medium.

When the colonies appeared to have matured (generally after 14–16 d of culture), the number of cells of small colonies consisting of <500 cells was estimated in situ, and the entire colony was lifted by use of an Eppendorf micropipette, and smears were prepared using a cytospin (Shandon Southern Instruments, Inc., Sewickley, PA) (9). Larger colonies of >500 cells were picked, made into single-cell suspensions, and aliquoted into two half-samples. One half of the sample was used for counting with a counting chamber, and the other half was spun in a cytocentrifuge for cytological examination. Smears were stained using the May-Grunwald-Giemsa method, and total cell differential counting was carried out on the smears. The accuracy of the latter method has been previously established by the concordance between the size of a diffuse basophil colony estimated in situ and that counted on the smears (5).

Results

Identification of candidate hemopoietic progenitors. First, we carried out several sequential observations (mapping studies) of the growth of small blast cell colonies to mono- and multilineage colonies in culture using the method we described for mouse blast cell colonies (14). By identifying smaller blast cell colonies each time and constructing a time course of colony formation, we deduced that the optimal time for isolating single progenitors is between days 2 and 5 of culture. The colony-forming cells are larger than lymphocytes and neutrophils, round, and evenly refractile. Often, they could be seen with pseudopod- or flagella-like structures (Fig. 1 *A* and *B*). We could identify noncolony-forming cells on the basis of small cell size, ragged contour, or granular appearance.

Colony formation from single cells. A representative time sequence of colony formation from a single cell to a multilineage colony and the companion May-Grunwald-Giemsa preparation are depicted in Fig. 1 *A–D*. We replated 88 single cells from cultures of cord blood mononuclear cells and observed colony formation in 27 cultures. Of these, 9 were pure erythroid colonies containing 28–150 cells/colony; 10 were monocyte-macrophage colonies containing 22–78 cells/colony; and 4 were pure eosinophil colonies containing 30–112 cells/colony. The remaining four were mixed colonies as presented in Table I. Of the 30 single cells replated from cultures of My-10-positive bone marrow cells, 12 yielded colonies, of which one was erythroid (220 cells/colony), 2 were monocyte-macrophage colonies (18–79 cells/colony), and 3 were pure eosinophil colonies (21–173 cells/colony). The remaining six were multilineage colonies as described in Table I. The highest incidence of colony formation was observed when 32 single cells were replated from cultures of My-10-positive umbilical cord blood mononuclear cells. 24 (75%) produced colonies, of which 9 were erythroid colonies (80–1,500 cells/colony), 5 were monocyte-macrophage colonies (60–400 cells/colony), and 2 were eosinophil colonies (90–360 cells/colony). The remaining eight were multilineage colonies as presented in Table I. In summary, a total of 150 single cells were replated, of which 63 produced colonies: 45 single-lineage and 18 multilineage colonies.

Total colony differential counting of the multilineage colonies is listed in Table I. Abbreviations used in this table for each hemopoietic lineage were based on the recommendation of a workshop at a University of California at Los Angeles

1. *Abbreviations used in this paper:* b, basophil; e, eosinophil; E, erythrocyte; m, monocyte-macrophage; n, neutrophil.

