Decrease in the mesenchymal stem-cell pool in the proximal femur in corticosteroid-induced osteonecrosis

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We have evaluated bone-marrow activity in the proximal femur of patients with corticosteroid-induced osteonecrosis and compared it with that of patients with osteonecrosis related to sickle-cell disease and with a control group without osteonecrosis. Bone marrow was obtained by puncture of the femoral head outside the area of necrosis and in the intertrochanteric region. The activity of stromal cells was assessed by culturing fibroblast colony-forming units (FCFUs).

We found a decrease in the number of FCFUs outside the area of osteonecrosis in the upper end of the femur of patients with corticosteroid-induced osteonecrosis compared with the other groups. We suggest that glucocorticosteroids may also have an adverse effect on bone by decreasing the number of progenitors. The possible relevance of this finding to osteonecrosis is discussed.

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The mechanism responsible for steroid-induced osteonecrosis is still unknown. In stromal-cell cultures of rat marrow, evidence for an inverse relationship between the differentiation of adipose and osteogenic cells has been demonstrated, and dexamethasone is known in certain circumstances to induce adipogenesis. Steroids have been shown to produce adipogenesis and to stimulate fat-specific genes in cloned bone-marrow cells. In patients with osteonecrosis secondary to corticosteroid therapy, abnormalities have been demonstrated in the bone marrow of the iliac crest, with a decrease in the stem-cell pool. Since adipocytes and osteoblasts share a common pool of stem cells, and since steroids induce their differentiation into adipocytes, a decrease in the stem-cell pool in the upper end of the femur, as observed in the iliac crest, may result in an insufficient number of stem cells to provide enough osteoblasts in the femoral head, eventually leading to osteonecrosis.

In order to examine the hypothesis that in patients with osteonecrosis related to corticosteroid therapy, abnormalities in the bone marrow are also present in the upper end of the femur, we compared the bone-marrow activity of adult patients with early osteonecrosis due to sickle-cell disease and with a control group of patients without osteonecrosis.

Patients and Methods

There were three groups of patients as follows:

Group 1. This consisted of 25 patients with a mean age of 34 years (18 to 45) who had osteonecrosis related to corticosteroid therapy. There were 17 men and eight women and the reasons for the administration of corticosteroid therapy included lupus erythematosus (8 cases), renal transplantation (7 cases), cardiac transplantation (2 cases), liver transplantation (1 case), allogeneic bone-marrow transplantation for leukaemia (3 cases), asthma (2 cases), uveitis (1 case) and other causes (1 case).

Group 2. There were 30 patients (13 men and 17 women) with a mean age of 28 years (19 to 33) with osteonecrosis related to sickle-cell disease. This group was selected since in sickle-cell disease the mechanism of osteonecrosis in bone is known and has no relation to a decrease in fibroblast colony-forming units (FCFUs).

Group 3. This was further divided into two subgroups. Group 3a consisted of seven normal patients (four men and three women) with a mean age of 37 years (35 to 45) who gave their informed consent to have aspiration of bone marrow from the femoral head and the intertrochanteric region during surgery under general anaesthesia. They had no underlying general or haematological disease.
In group 3b there were six patients (two men and four women) with sickle-cell disease but no osteonecrosis and a normal MRI. Their mean age was 28 years (35 to 31).

Measurement of bone-marrow activity. We used the FCFU as an indicator of stromal-cell activity. The fibroblast is not an osteogenic cell but, according to the theory of Friedenstein et al.\(^7\) and Owen et al.\(^8\) osteocytes develop from FCFU progenitor cells in the marrow. There seems little doubt that these colonies are clonal. We therefore used cultures of fibroblast progenitor cells\(^12\) obtained from aspirated samples from the proximal femur of patients having surgery for early osteonecrosis and compared them with bone marrow from the other two groups.

