Studies of the growth of human bone-derived cells in culture using aqueous two-phase partition

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Human bone cells, maintained in culture, have been subjected to partitioning in an aqueous two-phase system on a countercurrent distribution apparatus. A broad cell distribution was obtained indicating cell-surface heterogeneity. Two major cell populations were identified which appeared to be growing at different rates. The 'fast'-growing cells had a less hydrophobic cell surface than the 'slow'-growing cells. Possible relationships of these cell populations with osteoblast differentiation and the potential importance of this technique in studies of osteoblast differentiation are discussed.

Studies of osteoblast function currently involve the use of neonatal or fetal rodent calvarial cells or rodent osteosarcoma cell line. We have recently developed a system for culturing non-transformed cells derived from adult human bone. Cells thus derived display many osteoblastic characteristics including an increase in osteocalcin production and alkaline phosphatase activity in response to 1,25 dihydroxyvitamin D₃ (1,25(OH)₂D₃) (Beresford et al., 1983; Gallagher et al., 1983) and an increase in cyclic AMP content in response to parathyroid hormone (Gallagher et al., manuscript submitted).

Whilst this culture system has been used to investigate the activities and potencies of hormones and drugs on bone cells (Gowen et al., 1984; Gallagher et al., 1983; Beresford et al., 1983; MacDonald et al., 1984), its application is limited by the possible heterogeneity of constituent cell types. Heterogeneity certainly exists in terms of cellular differentiation, only a proportion of the cells exhibit high alkaline phosphatase activity (an enzyme associated with osteoblastic activity). Furthermore, the expression of this enzyme increases considerably with time in culture and also in response to 1,25(OH)₂D₃, a hormone known to stimulate the differentiation of a number of cell types (including osteoblasts) (Abe et al., 1981; Reitsma et al., 1983). In order to separate pure populations of cells and to investigate differentiation we have applied the technique of aqueous two-phase partition to separate different cell types present.

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Aqueous two-phase partition is a technique that can separate cells according to small differences in surface properties (Albertsson, 1971; Walter, 1977). Aqueous solutions of polymers (usually dextran and poly(ethylene glycol)), when mixed above certain critical concentrations, can produce two immiscible phases (Albertsson, 1971; Beijerinck, 1896). In the present instance the upper phase is rich in poly(ethylene glycol) and is more hydrophobic than the lower phase, which is rich in dextran (Albertsson, 1971). Cells partition between the interface and one of the phases, according to their surface character, and by conducting several partition steps, subtle cell separations can be achieved (Albertsson, 1981; Walter, 1977). Fifty-nine partition steps can be carried out automatically on a countercurrent distribution apparatus resulting in a cell 'profile' distributed over 60 fractions. The position of the profiles gives an indication of the relative surface hydrophobicity and the broadness of the profile an indication of cell-surface heterogeneity.

This technique has been used to separate and study the surface of a wide variety of cell types (Albertsson, 1971; Fisher, 1981) and is particularly useful for studying cellular differentiation since a cell distribution can be obtained which provides information on the cell-surface heterogeneity of the population (Sharpe et al., 1982).

**Materials and Methods**

*Cultures of human bone cells*

Human bone cells were cultured as previously described (Gallagher et al., 1984). Essentially, specimens of human trabecular bone obtained at surgery were extensively washed in phosphate-buffered saline (GIBCO Europe, Paisley, Scotland) and dissected into fragments 0.3-0.5 cm in diameter. These explants were plated out in 9-cm tissue-culture plates (Falcon, Oxford, England) containing Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum (FCS) (GIBCO). Explants were cultured at 37°C in an atmosphere of 95% air 5% CO₂.

Cells migrating from the explants formed a confluent monolayer culture after 4-6 weeks. All experiments were conducted on cells at first subculture. These cells reproducibly expressed the ability to synthesize osteocalcin in response to 1,25 dihydroxyvitamin D₃.

Cells were passaged by enzymic dispersion using 0.5% trypsin/0.2% EDTA (GIBCO) into 9-cm tissue-culture plates at 10⁶ cells/plate. These cells were then incubated in EMEM + 10% FCS for 24 h to allow attachment. A further 24-h incubation was carried out in EMEM + 10% FCS containing 2 μCi/ml [6-³H]thymidine (Amersham International plc), following which the cells were removed from the plate with trypsin/EDTA and applied to the phase system. In some cases sequential 24-h incubations with 2 μCi/ml [6-³H]thymidine followed by 0.4 μCi/ml [¹⁴C]thymidine were carried out prior to separation.

*Assessment of radiolabelled thymidine incorporation*

After phase separation 60 fractions were obtained. Previous experiments indicated that no radioactive material was present after fraction 40. A 1-ml aliquot of the fraction was taken to which was added 100 μl of 1% BSA and 100 μl of 50% trichloroacetic acid.
TCA-insoluble material was pelleted by centrifugation (1150 g 15 min) and, after removal of the supernatant, resuspended in 1 ml of H₂O. After repeating this process the TCA-insoluble radioactivity was dissolved in 500 μl of 0.1 M NaOH and counted on an LKB Rackbeta liquid-scintillation counter.