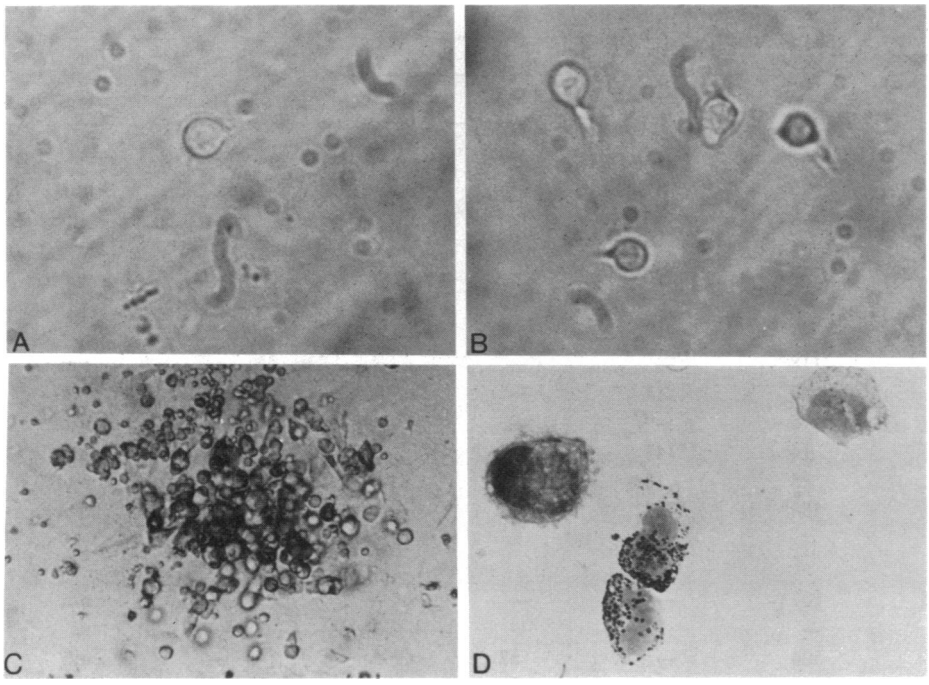

Figure 1. Time sequence of multilineage colony formation derived from a single cell (No. 17, Table I). (*A*) A candidate progenitor after micromanipulation and replating in complete methylcellulose medium. (*B*) Four cells derived from the single cell illustrated in section *A*. This picture was taken 36 h after replating. (*C*) Mixed hemopoietic colony revealing cells of various sizes and shapes. (*D*) A portion of the May-Grunwald-Giemsa stained preparation depicting a monocyte-macrophage, two basophils, and one eosinophil.

symposium (15): neutrophil (n)[1]; monocyte-macrophage (m); eosinophil (e); basophil (b); and erythrocyte (E). Seven types of bilineage colonies were seen, including mE, eb, nm, me, ne, mb, and eE. There was a total of eight multilineage colonies expressing terminal differentiation in more than two lineages. These combinations included ebE, nmE, meE, meb, mebE, and nmebE. Only one colony (mebE) revealed blast cells. We were unable to replate pure megakaryocyte or mixed megakaryocyte colonies from a single cell. We believe this is because the use of 30% fetal bovine serum is not optimal for megakaryocyte growth. Recently, Messner et al. (16) and Kimura et al. (17) suggested that the use of unfrozen human plasma and platelet-poor plasma supports megakaryocyte colony formation better than serum.

Discussion

The results presented in Table I were similar to our observations of murine hemopoietic colonies of single cell origin (1), and were consistent with the stochastic model of differentiation of hemopoietic stem cells. This model proposes that restriction of lineage potentials of a stem cell is a random process governed only by distributional parameters.

The proportion of each lineage in a multilineage colony was significantly variable. Colony no. 10 had ~1,200 cells but contained only two eosinophils. While colony no. 17 expressed three lineages in 500 cells, the monocyte-macrophage lineage consisted of only four cells. Colony no. 18 had 1,200 cells with five lineages expressed and had <3% erythrocytes. Colony no. 4 is an example of multiple lineage combinations (five lineages) with low proliferative expression by all members (120 cells). Previously, we documented varying proportions of cells in different lineages in murine multilineage colonies (1). In the analysis of paired progenitors from single cell origin, we noted that there were discordances among the apparently homologous pairs (18). Proportions of lineage expression as well as colony size differed significantly between members of some of the pairs. In another report, we also noted one eosinophil in a multilineage colony of single-cell origin consisting of 732 cells (19). Documentation of the presence of a very small number of cells in one lineage in a multilineage colony may possess serious implications for the cellular model of stem cell differentiation. These colonies were seen in cultures that are permissive to all lineages expressed in Table I as exemplified by large single-lineage colonies in the same culture.