Collection of bone marrow. Bone marrow was collected from the proximal femur using a technique of autologous bone-marrow grafting from the iliac crest associated with core decompression.\(^13\) Under general anaesthesia, it was obtained from the area of osteonecrosis, the intertrochanteric region of the proximal femur and the femoral head. The patient was placed on a fracture table in the supine position and two image intensifiers were positioned to allow visualisation of the femoral head in the antero-posterior and lateral planes. Bone-marrow punctures were carried out under fluoroscopic control and an aspiration performed.\(^14\) The needle was rinsed with a heparin solution, introduced by hand through the lower aspect of the greater trochanter in the intertrochanteric region and 2 to 4 ml were aspirated within two to eight seconds. The obturator and needle were then pushed in the direction of the anterosuperior quadrant of the femoral head under the ‘sequestrum’. Marrow was then aspirated from the normal bone of the femoral head, as seen by MRI, outside the necrosis by the same technique. We used the coronal MRI cut with the greatest area of abnormality. Since the validity of the results obtained depended on the ability to aspirate bone marrow from areas of the femoral head not affected by osteonecrosis, we drew up less than 4 ml and the position of the extremity of the needle was assessed on the anteroposterior and lateral views on the image intensifier.

To determine the number of FCFUs quadruplicate aliquots of 2 × 10\(^5\) cells were inoculated in 25 ml tissue-culture flasks containing 10 ml of culture medium supplemented with 20% fetal calf serum, 100 U/ml of penicillin and 100 mg/ml of streptomycin. Culture flasks were placed in a humidified incubator with 5% CO\(_2\) and maintained at 37°C. The growth medium was completely renewed every three to four days. Fibroblastic colonies were stained with Giemsa and counted under an inverted microscope at a magnification of ×25. An aggregate of cells containing more than 50 fibroblasts was scored as a colony. The results were expressed as the mean number of FCFUs per 10\(^5\) bone-marrow cells. The fibroblastic nature of the colonies was shown by immunofluorescence staining with antibodies against fibronectin and type-I and type-III collagen.

Volumetric analysis of the femoral head. The results were expressed as the mean number of FCFUs per 10\(^5\) bone-marrow cells. To determine if there was a difference in the number of FCFUs between small and large osteonecrotic lesions, we adjusted the result to the volume of the femoral head outside the osteonecrosis. The volume of the osteonecrosis and that of the femoral head outside the area of osteonecrosis were measured on the MR scans.

Although the MR image is two-dimensional, it provides information from a three-dimensional block. Summing these blocks approximates to the respective total volumes of the necrotic bone. The number of blocks per femoral head depends on the thickness selected (5 mm) and on the choice of a contiguous or step section protocol. Thus, the sector of osteonecrosis was calculated on each slice. The volume of the necrosis was the sum of all the elementary surfaces multiplied by their thickness.

T1-weighted coronal slices with a 1.5 Tesla superconducting unit were contiguous and included all of the femoral head; the thickness of each cut was always 5 mm. For each coronal slice (mean 9.4 slices per hip), the sector of the necrosis was considered as the area demarcated by the serpiginous line corresponding to the band-like hypointense line. The volume of the necrosis was the sum of all the elementary surfaces multiplied by their thickness. In practice, each coronal slice was digitised and the area of the osteonecrosis outlined using an image-analysis program (public-domain National Institutes of Health Image program). To assess the validity of the method, 30 hips were evaluated independently by the two authors. Each measured the volume on the MR scan twice, with an interval of at least one month between the first and second readings. The interobserver variability was measured by defining a tolerance interval using an analysis of variance.\(^15\) The mean difference between the two observers was estimated to be 0.25 cm\(^3\) (95% CI 2.50 to 3.00) and the mean difference between the first and the second observation in each observer was 0.05 cm\(^3\) (95% CI 2.65 to 2.75). We therefore assumed that both of these differences were zero, and estimated the intraobserver and interobserver variance com-
ponents as 0.157 and 0.065, respectively. Under this assumption, the difference between two different readings made by the same observer had a variance of $2 \times 0.157 = 0.314$, and therefore a standard deviation of 0.560 cm$^3$. The difference between two different readings made by different observers had a variance of $2 \times (0.157 + 0.065) = 0.444$, giving a standard deviation of 0.666 cm$^3$. We therefore expected that 95% of intraobserver differences would have a magnitude of no more than 1.5 cm$^3$ and 95% of interobserver differences a magnitude of no more than 1.7 cm$^3$. Thus, we could be 95% confident that 95% of the time, one observer’s reading would be no more than 2 cm$^3$ of the other observer’s reading due to observer error alone. This value was only 1 cm$^3$ for osteonecrosis with a volume less than 15 cm$^3$, but was equal to 3 cm$^3$ for osteonecrosis with a volume greater than 30 cm$^3$.

