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Cell Biology

Long-term cultivation and differentiation of human erythroleukemia cells in a protein-free chemically defined medium

(serum-free culture/hemoglobin/leukemia)

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ABSTRACT **To examine whether a human erythroleukemia cell line, K-562, can proliferate and differentiate without protein supplements, long-term cultivation of the cells was carried out in a protein-free chemically defined medium. By the use of stepwise decreases in the fetal bovine serum concentration, continuous growth of K-562 cells was established in a protein-free F-10 medium. The cells have been propagated in this medium for 3 years. The population-doubling time of the cells is about 30 hr. Growth was not stimulated by the addition of insulin, epidermal growth factor, fibroblast growth factor, multiplication-stimulating activity, transferrin, platelet-derived growth factor, or dexamethasone. Addition of serum stimulated the cell growth slightly and increased the saturation density. The saturation density of the cells could be increased to that seen with serum-supplemented cultures by changing the serum-free medium daily. The cells synthesized significant amounts of hemoglobin in the presence of hemin without serum supplementation. The results suggest that the human erythroleukemia cells grown in a protein-free medium do not require serum components for their growth or hemin-induced hemoglobin synthesis and provide an excellent model for better understanding of the growth and differentiation of human leukemia cells.**

Learning how cells of multicellular animals control their proliferation rates has long been a key objective of experimental biology. Nutritionally simple microbes offer several advantages for biosynthetic studies on animal tissues. Compared to *Escherichia coli,* our knowledge of the structural organization and biochemistry of eukaryotic cells is still pitifully meager. The greater potential of tumor cells to yield cell lines has led to the belief that all cells that grow as cell lines perhaps do so because they have acquired many of the essential properties of the cancer cell by mutation(s). In general, the growth in culture requires not only a well-defined collection of amino acids and vitamins but also depends upon protein factors present in blood serum. In the absence of such growth-promoting factors, most cells enter a resting state of G_1 phase of cell cycle, with a substantial reduction of cell numbers or complete cell death within a few days. The mechanisms by which such factors affect cell functions have not been understood fully. Among these factors, even the effects of insulin on cell growth and functions have not been clarified (1). The complex nature of such factors represents an undefined variable when assessing the biochemical changes within the cell during proliferation or differentiation. To circumvent this problem, long-term cultivation of human erythroleukemia cells was performed in a protein-free chemically defined medium. This protein-free culture may provide advantages similar to bacterial cultures, especially for genetic research of cells, because mutations to auxotrophy in this system are possible in the absence of serum factors.

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MATERIALS AND METHODS

Cell Culture. A human erythroleukemia cell line, K-562, was kindly provided by M. Mori (Tokyo Metropolitan Geriatric Hospital, Tokyo). The cells were cultivated in F-10 synthetic medium supplemented with 5% fetal bovine serum (Flow Laboratories), penicillin (100 units/ml), and streptomycin (100 μg/ml) (2). Triple-distilled water was used to prepare media. Stock cultures were grown in flasks (Falcon 3013, 25 cm^2 surface area) in 8 ml of medium in humidified 5% CO_2/95% air at 37°C and subcultured every 2–4 days. The initial cell number was 1–2 × 10^5 cells per flask. The cells were counted with the aid of a hemocytometer.

Morphological Examination. Cultured cells were photographed without stain by using a phase-contrast microscope or were stained with Wright's dye. For electron microscopic examination, the cells were fixed with 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), postfixed in 1% OsO_4 in the same buffer, and embedded in Epon 812 after dehydration. Thin sections were cut, stained with uranyl acetate and lead citrate, and examined in a Hitachi HU-12 electron microscope.

Assay of Hemoglobin Content. Hemoglobin was measured by optical absorption. After growth in the presence of inducing agents, the cultures were chilled on ice, and the cells were pelleted by low-speed centrifugation and washed twice in ice-cold phosphate-buffered saline. The washed cell pellet was resuspended in 1 ml of lysing buffer containing 0.81% NaCl, 0.03% Mg acetate, and 0.12% Tris (pH 7.4), and Nonidet P-40 (Nakarai Chemical, Kyoto, Japan) was added to yield a final concentration of 0.5%. After 15 min on ice, the nuclei were removed by centrifugation (1,500 rpm), and the visible absorption spectrum of the supernatant was scanned on a spectrophotometer. Absorption was measured at 414 nm, and readings were taken at 403 and 425 nm to correct for nonspecific absorption due to light scattering (3). The absorption material was identified as hemoglobin by the presence of absorption maxima at 414, 540, and 576 nm, which shifted to 417, 537, and 568 nm after treatment with carbon monoxide. An optical density of 0.1 at 414 nm corresponds to a hemoglobin concentration of 1.25 μg/ml.

