Clonal analysis \textit{in vitro} of osteogenic differentiation of marrow CFU-F

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Summary

Fibroblastic colonies, each of which is derived from a single precursor cell (CFU-F), are formed when suspensions of marrow cells are cultured \textit{in vitro}. The ability of marrow CFU-F to differentiate \textit{in vitro} was investigated using the expression of alkaline phosphatase activity as a marker for osteogenic differentiation. In cultures of rabbit marrow cells the colonies formed varied in size, morphology and expression of enzyme activity, indicating that marrow stromal CFU-F are a heterogeneous population. Growth and differentiation of marrow CFU-F can be modified \textit{in vitro}. Epidermal growth factor increased average colony size and reduced clonal expression of alkaline phosphatase activity to very low levels. Hydrocortisone activated the osteogenic differentiation programme within the cellular progeny of a wide spectrum of CFU-F. The results support the possible development of \textit{in vitro} clonal methods for the study of differentiation and regulation of the osteogenic and other fibroblastic cell lines of the marrow stromal system.

Key words: marrow CFU-F, clonal analysis, osteogenesis.

Introduction

The two main cell systems associated with bone and marrow are the haemopoietic and stromal systems. By comparison with the haemopoietic system relatively little is known about cell lineage in the stromal system. According to current hypothesis the fibroblastic components of bone marrow stroma contain stromal stem cells that are able to generate several cell lines, including osteogenic, adipocytic and other fibroblastic lines (Owen, 1985). Whereas clonal methods for assay of early haemopoietic precursors have been used extensively in investigating cell lineage in haemopoiesis (Metcalf, 1984), they have scarcely been applied in studies of the stromal system. The present work demonstrates the possibility of developing clonal analysis \textit{in vitro} for the study of differentiation of marrow stromal precursors.

Fibroblastic colonies were formed when suspensions of dispersed marrow cells were cultured \textit{in vitro} (Friedenstein, 1973; Castro-Malaspina \textit{et al.} 1980; Ashton \textit{et al.} 1980, 1984). Friedenstein (1976) demonstrated that each colony is derived from a single fibroblastic colony-forming cell (FCFC) or colony-forming unit-fibroblastic (CFU-F). Previous observations suggest that marrow CFU-F may contain putative stem cells of the stromal system. When individual fibroblastic colonies were transplanted \textit{in vivo} under the renal capsule a proportion of them formed the mixture of stromal tissues that provides the microenvironment necessary to support haemopoiesis, indicating pluripotency of the initiating CFU-F (Friedenstein, 1980). The fibrous and osteogenic connective tissues formed from marrow cells cultured in diffusion chambers \textit{in vitro} are generated by a small number of stromal precursors with stem cell characteristics (Bab \textit{et al.} 1986). The sequence of events in the chambers resembles the normal developmental process for osteogenesis (Mardon \textit{et al.} 1987).

The influence of marrow stromal tissue on haemopoiesis is well known (Trentin, 1976; Dexter, 1982). Variation in the number of marrow CFU-F in haemopoietic diseases has been reported (Castro-Malaspina \textit{et al.} 1982; Nagao \textit{et al.} 1981; Haworth & Testa, 1983), but knowledge of the nature of CFU-F and of factors regulating their differentiation is lacking. The purpose of the present study was to investigate whether CFU-F are able to differentiate \textit{in vitro} and if this can be modified by environmental factors. Expression of alkaline phosphatase (AP) activity is used as a marker for osteogenic differentiation. Rabbit marrow cells are cultured and fibroblastic colonies are formed that vary in size, morphology and level of expression of the enzyme activity. Receptors for epidermal growth factor...
and hydrocortisone have been demonstrated in osteogenic cells (Ng et al. 1983; Rodan & Rodan, 1984). Addition of these factors to the culture medium modulates growth and expression of AP activity in the present system. The results support the feasibility of developing in vitro clonal assays for investigating cell lineage and regulatory mechanisms in the marrow stromal system.