**Cell counting**

A 1-ml aliquot was removed from each fraction unloaded from the machine. This was diluted to 10 ml with Isoton (Coulter Electronics Ltd.) and the cell number counted on a Coulter Counter ZM with gate set for counting of cells of 12-30 μm diameter.

**Phase system**

Dextran T500 batch FD 16027 was supplied by Pharmacia (Uppsala, Sweden). Poly(ethylene glycol) M₉ 4000 was obtained from BDH (Poole, England). The phase system used contained: 4.9% (w/w) dextran / 4.9% (w/w) poly(ethylene glycol), 0.05 mol/kg NaCl, 0.01 mol·kg⁻¹ potassium/potassium phosphate buffer pH 7.8, 5.3% (w/w) sucrose, 0.1 mmol/kg CaCl₂, 5% (w/w) fetal calf serum, and 1% penicillin/streptomycin solution (10 000 units/ml, GIBCO).

Countercurrent distribution was carried out at 4°C on a Bioshef MK1, thin-layer countercurrent distribution apparatus. Cells were unloaded by the addition of 1.54 ml/phosphate-buffered saline pH 7.4 into each chamber, giving a total volume of 3.0 ml.

**Results**

Fig. 1 shows the partition profile provided by human bone cells in the phase system described. The profile consisted of a broad distribution of cells between fractions 7 and 43, indicating a high degree of cell-surface heterogeneity. A peak of cells was detectable in fraction 15 which appeared to represent a discrete cell population, with respect to surface properties. This cell population was termed Peak I. The profile broadened considerably to the right from fraction 15 to fraction 43. There were clearly many cell populations within

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**Fig. 1.** (---) CCD profile of human bone cells after 59 transfers.
(---) TCA-precipitable [³H]thymidine.
this broad area but for convenience the broad area between fractions 15 and 43 was considered as a single population and termed Peak II. Cells occupying Peak II had a slightly higher affinity for the poly-(ethylene glycol)-rich top phase and hence have a more hydrophobic surface than cells occupying Peak I. When cells were labelled with \[^{3}H\]thymidine for a period of 24 h prior to countercurrent distribution, the amount of radiolabel incorporated into acid-precipitable material followed the pattern shown in Fig. 1. There was more incorporation of thymidine into the DNA of cells from Peak I than cells from Peak II, indicating that cells in Peak I divide more rapidly than cells in Peak II. An alternative explanation, however, could have been that cells occupying Peak I were enriched with cells passing through the S phase during the 24 h prior to separation.

In order to determine whether the separation of cells into two populations was on the basis of the cell cycle, cells were grown in the presence of \[^{14}C\]thymidine for 24 h followed by a further 24 h with \[^{3}H\]thymidine. Fig. 2 shows the ratio of the incorporation of \[^{14}C\]thymidine/\[^{3}H\]thymidine per cell. The ratio remained constant for each countercurrent distribution fraction, indicating that cells in different phases of the cell cycle did not occupy different positions in the cell profile. It is unlikely that cells would be present in the same phase of the cell cycle in two successive 24-h incubations with radiolabel. Separate studies have revealed that cells derived from human bone are relatively slow-growing with a doubling time of 4.08 d (Gallagher et al., manuscript submitted).

Discussion

Human bone cells produced a broad countercurrent distribution profile when partitioned as described, which is an indication that there is a large degree of surface heterogeneity within the cell population. The distribution of cells (fractions in which cell counts were obtained)
was essentially the same for a number of cell cultures obtained from different donors. Figures shown are representative of these experiments. The resulting profile can be considered to be essentially two major cell populations although there are certainly more sub-populations present. The separation of these cell populations did not appear to be on the basis of phase of the cell cycle or at least on the basis of any enrichment of cells in the $S$ phase. Previous studies have demonstrated that aqueous two-phase partition has been shown to separate cells on the basis of cell cycle (Sharpe & Watts, 1984; Walter et al., 1973).

The cells separated into the sub-populations had different surface properties and different rates of growth. Cells occupying Peak I had the fastest growth rate, incorporating almost ten times the amount of $[^3]$H]thymidine per cell than cells occupying the far right of peak II (fractions 30-40). The amount of incorporation of thymidine per cell appeared to gradually decrease with increase in cell-surface hydrophobicity. Thus, increase in surface hydrophobicity may be related to a decrease in growth rate.

It is possible that the cell-surface heterogeneity and altered growth rates are related to osteoblast maturation/differentiation. Osteoblasts are derived from primitive mesenchymal stem cells and it is likely that, in these heterogeneous cell cultures, cells from all stages in this cycle may be present. The possibility of reculturing cells from different parts of the countercurrent distribution profiles makes this technique potentially useful in the study of osteoblast differentiation, the biochemical changes associated with this differentiation, and the isolation of pure populations of mature osteoblast cultures from human bone-cell cultures.

References