Skeletal progenitor cells and ageing human populations

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1. Stem and progenitor cells present within bone marrow give rise to colony forming units-fibroblastic (CFU-F) which can differentiate into fibroblastic, osteogenic, myogenic, adipogenic and reticular cells. The decrease in skeletal bone formation and rate of fracture repair observed with ageing and in osteoporosis has been suggested to be due to a decrease in numbers of these progenitors, but human studies are limited.

2. We have tested the potential to form CFU-F in a total of 99 patients undergoing corrective surgery (16 controls, 14-48 years of age) or hip arthroplasty for osteoarthritis (57 patients, 28-87 years of age) or osteoporosis (26 patients, 69-97 years of age). Total colony number, alkaline phosphatase-positive colony number and colony size were determined.

3. No decrease in colony forming efficiency under the culture conditions used was observed in all populations examined irrespective of age, disease or gender, as determined by the lack of correlation between colony formation and age.

4. Examination of colony sizes showed a significant reduction in colony size with age in osteoarthritic and control populations indicating a change in cellular proliferative potential with age.

5. Examination of number and percentage of alkaline phosphatase-positive CFU-F showed a significant decrease in osteoporotic patients compared with controls and osteoarthritic patients, indicating altered differentiation potential.

6. These results suggest that the reduction in bone mass with ageing may be due to reduction of the proliferative capacity of progenitor cells or their responsiveness to biological factors leading to alteration in subsequent differentiation. The maintenance of CFU-F number and alkaline phosphatase activity in these osteoarthritic patients may, in part, explain the inverse relationship observed for the preservation of bone mass between generalized osteoarthritis and primary osteoporosis.

INTRODUCTION

Bone structure is maintained by a delicate balance between bone resorption and bone formation during skeletal remodelling in the young individual [1-3]. However, in aged individuals this process is altered, resulting in net bone loss as a consequence of increased trabecular and cortical bone resorption and decreased osteoblast number and bone formation [4-9]. Stem cells within bone marrow give rise to colony forming units-fibroblastic (CFU-F), which can differentiate into cells of the osteogenic, adipogenic, fibroblastic and reticular lineages [10-13]. A proportion of these CFU-F cells have high proliferative and differentiation capacity, as shown by the formation of bone in diffusion chambers in rat, mouse, rabbit, porcine and human marrow populations, which has led to interest in the use of these stem cells for skeletal repair [14-20]. However, there is little known about the roles of the osteoblast stem cells, osteoblast progenitors or their changes in number or regulation during the process of ageing in normal, osteoarthritic or osteoporotic populations. Osteoarthritis (OA) and osteoporosis affect a majority of the population over 70 years of age [21,22]. However, these two common age-related musculo-skeletal diseases rarely co-exist and a number of clinical studies have suggested an inverse relationship between OA and osteoporosis with the increased bone mineral density observed in OA providing protection against osteoporosis [22,23].

To date, the culture of marrow cells and quantification of osteoprogenitors with ageing has been examined in predominantly animal models with contradictory results. Bergman et al. [24] reported that in mouse marrow cells from 4- and 24-month-old mice, the numbers of osteoblast progenitors were decreased, although the basal proliferative rate of these cells in older animals was increased. No differences in alkaline phosphatase expression were observed at the time points studied. Tsuji et al. [25], using marrow cultured from young and old rats, found reduced nodule formation and reduced alkaline phosphatase-positive cells in older animals, and, similarly, Quarto et al. [26] and Egrise et al. [27] reported reduced osteoprogenitor number and reduced alkaline phosphatase number in aged rats. In contrast, Roholl et al. [28] in an ultrastructural analysis study found the numbers of osteoblasts in ageing rats decreased 10-fold, but the numbers of pre-osteoblasts were age independent suggesting that the diminished maturation of osteoprogenitors to osteoblasts is the main factor in bone loss associated with ageing. In studies looking at stromal colony formation from mouse marrow, Xu et
al. [29] observed colony formation was 50% higher in 14–15-month-old mice compared with that in 3–4-month-old mice.

The number of human studies on osteoprogenitor number and age are few and conflicting. Evans et al. [30] observed no correlation in cell growth and donor age from in vitro studies using trabecular bone explant cultures, and Knight and Gowen [31] found no relationship between osteoblast function and age. However, preliminary reports from Nishida et al. [32] and Endo et al. [33] showed that CFU-F number and alkaline phosphatase-positive CFU-F number were decreased with age, while Shigeno and Ashton [34] found cell proliferation was decreased in populations under 30 years of age.