Materials and methods

In vitro culture

Ten New Zealand White male rabbits, 800 g to 1·1 kg weight, were used in the experiments. Marrow cells were flushed from the mid shafts of the femur with a syringe containing 0·5 ml of serum-free medium and a suspension of single cells was prepared by drawing the cells into the syringe through 19, 21 and 23 gauge needles in sequence. Any remaining cell aggregates were removed by passage of the cells through 90 µm nylon mesh (Bolting cloth, H. Simon Ltd, Stockport, UK). Samples of the cell suspension were diluted with 0·5 % (w/v) Trypan Blue prepared in 0·16 M-ammonium chloride and the number and viability of nucleated cells were counted in a haemocytometer. About 2·5 x 10^6 cells were obtained per femur. The cells were plated out in 25 cm² plastic tissue culture flasks (Nunc, Gibco Ltd) 10^7 cells/flask. Culture medium was BGM (Gibco Ltd) supplemented with 10% foetal calf serum (FCS) (Flow Labs, Irvine, UK), glutamine (10 µg/ml), vitamin C (5 mg/ml), penicillin (5000 units/100 ml) and streptomycin sulphate (5 mg/ml). The medium was prepared 16—24 h before plating out the cells and before each change of medium. Incubation took place at 37°C in a gassed incubator, 5 % CO₂ in air. The medium was first changed at 7 days and then every 3 or 4 days.

Experiments with epidermal growth factor (EGF) and hydrocortisone (HC)

EGF (rat submaxillary gland, Sigma Chemical Co. Ltd, Poole, UK) was dissolved in distilled water and HC (hemisuccinate salt, Sigma) was dissolved in absolute ethanol. Both were stored in samples until required, EGF at −20°C and HC in the refrigerator. EGF and HC were added either separately or together to treated flasks at final concentrations of 40 ng/ml and 10⁻⁶ M, respectively. Control flasks without added factors were cultured under identical conditions.

Colony counting

All flasks were fixed with 95 % ethanol and stained for alkaline phosphatase (AP) activity (Sigma kit no. 85). A few flasks were also stained with Mayer’s haematoxylin and for acid phosphatase activity (Sigma kit no. 386A). Two control flasks and two from each treatment were fixed and stained at 7 days and examined under the microscope. The remaining cultures were stopped at 16 days and the total number of colonies formed and the number positive for AP activity were counted by eye using an Anderman colony counter (Anderman & Co. Ltd, Kingston-on-Thames, UK). All colonies were recorded according to diameter in four size ranges: 1·0—2·0 mm, 2·0—4·0 mm, 4·0 to 6·0 mm and >6·0 mm. Staining for AP activity was designated by eye in four categories (+ to ++++) according to intensity. Addition of vehicle to control flasks had no effect on formation of colonies or expression of enzyme activity. Counting of the flasks was done blind by two independent observers.

Statistical analysis of results

For each experimental schedule the results for several rabbits were pooled. Comparison between treated flasks and controls was performed using the Wilcoxon rank-sum test; P < 0·05 was considered to be significant. All data are expressed as mean ± S.E.M.

Results

Total colonies formed and alkaline phosphatase-positive colonies per number of cells inoculated

The total number of fibroblastic colonies formed and the number positive for AP activity in control cultures at 16 days are directly proportional to the number of nucleated cells inoculated. A typical result is shown in Fig. 1. For 10^7 marrow cells per flask the colony-forming efficiency, CFE (total colonies per number of cells inoculated), and percentage of colonies positive for AP activity in control cultures ranged from
Fig. 2. Colonies in control culture fixed and stained for AP activity at 7 days. Colonies vary in morphology and size and are negative for the enzyme; Zeiss ICM 405 microscope and M 35 camera. ×16.

1.6×10⁻⁶ to 8.5×10⁻⁶ and from 10 to 45%, respectively, for the ten rabbits.