Materials. Hemin was prepared by dissolving 13 mg of hemin (bovine, Sigma) in 0.2 ml of 0.5 M NaOH, buffering with 0.25 ml of 1 M Tris (pH 7.8) and making the volume up to 5 ml with triple-distilled water. This 4 mM stock solution was sterilized by passage through a 0.22-μm membrane (Millipore) and was used fresh or stored at −20°C. n-Butyric acid (Sigma) was dissolved in Hanks' balanced salt solution, buffered to pH 7.2 with NaOH, made up to a concentration of 1 M, and filtered through a 0.22-μm membrane (Millipore). Crystalline bovine insulin and human transferrin were obtained from Sigma. Epidermal growth factor, multiplication-stimulating activity, platelet-derived growth factor, and fibroblast growth factor were obtained from Collaborative Research (Waltham, MA).

RESULTS

Adaptation of Cells to Protein-Free Medium. Stationary cultures of K-562 cells in Ham's F-10 medium containing 5% serum were subcultured at a 1:3 ratio into F-10 medium with 2% fetal bovine serum. An attempt to subculture the cells directly into serum-free medium resulted in some initial cellular proliferation but no sustained growth. Stepwise decreases in the fetal bovine serum concentration at weekly or biweekly intervals were then used until the serum was eliminated from the medium. This total time interval could be as short as 3 months by the use of high seedings for subcultures. Although the most of the cells were round in shape, during the process of adaptation a few cells adhered to the bottom of the culture vessels and showed a spindle-like appearance.

Characteristics of Adapted Cells. The cells established in protein-free medium, designated K-562-T-1, were morphologically similar to the original K-562 cells. Most of the cells had a large round nucleus and scant, basophilic cytoplasm (Fig. 1). Some cells had cytoplasmic processes and showed a spindle-like or polygonal appearance. The nuclei of these cells were elongated and indented. Electron microscopic examination revealed a large indented nucleus, many mitochondria, and glycogen vacuoles. Spindle-like cells contained an elongated bizarre nucleus, cytoplasmic filaments, and microtubules (Fig. 2).

The cells have been propagated continuously in protein-free F-10 medium for about 3 years. A kinetic study on the *in vitro* growth of the cells is shown in Fig. 3. The cells used for these experiments were of the 86th-transfer generation that had been adapted to the protein-free medium. The experimental cultures were initiated with an inoculum of 3×10^4 or 6×10^4 cells and incubated in a CO_2 chamber at 37°C. The growth curve was obtained by counting the number of cells per dish as a function of time. From the curve in Fig. 3, the population-doubling time was estimated as 30 hr. Addition of serum stimulated the cell growth and increased the saturation density of the culture. The population-doubling time in the presence of 5% serum was about 24 hr. Insulin, epidermal growth factor, fibroblast growth factor, multiplication-stimulating activity, transferrin, dexamethasone, or platelet-derived growth factor did not cause significant growth stimulation (Fig. 4). In protein-free cultures, the saturation density exceeded that in serum-supplemented cultures when the medium was changed every day (Fig. 5). Addition of the conditioned medium from dense cultures inhibited the growth of cells in a protein-free or in serum-supplemented medium.

Induction of Differentiation in Protein-Free Culture. The original cell line has been shown upon induction by butyrate or hemin in serum-supplemented medium to differentiate into erythroid cells synthesizing hemoglobin. The strain established in protein-free F-10 medium showed erythroid differentiation in response to hemin. After incubation of the cells in the presence of hemin for 4 days, a significant amount of hemoglobin was observed (0.5 pg per cell) with or without serum supplementation. Treatment of the cells with sodium butyrate did not stimulate hemoglobin production in the presence or the absence of serum (Fig. 6). Growth factors such as insulin, epidermal growth factor, fibroblast growth factor, multiplication-stimulating activity, transferrin, platelet-derived growth factor, or dexamethasone did not cause significant stimulation of hemoglobin production.

The results suggest that the human erythroleukemia cells are capable of growing continuously in a protein-free medium and can differentiate in the absence of serum or other humoral factors.