It has generally been held that differentiation of hemopoietic stem cells consists of two compartments: commitment of multipotent progenitors to monopotent progenitors, and maturation of monopotent progenitors to mature cells. Erythroid burst-forming units, colony forming unit in culture, and megakaryocyte colony forming unit are depicted as the early (committed) erythroid, granulocytic, and megakaryocytic precursors, respectively, with each possessing significant proliferative capabilities (20). This model dictates that a relatively constant number of cells represents a lineage in a multilineage colony as a consequence of cell proliferation of early committed progenitors, and that there is a correlation between the number of lineages expressed and the size of colonies. Previous cell

Table I. Composition of Human Multilineage Colonies of Single Cell Origin

Colony	Estimated colony size	Differential counts						Colony type
		n	m	e	b	E	bl*	
Umbilical cord blood cells								
1	350	—	7	—	—	365	—	mE
2	100	—	—	87	1	—	—	eb
3	200	—	—	17	2	175	—	ebE
4	120	5	2	20	2	77	—	nmebE
My-10$^+$ bone marrow cells								
5	250	115	118	—	—	—	—	nm
6	240	—	10	217	—	—	—	me
7	240	12	6	—	—	187	—	nmE
8	200	—	14	143	—	34	—	meE
9	150	—	28	18	12	59	—	mebE
10‡	1200	—	588	2	58	492	4	mebE
My-10$^+$ cord blood cells								
11	180	17	107	—	—	—	—	nm
12	100	3	—	76	—	—	—	ne
13‡	600	—	504	—	52	—	—	mb
14‡	1000	—	—	616	46	—	—	eb
15	100	—	—	42	16	—	—	eb
16‡	1400	—	—	4	—	1340	—	eE
17	500	—	4	262	192	—	—	meb
18‡	1200	120	464	200	100	28	—	nmebE

* bl, blast cells. ‡ The estimated colony size and the differential counts have been multiplied by a factor of 2 (see Methods).

culture studies of hemopoiesis indicated that multilineage colonies are large and macroscopic, while colonies derived from monopotent progenitors, such as erythroid burst-forming units and erythroid colony-forming units, are significantly smaller.

Based on the observations presented in this report, we propose a modification of the stochastic model of stem cell differentiation schematically depicted in Fig. 2. In this model, lineage restriction and loss of proliferative potential are both stochastic processes. There are precedents to predict that restriction of proliferative potential at the level of committed progenitors is a stochastic process. In a recent study of human T lymphocyte colony-forming units, Wu (21) noted that the replating ability of the individual T lymphocyte colony-forming units was extremely variable. Pharr et al. (19) reported that the replating efficiencies of primary mast cell colonies varied over a wide range, and that the size of the secondary colonies was heterogeneous. Independent and random restrictions in lineage potentials and proliferative potentials of the progenitors would account for the extreme heterogeneity in the differential counts of the multilineage colonies.

Two decades ago, applying Monte Carlo simulation on spleen colony formation, Till and his associates (22) proposed that the decision of a stem cell to self-renew or to differentiate is a stochastic process. Subsequently, we have presented experimental data suggesting that self-renewal and commitment of multipotent progenitors to monopotent progenitors is a

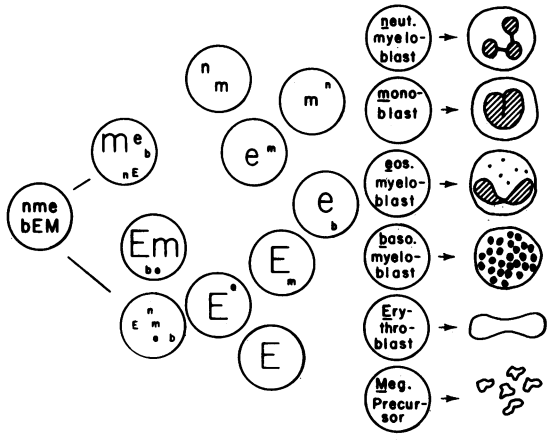

Figure 2. A schematic presentation of a model of stem cell differentiation. Progressive lineage restriction and loss of proliferative capacity of individual progenitors are depicted as separate stochastic processes. The size of the lettering is representative of the apparent proliferative potential of an individual lineage.

stochastic process (1, 18, 23). We now propose that the entire process of stem cell differentiation and proliferation is a stochastic process.

Acknowledgments

This work was supported by the Veterans Administration, National Institutes of Health grants HL20913, AM32294, and CA32318; and by grant 1418 from the Tobacco Research Council USA. Dr. Ogawa is a Veterans Administration Medical Investigator. Dr. Strauss is a Fellow of the Leukemia Society of America.

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