We have examined the effects of ageing on human osteogenic potential in control, osteoarthritic and osteoporotic populations and tested the hypotheses that (i) reduced CFU-F numbers are responsible for reduced osteoprogenitor and osteoblast number and consequently reduced bone formation observed with age in control and osteoporotic populations, and (ii) that maintained or increased CFU-F numbers or CFU-F activity are responsible for the maintenance of osteoprogenitor and osteoblast number/activity in generalized OA and thus explain, in part, the increased bone mineral density observed in OA.

METHODS

Materials

Cell culture reagents were obtained from Gibco/BRL (Paisley, Scotland). Alkaline phosphatase kits and other reagents were from Sigma Chemical Company (Poole, Dorset).

Cell culture

Primary cultures of bone marrow cells obtained from haematologically normal patients undergoing routine total hip replacement surgery or corrective surgery (Table 1) were established as previously described [35]. Only tissue which would have been discarded was used with the approval of the local hospital management and ethical committee. In brief, marrow cells were harvested using a minimal essential medium (αMEM) from trabecular bone marrow samples and pelleted by centrifugation at 500 g for 5 min at 4 °C. The cell pellet was resuspended in 10 ml of αMEM and passaged through nylon mesh (90 μm pore size; Lockertex, Warrington, U.K.). Samples of cell suspension were diluted with 0.5% (w/v) Trypan Blue in 0.16 M ammonium chloride and the number and viability of nucleated cells determined. Cells were plated out in 25 cm² plastic tissue culture flasks at 2 × 10⁴ nucleated cells/flask in αMEM supplemented with 10% (v/v) foetal calf serum, penicillin (5000 units/100 ml) and streptomycin sulphate (5 mg/100 ml). To avoid problems that can be encountered with batch to batch serum variation, all studies were conducted using the same serum batch, known from laboratory studies to promote stromal cell adhesion and cell proliferation in human bone marrow cultures.

Cultures were maintained at 37 °C in a gassed incubator, 5% CO₂ in air. The medium was changed after 6 days and then at day 10 and cultures were stopped at day 12. At the completion of cell culture, the medium was removed, the cell layer washed in PBS and cultures fixed in 95% (v/v) ethanol. Histological examination of the human bone marrow cultures was performed using a Zeiss Axioshot photomicroscope (Carl Zeiss Ltd, Welwyn Garden City, Herts, U.K.), and recorded on Kodak tungsten film (Kodak, Hemel Hempstead, U.K.).

Colony counting and colony size

A range of plating densities (from 5 × 10⁴ to 5 × 10⁶ cells/25 cm²) was examined in extensive preliminary studies to determine the optimal seeding density. A seeding density of 2 × 10⁴ cells provided clear distinct colonies and reproducible stable colony numbers (results not shown). Total and alkaline phosphatase-positive colonies were counted by eye using an Anderman colony counter (Anderman and Co. Ltd, Kingston-on-Thames, U.K.) as previously reported [36]. All counts were performed blind by an independent observer. Counts were repeated randomly to confirm reproducibility of counts obtained. Mean values for each age point from each patient were derived from 3–6 flasks. For quantitative measurement of colony sizes, CFU-F colony images were captured with a video camera, digitized and analysed using OPTIMAS image analysis software (DataCell Ltd, Maidenhead, Berks, U.K.). Mean colony sizes (± S.E.M.; cm²) for each patient were determined from 10 colonies selected at random [37], using a grid of the flask area drawn up using random number tables to determine x–y coordinates, and placed over the surface of the flask (3–6 flasks per patient).

<table>
<thead>
<tr>
<th>Patient no. (Male + Female)</th>
<th>Patient no. (Male)</th>
<th>Patient no. (Female)</th>
<th>Age (years)</th>
<th>Clinical information</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>2</td>
<td>10</td>
<td>14–31</td>
<td>Costoplasty/Secliosis</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>2</td>
<td>21–48</td>
<td>Fracture of femur</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>1</td>
<td>47</td>
<td>Fusion of spine</td>
</tr>
<tr>
<td>57</td>
<td>23</td>
<td>34</td>
<td>28–67</td>
<td>Coxarthrosis</td>
</tr>
<tr>
<td>26</td>
<td>4</td>
<td>22</td>
<td>69–97</td>
<td>Osteoporosis—Thompson Fracture</td>
</tr>
</tbody>
</table>
Histochemical staining—alkaline phosphatase activity

Cultures were rinsed three times in PBS, fixed in 95% (v/v) ethanol and stained using an alkaline phosphatase kit (Sigma kit no. 85) according to the manufacturer’s instructions. Colonies were determined to be alkaline phosphatase-positive if they showed any observable staining by light microscopy. The percentage of alkaline phosphatase-positive colonies (for each patient) was based on the mean for each flask.