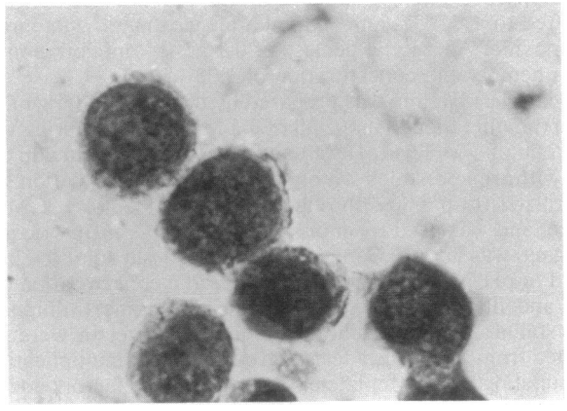

FIG. 1. Morphological features of K-562 cells grown in a protein-free medium. (Giemsa stain, ×600.)

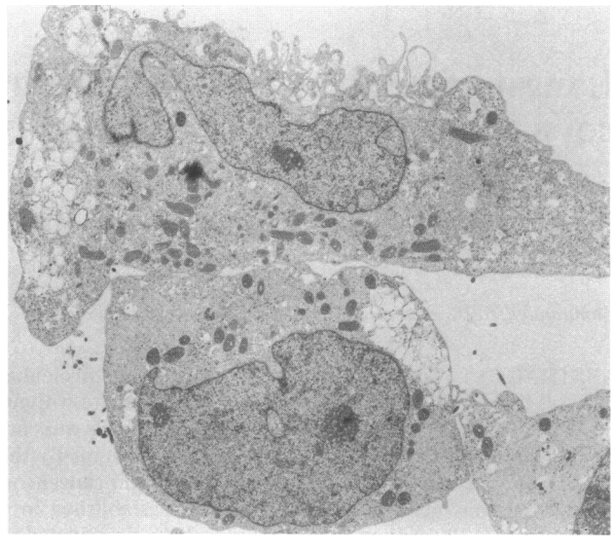

FIG. 2. Ultrastructure of K-562 cells grown in a protein-free medium. (×3,500.)

DISCUSSION

The long-term growth of human erythroleukemia cells, K-562, has been established in a protein-free chemically defined medium. The cells have been propagated continuously

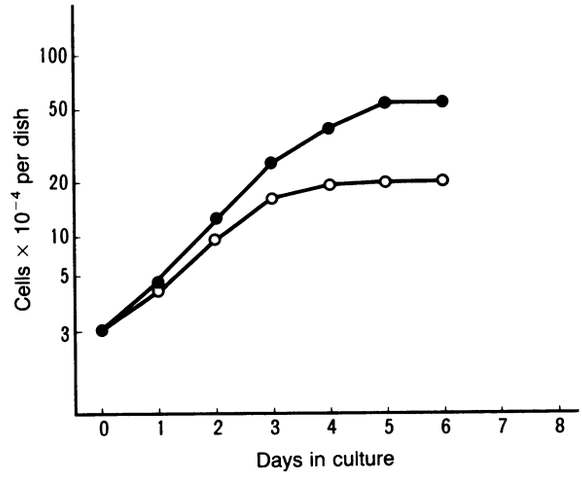

FIG. 3. Growth curve of K-562 cells grown in protein-free F-10 medium. The experimental cultures were initiated with an inoculum of 3×10^4 cells and incubated in a 5% CO_2 chamber at 37°C. The cells of passage 86 were grown in 2 ml of medium with (●) or without (○) fetal bovine serum. The medium was not changed in these cultures. Each point represents the mean value from two dishes.

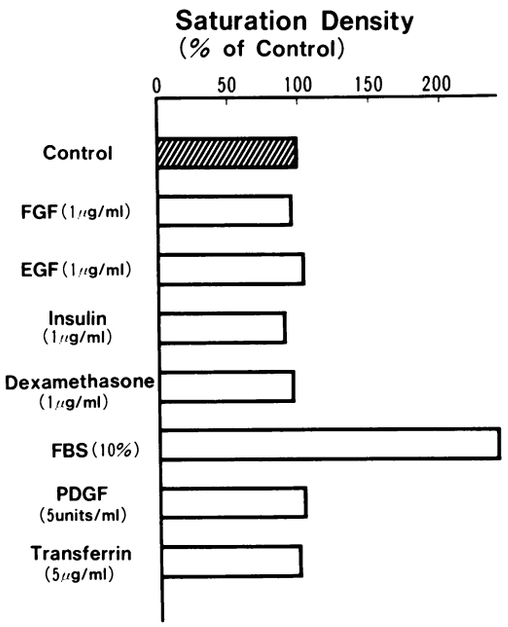

FIG. 4. Effect of growth factors and serum on the growth of K-562 cells grown in a protein-free medium. The initial cell number was 3×10^4 cells per dish. A cell count was taken on day 6 in each dish. Medium renewal was not performed. FGF, fibroblast growth factor; EGF, epidermal growth factor; FBS, fetal bovine serum; PDGF, platelet-derived growth factor. Each column represents the mean value from two dishes.