Light-microscope observations
At the first change of medium at 7 days small fibroblastic colonies are visible under the microscope. They vary in morphology and size, from a few cells to about a hundred cells (Fig. 2) and rarely contain cells that stain for AP activity at this stage. Haemopoietic cells do not survive well in BGJ medium and die or are removed with medium changes. At 16 days there was no evidence of acid-phosphatase-stained cells (macrophages), and other haemopoietic cells in the cultures and the background of cellular debris seen at 7 days had disappeared.

In both control and treated cultures fixed at 16 days, there is a wide variation in colony size and in the level of expression of AP activity (Fig. 3). Colonies are either completely unstained for the enzyme or contain varying numbers of stained cells that predominate in the centre or oldest part of the colony and appear to spread from there (Fig. 3). Two adjacent colonies from a control flask, one negative and the other positive for the enzyme (+ + + +), are shown in Fig. 3A and another positive at the + + level in Fig. 3B. A typical large colony in an EGF-treated flask positive at the + level is shown in Fig. 3C. Fig. 3D,E illustrates colonies from HC-treated cultures that differ in morphology and are strongly positive for the enzyme (+ + + +).

Quantitative measurements of the effect of EGF and HC
Results for total colonies formed, the number expressing AP activity, colony size and staining intensity are given in Figs 4, 5, 6, and Tables 1 and 2. EGF and HC were added to the cultures either from day 7 when small fibroblastic colonies are already established or from day 0.

When EGF is added at day 7, the total number of colonies formed is about 70% of control, $P<0.01$, and the number of AP-positive colonies is decreased to about 12%, $P=0.001$ (Fig. 4). When HC is added at day 7 there is no effect on total colonies formed but the number of AP-positive colonies is increased by about 50%, $P=0.05$ (Fig. 4). When HC + EGF are added together at day 7 there is no effect on total colonies formed but the number of AP-positive colonies is significantly reduced, $P=0.01$ (Fig. 4). Hence, EGF appears largely to override the effect of HC on enzyme expression.

When EGF is present throughout the culture period there is a decrease of about 30% in total colonies formed, $P=0.05$, and the number expressing AP activity is reduced to about 5% of control, $P=0.001$ (Fig. 5). When EGF is added at day 0 only, there is no effect on total colonies and the number positive for AP activity is reduced to 50%, $P=0.05$ (Fig. 5). When EGF is added at day 0 and EGF + HC and HC from day 7, there is no effect on total colonies, but there is a decrease in the number expressing the enzyme activity when EGF and HC are added together, $P=0.001$, which is intermediate between the effect of the continuous presence of EGF and control (Fig. 5).

When HC is present throughout the culture period there is a significant increase of about 30% in total colonies formed, $P=0.01$, and in the number expressing AP activity is reduced to about 50%, $P=0.05$ (Fig. 6). When HC is added at day 0, and HC + EGF and EGF from day 7, there is no effect on total colonies formed but there is a decrease in the number expressing the enzyme activity when EGF and HC are added together, $P=0.001$, which is intermediate between the effect of the continuous presence of EGF and control (Fig. 5).

In vitro differentiation of marrow CFU-F
Fig. 3. A. Two adjacent colonies in control culture: colony on left completely negative for enzyme, colony on right positive at +++ level. B. Colony in control culture positive at ++ level. C. Colony in culture treated with EGF from day 0, positive at + level. D, E. Colonies in culture treated with HC from day 0: both positive at ++++ level. Note different morphologies: in D the majority of the cells right to the edge of the colony are well stained, in E colony is relatively small with cells intensely stained piled up in the centre. Flasks fixed and stained for AP activity (black) at 16 days. Zeiss SR stereomicroscope and MC 63 camera. ×12.
but the addition of EGF significantly reduces the number positive for AP activity (Fig. 6).