Statistics

Values are expressed as means ± S.D. or S.E.M. Linear regression was performed on all data sets and statistical significance (critical value of t) calculated on n − 2 degrees of freedom at the two-sided significance level using the following equation from Altman [38]:

To test under the null hypothesis Ho that there is no correlation with the parameter in the population, the population correlation coefficient \( p = 0 \)

\[ t = \frac{(n-2)}{\sqrt{1-r^2}} \sim t_{(n-2)} \]

where \( r \) = correlation coefficient and \( n \) = sample number. If \( 0 - t_c < t < t_c \), conclude \( p = 0 \). The minimum number of samples required to provide a valid result at the correlation observed was calculated using the following equation [37]:

\[ n = \left( \frac{1.96 \sqrt{1-r^2}}{r} \right)^2 + 2 \]

where \( n \) gives the minimum sample size at the corresponding correlation coefficient \( r \) required to give a significant two-sided result.

RESULTS

Colony forming efficiency

At the completion of culture on day 12, discrete, individual alkaline phosphatase-positive and negative colonies were observed by eye and microscopy and demonstrated a wide variation in colony size. Colonies were observed to be either completely unstained or to contain varying numbers of stained cells. Colonies containing any alkaline phosphatase-positive cells were counted as alkaline phosphatase-positive CFU-F. The mean fibroblast colony forming efficiency from the whole patient group (99 patients; 14–97 years of age) was found to be \( 2.75 \times 10^{-3} \pm 1.45 \times 10^{-5} \), giving approximately 1 colony/40000 plated nucleated marrow cells. The colony forming efficiency, as shown in Table 2, was similar whether analysed as a whole or in individual control (16 patients, 14–48 years of age), osteoarthritic (57 patients, 28–87 years of age) and osteoporotic populations (26 patients, 69–97 years of age).

Variation of CFU-F number with age

The variation in colony number in control, osteoarthritic and osteoporotic populations is shown in Figure 1a. Examination of colony number as a function of age showed no correlation of CFU-F number (regression value, \( r = -0.16 \)) in vitro with increasing age in control populations (Table 2). Similarly, examination of CFU-F number with age in the osteoarthritic (\( r = 0.03, 28–87 \) years of age) and osteoporotic (\( r = -0.02, 69–97 \) years of age) populations showed no significant correlation as a function of age (Table 2). It should be noted that the significance level of the correlation coefficient and the sample size are inversely related and thus a larger sample size ensures that a smaller observed correlation value is required to have a significant result. None of the above correlation coefficients are significant on this basis.

Variation with gender and age

The current data were analysed for variation in CFU-F number within the female population sampled to determine whether variation in CFU-F number in control, osteoarthritic or osteoporotic patients was gender specific. There was a wide variation in CFU-F numbers, when examined as a complete group or subdivided into disease categories; no significant correlation in CFU-F number with age was observed whether from control (\( r = -0.195 \)), osteoarthritic (\( r = 0.01 \)) or osteoporotic populations (\( r = 0.120 \)).

Expression of alkaline phosphatase-positive CFU-F

Examination of alkaline phosphatase-positive CFU-F in control subjects and patients with OA revealed no significant age-related differences in alkaline phosphatase expression. Alkaline phosphatase-positive CFU-F colonies were detected in both young and old marrow samples with a high degree of variation across all age groups, whether expressed as numbers of total alkaline phosphatase-positive CFU-F colonies or the percentage of alkaline phosphatase-positive colonies irrespective of age or gender (Figures 1b and 1c). In contrast, in osteoporotic patients, although there is no change with age in colony number, the mean number of alkaline phosphatase-positive CFU-F colonies and the percentage of alkaline phosphatase-positive CFU-F colonies was significantly reduced (\( P < 0.001 \)) compared with control subjects and patients with OA (Figures 1b and 1c and Table 2), indicating altered differentiation potential of CFU-F colonies from osteoporotic populations.