in nutrient medium F-10 for more than 3 years. No supplements were used for the culture. The cells grew in protein-free F-10 medium with a 30-hr population-doubling time. Intensive efforts have been made to develop human cell lines in a protein-free chemically defined medium (4, 5). However, only a few human cells have been shown to proliferate in protein-free media for a long time. In addition, they have not expressed their differentiated functions in the protein-free media. On the other hand, many cell lines grow in serum-free media supplemented with several growth-promoting factors such as insulin, transferrin, albumin, or other growth factors and hormones (6, 7). Because the biological actions of these factors have not been elucidated fully and many of them show several different biological effects on cultured cells (8,

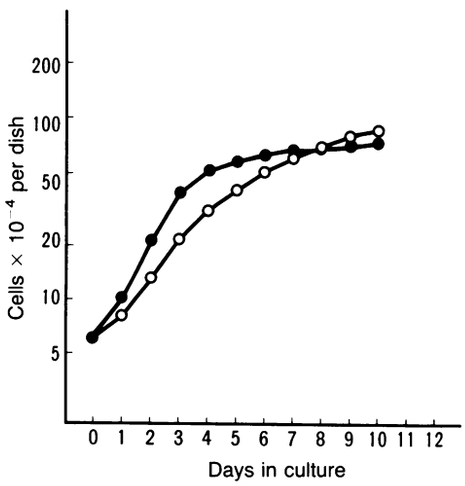

FIG. 5. Growth curve of K-562 cells in a protein-free medium. The cells of passage 86 were grown in 2 ml of protein-free F-10 medium. The medium was changed every day. A cell count was taken each day in two dishes. Each point represents the mean values from two dishes. ○, Without serum; ●, with 5% fetal bovine serum.

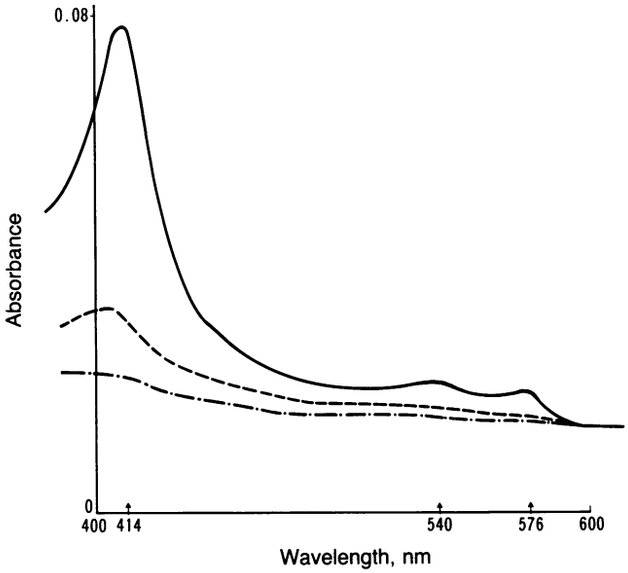

FIG. 6. Induction of hemoglobin synthesis in K-562 cells grown in a protein-free medium. A significant amount of hemoglobin (0.5 pg per cell) was demonstrated in K-562 cells treated with hemin (—) in the absence of serum or other supplements. ---, Untreated K-562 cells; -·-, K-562 cells treated with butyric acid (1.4 mM) for 4 days; ——, K-562 cells treated with hemin (100 μM) for 4 days.

9), the inclusion of these factors can cause confusion when we assess the biochemical changes occurring during proliferation and differentiation of the cells. Our studies have shown that human leukemia cells can be propagated in a protein-free chemically defined medium and can synthesize hemoglobin in response to hemin without serum or other growth-promoting factors. Numerous studies have demonstrated that K-562 cells can serve as a useful model system for studying human erythroid differentiation (10, 11). Not only do these cells differentiate *in vitro* into erythroid cells or myeloid cells, but also they can mimic early stages of human erythropoiesis when cultured under appropriate conditions. These findings demonstrate that the versatility of K-562 cells is affected by their environment. All of the studies cited above were conducted, however, in serum-containing media wherein miscellaneous factors in serum may affect the cell functions. By the use of the cell line grown in a protein-free medium, many *in vitro* studies on human erythropoiesis can be done without interference by serum or other growth-supporting factors.

Electron microscopic studies were done in collaboration with Dr. H. Yorifuji, Department of Anatomy, Faculty of Medicine, University of Tokyo.

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