**Colony size and expression of AP activity**

Total colonies and percentage positive for AP activity in the different colony size ranges for control and treated cultures are shown in Table 1. The percentage positive for AP activity is higher for larger than for smaller colonies. In EGF-treated cultures the size distribution of colonies differs from that of controls and the average colony size is greater. The number of colonies in the smallest size range is less in controls, \( P \leq 0.01 \), and in the largest size range greater, though significant only for EGF added from day 7, \( P \leq 0.05 \). In EGF-treated cultures the decrease in colonies expressing the enzyme occurs in all size ranges (Table 1). In HC-treated cultures the size distribution of colonies is similar to that in controls and the average colony size is comparable. When HC is present from day 0 total colonies are increased over control and the increase is in all size ranges (Table 1). In HC-treated cultures the increase in the number of colonies expressing the enzyme occurs in all size ranges but is relatively greater for smaller colonies (Table 1).

The distribution of AP-positive colonies amongst the four staining levels for the same cultures as in Table 1 is shown in Table 2. In both EGF- and HC-treated cultures the effect on expression of AP activity occurs at all staining levels. The largest number of colonies express the enzyme at the lower levels. Colonies in the two higher staining categories are very few in number, 2-5% and 5-7% of total colonies or 6-7% of AP-positive colonies, for control and HC-treated cultures, respectively, calculated from data in Tables 1 and 2.

**Discussion**

It has been shown previously that fibroblastic colonies formed in cultures of marrow cells are each derived

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**Figs 4, 5, 6.** Vertical axis; total colonies per flask (□) and AP-positive colonies per flask (●) (mean ± S.E.M.).

40 ng/ml EGF, 10⁻⁶ M-HC. Medium changes at 7, 10 and 13 days. Flasks fixed and stained for AP activity at 16 days. Comparison with control: (●) \( P \leq 0.05 \), (■) \( P \leq 0.01 \), (★★) \( P \leq 0.001 \); comparison with HC, (▲▲▲) \( P \leq 0.001 \).

**Fig. 4.** Data for five rabbits; four or five flasks per rabbit in each control and treated group. From left to right, control flasks and three groups of treated flasks: EGF, HC and HC + EGF added at day 7 and at subsequent medium changes.

**Fig. 5.** Data for four rabbits; two–five flasks per rabbit in each control and treated group. From left to right, control flasks and four groups of treated flasks: EGF added at day 0, day 7 and at subsequent medium changes; EGF added at day 0 and not at subsequent medium changes; EGF added at day 0, EGF + HC added at day 7 and at subsequent medium changes; EGF added at day 0, HC added at day 7 and at subsequent medium changes.

**Fig. 6.** Data for four rabbits, three–five flasks per rabbit in each control and treated group. From left to right, control flasks and four groups of treated flasks: HC added at day 0, day 7 and at subsequent medium changes; HC added at day 0 and not at subsequent medium changes; HC added at day 0, HC + EGF added at day 7 and at subsequent medium changes; HC added at day 0, EGF added at day 7 and at subsequent medium changes.

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Table 1. Total number of colonies/flask (TC), mean ± S.E.M., percentage of colonies positive for AP activity (% AP) in each colony size range