Variation in colony size

Colony size and thus proliferation of putative osteoprogenitors was examined in controls (\( n = 10 \)), osteoarthritic (\( n = 22 \)) and osteoporotic (\( n = 22 \)) populations to examine if this parameter was altered during ageing. Colony size was significantly reduced (\( P < 0.001 \)) with increasing age irrespective of whether the donors were control subjects (\( r = -0.618 \)) or...
Table 2: Analysis of CFU-F number and colony size as a function of age in normal subjects or patients with skeletal disease

Fibroblast colonies from normal, osteoarthritic or osteoporotic male and female patients of differing ages were analysed by counting colony number and measuring colony size using image analysis. Correlations of the changes with age within populations of normal, osteoarthritic and osteoporotic patients were calculated. *P < 0.001 versus control using Student's t-test; †P < 0.001 versus OA group using Student's t-test, ††P < 0.001, significant correlation in colony size with age.

<table>
<thead>
<tr>
<th>Age group (years)</th>
<th>Patient no.</th>
<th>Colony number (mean ± S.D.)</th>
<th>Age correlation (r value)</th>
<th>% Alkaline phosphatase-positive CFU-F (mean ± S.E.M.)</th>
<th>Alkaline phosphatase-positive CFU-F (mean ± S.E.M.)</th>
<th>Colony forming efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony no.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (14–48)</td>
<td>16</td>
<td>62 ± 27</td>
<td>−0.16</td>
<td>37 ± 3</td>
<td>56 ± 4</td>
<td>3.1 × 10⁻⁵ ± 1.4 × 10⁻⁵</td>
</tr>
<tr>
<td>OA (28–87)</td>
<td>57</td>
<td>48 ± 28</td>
<td>+0.03</td>
<td>33 ± 2</td>
<td>62 ± 3</td>
<td>2.4 × 10⁻⁵ ± 1.4 × 10⁻⁵</td>
</tr>
<tr>
<td>Osteoporosis (69–97)</td>
<td>26</td>
<td>66 ± 22</td>
<td>−0.02</td>
<td>12 ± 1††</td>
<td>18 ± 2††</td>
<td>3.3 × 10⁻⁵ ± 1.1 × 10⁻⁵</td>
</tr>
<tr>
<td>Colony size</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal (14–48)</td>
<td>10</td>
<td>0.094 ± 0.033</td>
<td>−0.618‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OA (48–83)</td>
<td>22</td>
<td>0.062 ± 0.035</td>
<td>−0.645§</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteoporosis (69–97)</td>
<td>22</td>
<td>0.073 ± 0.019</td>
<td>−0.096</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Patients with OA (r = −0.646) (Table 2). Similarly, colony size was reduced in osteoporotic patients compared with controls and thus an effect of age is apparent as the osteoporotic population has a mean age (83.6 ± 7.1 years) greater than the control population (23.8 ± 9.5 years). However, colony size within the osteoporotic population was not age dependent (r = −0.096) over the narrow age range of 69–97 years (Table 2). Calculation of the minimum number of samples required to give a significant result, at the correlation values observed for colony size change with age for the control subjects and patients with OA (r = −0.618 to −0.645 respectively), indicates that only 4–8 patient samples were required.

DISCUSSION

The present studies indicate that no correlation between age and CFU-F number or colony forming efficiency was observed in all age groups of the patients examined irrespective of disease state or gender. Thus the potential to form CFU-F, examined in this study, was found to be independent of age in these situations. This indicates the possibility that the reduction in bone formation associated with ageing is not a consequence of reduced numbers of early progenitor cells as shown by measurement of colony forming efficiency or colony number. Rather, it appears more probable that it is a consequence of alteration of osteoprogenitor proliferation and differentiation and possibly altered responsiveness of these cells to biological factors affecting these processes. In the populations examined, a significant reduction in colony size with age was observed within control subjects and patients with OA. When compared with controls this was also seen in osteoporotic patients, indicating a general lowering of proliferative capacity of CFU-F with development of the disease. Furthermore, in osteoporotic patients the number and percentage of alkaline phosphatase-positive CFU-F colonies was reduced compared with osteoarthritic and control populations. It has been observed that there is a general and inverse relationship between OA and osteoporosis [22,23], and this could be attributable, in part, to a maintenance of CFU-F number and activity in OA. The present studies, however, are more supportive of the hypothesis that the differences in these two populations are a result of elevated growth factor content in OA which may contribute to the altered bone turnover, increased bone density and decreased incidence of skeletal fracture seen in this condition [39,40].