<table>
<thead>
<tr>
<th></th>
<th>1–2 mm</th>
<th>2–4 mm</th>
<th>4–6 mm</th>
<th>&gt;6 mm</th>
<th>Average colony size (mm)†</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>TC 18.8 ± 1.7</td>
<td>18.5 ± 1.5</td>
<td>10.2 ± 2.1</td>
<td>1.4 ± 0.5</td>
<td>3.0 ± 0.10</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>% AP 7.8</td>
<td>35.7</td>
<td>71.8</td>
<td>68.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ EGF</td>
<td>TC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 7</td>
<td>% AP 4.4 ± 1.1</td>
<td>12.1 ± 1.3</td>
<td>11.9 ± 1.9</td>
<td>2.8 ± 0.7</td>
<td>3.6 ± 0.21</td>
<td>21</td>
</tr>
<tr>
<td>+ HC</td>
<td>TC 18.7 ± 2.7</td>
<td>18.6 ± 1.5</td>
<td>12.3 ± 2.1</td>
<td>1.7 ± 0.7</td>
<td>3.1 ± 0.20</td>
<td>20</td>
</tr>
<tr>
<td>day 7</td>
<td>% AP 20.5</td>
<td>60.9</td>
<td>66.0</td>
<td>70.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>TC 15.4 ± 1.6</td>
<td>15.2 ± 1.9</td>
<td>13.5 ± 2.6</td>
<td>1.9 ± 0.6</td>
<td>3.3 ± 0.18</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>% AP 6.4</td>
<td>28.9</td>
<td>71.6</td>
<td>64.0</td>
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<td></td>
</tr>
<tr>
<td>+ EGF</td>
<td>TC 9.6 ± 0.9</td>
<td>9.6 ± 0.8</td>
<td>11.1 ± 2.9</td>
<td>4.5 ± 1.4</td>
<td>4.0 ± 1.38</td>
<td>18</td>
</tr>
<tr>
<td>day 0</td>
<td>% AP 0.7</td>
<td>1.6</td>
<td>13.6</td>
<td>4.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>TC 12.7 ± 1.3</td>
<td>14.2 ± 1.9</td>
<td>9.9 ± 2.7</td>
<td>1.3 ± 0.6</td>
<td>3.2 ± 0.17</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>% AP 9.9</td>
<td>40.3</td>
<td>79.5</td>
<td>54.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ HC</td>
<td>TC 15.6 ± 1.6</td>
<td>17.8 ± 0.8</td>
<td>16.1 ± 2.4</td>
<td>2.3 ± 0.8</td>
<td>3.3 ± 0.17</td>
<td>17</td>
</tr>
<tr>
<td>day 0</td>
<td>% AP 26.8</td>
<td>57.4</td>
<td>78.9</td>
<td>62.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Control cultures and cultures treated with EGF and HC from day 7 and day 0; first three groups (Fig. 4), first two groups (Figs 5, 6). n, no. of flasks. Comparison with control: * P < 0.05, ** P < 0.01, *** P < 0.001.
†Calculated assuming average values of 1.5 mm, 3 mm, 5 mm and 7 mm for each size range, respectively.

from a single cell, using thymidine labelling, chromosome markers and time-lapse photography (Friedenstein, 1976; Friedenstein et al. 1987). In the present study the number of colonies formed is directly proportional to the number of cells inoculated, which is consistent with clonally derived colonies.

Expression of alkaline phosphatase activity is widely accepted as a marker of osteogenesis in bone-forming systems (Rodan & Rodan, 1984). The appearance of the enzyme just prior to mineralization in developing osteogenic tissue formed when rabbit marrow cells were cultured in diffusion chambers in vivo (Ashton et al. 1985; Beckstead et al. 1981; Westen & Bainton, 1979).

In the current hypothesis of lineage in the marrow stromal system, stem cells give rise to committed progenitors and different cell lines (Fig. 7) (Owen, 1985) the number and hierarchy of which have not been fully elucidated. Fibroblastic colonies are formed in culture from CFU-F and differentiation along one or several lineages may occur within the colonies. Clonal expression of AP activity indicates differentiation in an osteogenic direction. Markers for differentiation along fibroblastic and reticular lines are not available and

Table 2. Number of AP-positive colonies/flask in each staining category, mean ± S.E.M., for same cultures as Table 1

<table>
<thead>
<tr>
<th></th>
<th>+</th>
<th>++</th>
<th>+++</th>
<th>++++</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.5 ± 1.7</td>
<td>4.9 ± 0.9</td>
<td>0.9 ± 0.3</td>
<td>0.0</td>
<td>24</td>
</tr>
<tr>
<td>+ EGF</td>
<td>2.0 ± 0.5</td>
<td>0.1 ± 0.1</td>
<td>0.0</td>
<td>0.0</td>
<td>21</td>
</tr>
<tr>
<td>day 7</td>
<td>13.2 ± 1.0</td>
<td>8.3 ± 0.9</td>
<td>1.7 ± 0.5</td>
<td>0.2 ± 0.1</td>
<td>20</td>
</tr>
<tr>
<td>+ HC</td>
<td>9.8 ± 2.3</td>
<td>5.3 ± 1.1</td>
<td>1.1 ± 0.4</td>
<td>0.0</td>
<td>18</td>
</tr>
<tr>
<td>day 0</td>
<td>1.2 ± 0.6</td>
<td>0.2 ± 0.9</td>
<td>0.05 ± 0.06</td>
<td>0.05 ± 0.06</td>
<td>18</td>
</tr>
<tr>
<td>Control</td>
<td>9.7 ± 2.2</td>
<td>5.1 ± 1.1</td>
<td>1.3 ± 0.4</td>
<td>0.0</td>
<td>18</td>
</tr>
<tr>
<td>+ HC</td>
<td>16.1 ± 0.9</td>
<td>8.8 ± 2.1</td>
<td>3.1 ± 0.4</td>
<td>0.4 ± 0.1</td>
<td>17</td>
</tr>
</tbody>
</table>

n, no. of flasks.

Fig. 7. Hypothetical diagram for lineage in the marrow stromal system. In analogy with the haemopoietic system it is proposed that: (1) stromal stem cells generate progenitors committed to one or more cell lines; (2) the cells form a continuum where capability for self-renewal and multipotentiality decrease as lineage commitment increases; (3) CFU-F are components of the stem and progenitor cell population.
adipogenesis is rarely seen in cultures with FCS (Dexter, 1979), and this is also true in the present experiments.

The stem cell characteristics of CFU-F were first suggested by the work of Friedenstein (1976, 1980). Recently it has been shown that CFU-F have a high capacity for self-renewal in culture and retain their ability, after many passages, for differentiation into developing osteogenic tissues in diffusion chambers in vitro (Friedenstein et al. 1987). The variation in size, morphology and level of expression of AP activity among colonies in the present cultures suggests a heterogeneous stem and progenitor population for the stromal system similar to that found for the haemopoietic system (Metcalf, 1984). Much of the heterogeneity may be explained if, as proposed, CFU-F are at different stages in a tissue developmental system (Fig. 7). Furthermore, since only 2.5% of colonies in control flasks are in the higher staining categories, i.e. approach committed progenitor status for osteogenesis, it seems likely that a large proportion of CFU-F in the present cultures are early stem and progenitor cells of the stromal system.

The main effect of EGF in the present experiments was to increase average colony size concomitant with depression of AP activity to negligible levels. This is in agreement with the stimulation of [3H]thymidine uptake and inhibition of the enzyme expression by EGF in an established osteogenic cell line (Kumegawa et al. 1983). The continuous presence of EGF is necessary in the present system for its effect on AP expression (Fig. 5) and might suggest that the presence of the factor keeps the cells in cycle, preventing them from moving into the differentiation pathway. Stimulatory effects of HC on AP activity have been observed in many osteogenic systems in vitro (Rodan & Rodan, 1984; Tenenbaum & Heersche, 1985). In the present system the number of colonies that express AP activity is increased by HC at all levels of expression of the enzyme (Table 2). These results indicate that osteogenic differentiation is being activated within colonies from a wide spectrum of CFU-F.

The present work demonstrates that differentiation occurs in vitro within single colonies derived from marrow CFU-F and that this can be modified by environmental factors. The current model of the marrow stromal stem cell is based primarily on data from in vitro assay of CFU-F (Friedenstein, 1980; Bab et al. 1986; Friedenstein et al. 1987). The present results support the model and encourage further development of in vitro clonal methods with the object of elucidating the lineage of the stromal system and identifying mechanisms involved in commitment of stromal stem cells to a particular lineage and promotion of specialized functional cell lines. However, production of specific markers for identification of the different stromal cell lines is an urgent requirement for future application of the methods.

References


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