To examine variations in CFU-F number in human populations, the site and reproducibility of marrow extraction and the culture conditions employed are crucial. In all OA samples used in this study, marrow was from the trabecular bone within the femoral head and neck. For patients undergoing corrective surgery marrow was from the rib, and for osteoporotic samples (all classified as Thompson fractures) it was extracted from the femoral head and neck. Although only limited comparisons can be drawn between patients undergoing corrective surgery and patients presenting with OA or osteoporosis, as these groups have different patient age ranges, numbers and pathophysiology, no significant differences were observed in CFU-F number, indicating the site of marrow extraction was not an issue. In the current study a wide variation in alkaline phosphatase-positive CFU-F colonies was observed in patients presenting with OA. Whether this
reflected differences in the phenotype of the precursor pool in OA is unclear. Furthermore, in the present studies no dexamethasone or other additional osteogenic factors were added to the cultures, as, particularly with dexamethasone, almost all human CFU-F become alkaline phosphatase-positive [35,41,42]. Thus this parameter appears to be less indicative of osteogenic capacity in the presence of dexamethasone in the human system.

The results presented here differ from some of the data obtained by using mouse and rat marrow stromal cells from young and aged animals [4,27,36,43] in which a negative correlation is observed between the number of osteoblast progenitors and age. However, other studies in rodent systems are in conflict with these observations. Xu et al. [29] found an increase in CFU-F formation in old mice compared with young mice, and Roholl et al. [28] found that the changes in
bone mass observed between young and old mice were a consequence not of altered osteoblast precursor number, but rather a failure of osteoblast differentiation into mature osteoblasts. They observed no evidence, from morphometric examination of bone surfaces, for a reduction in number of osteoblast precursors in old mice. Shigeno and Ashton [34] examined cell outgrowths from human trabecular explants and noted a decrease in proliferation in trabecular bone cells as a function of age, but only in the second and third decades. In the present study, CFU-F number from controls over five decades showed no significant correlation \((r = -0.16)\) with age over the age range 14-48 years.

The difference between these results and those in abstracts from Nishida et al. [32] and Endo et al. [33], reporting a correlation in CFU-F and alkaline phosphatase-positive CFU-F in their human marrow populations, is difficult to reconcile. However, their studies provided no clinical details of the patients studied. The large number of patients examined in the present study argues against limited sample size and numbers of observations as the cause for discrepancy. It is particularly relevant that studies looking at haemopoietic status and function in young (2–3 months) and old (2–2.5 years) mice, where total marrow cellularity and stem cell numbers for granulocytes, fibroblasts and erythroblasts were examined, found no evidence for a decrease in numbers of these cells with ageing [43]. The current results also indicate that in the stromal system, like the haemopoietic system, ageing has no effect on the efficiency of colony formation, or colony numbers. In support of the possibility of altered responsiveness of progenitor cells and a decrease in osteoprogenitor proliferation, Marie et al. [44] found a reduction in cell proliferation capacity in trabecular bone explant cultures from women with postmenopausal osteoporosis.

The current results emphasize an important point made by Friedenstein [45], namely that changes in observed marrow CFU-F quantity or colony forming efficiency may be dependent on sensitivity to a variety of growth factors rather than an actual reduction of stem cell number \(in vivo\). The present results do not preclude the possibility that changes in CFU-F number may occur within the first few years of development, but in the population examined here the stem cell–progenitor pool appears to remain constant from 14 to 97 years of age. The lack of evidence to substantiate a decrease in CFU-F number but the evidence of a decrease in proliferation of osteoprogenitors with age and in osteoporotic patients, suggests alternative reasons for the reduced bone mass associated with ageing. Thus either CFU-F are not altered as a function of age, but the responses to growth factors and hormones are decreased and/or the progeny of the osteogenic stem cells have altered differentiation potential, or, differentiation into alternative stromal fibroblastic lineages, such as adipocytes, occurs. It is well known that there is an association between an increase in adipogenesis in osteoporotic patients and the elderly and a progressive loss of bone [6,46,47].

Pfeilschifter et al. [48], using trabecular bone explant cultures, have presented data in support of a decreased response of osteoblasts to growth factors and hormones in osteoporosis. In an extension of these studies Bismar et al. [49], and in separate studies Marie et al. [50], have also shown that the combination of altered cytokine production and osteoprogenitor responsiveness may play a major role in the pathogenesis of bone loss with age. In summary, the known reduction in fracture repair and bone loss with ageing [51] or in osteoporosis may be due to reduction of the proliferative capacity of progenitor cells and/or a reduction in responsiveness of osteoprogenitor cells to biological factors resulting in alteration in their subsequent differentiation. Furthermore, our results indicate that the inverse association between OA and osteoporosis may be partially explained by the maintenance of CFU-F number and activity in osteoarthritic patients with age. These results indicate that examination of CFU-F proliferation and activity and identification of the factors and mechanisms that regulate these processes may provide useful insights into bone formation in disease states or in ageing populations.

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REFERENCES