**Statistical analysis.** Because a normal model could not be assumed for the data for control patients, we used non-parametric tests for statistical analysis. The Mann-Whitney U test (equivalent to the Wilcoxon rank-sum test) was used with each group of patients being an independent variable and the number of FCFUs and the volume of the osteonecrosis the dependent variables.

**Results**

Table I gives the volume of the osteonecrotic areas and the number of FCFUs for each group. The p values for the group comparisons are presented in Tables II and III.

The main conclusion of Table II is that group 1 has CFU counts spectacularly below all the other groups. The null hypothesis that group 1 is no different from other groups is refuted, but the high p values observed elsewhere do not prove the null hypothesis since the data may also be compatible with more modest differences, especially when numbers are low.

The Wilcoxon test determines a difference between two probabilities. Given two randomly chosen patients from groups A and B, these are that the group-A patient has a higher value than the group-B patient and that the group-B patient has a higher value than the group-A patient. The difference between these two probabilities is called Somers’D, and is estimated in Table II (with approximate 95% confidence limits) for all the non-significant CFU differences in this Table. For instance, given randomly chosen patients from groups 2 and 3b, the group-2 patient is 21% less likely than the group-3b patient to have the higher of the two CFU counts in the femoral head, but the true population value of the difference could be anything from 69% less likely to 28% more likely. The data are compatible with the null hypothesis of equal probabilities, but also with sizeable differences in either direction.

**Group 1 (corticosteroid-induced osteonecrosis).** No FCFUs were found in the area of osteonecrosis. In the femoral head outside the osteonecrosis, the mean number of FCFUs (sd 4.02) was 1.88 per 10$^6$ bone-marrow cells. For the intertrochanteric region it was 1.20 (sd 2.27) per 10$^6$ bone-marrow cells.

There was a significant difference in the number of FCFUs both in the femoral head and in the intertrochanteric region between group 1 and groups 3a and 3b and group 3 as a whole.

**Group 2 (osteonecrosis related to sickle-cell disease).** No FCFUs were found in the area of the osteonecrosis. For the 20 patients with osteonecrosis related to sickle-cell disease, the mean number of FCFUs was 30.9 (sd 33.1) in the femoral head outside the necrosis and 37.8 (sd 33.2) in the intertrochanteric region. These were significantly different from group 1, but no significant difference was seen between group 2, groups 3a and 3b and group 3 as a whole.

**Group 3 (control patients).** In the control group the mean number of FCFUs was 33.5 (sd 21.7) per 10$^6$ bone-marrow cells. For the intertrochanteric region it was 43.7 (sd 26.8) per 10$^6$ bone-marrow cells.

**Number of FCFUs and volumetric analysis of the femoral head.** The mean volume of osteonecrosis was 15.7 cm$^3$ (sd 7.34) for the group with osteonecrosis related to sickle-cell disease and 19.1 cm$^3$ (sd 11.5) for those in the corticosteroid group; this difference was not significant (p = 0.464). The number of FCFUs in the femoral head outside the osteonecrosis or in the intertrochanteric region was not related to the volume of the osteonecrosis or to the percentage of the volume of the femoral head involved in the osteonecrosis.

**Discussion**

During normal fetal development, bone marrow has an entirely active haematopoietic function. Conversion from red to yellow marrow begins just before birth in the distal extremities, followed by the epiphysis and midshafts of the long bones. The process continues until 25 years of age when the adult distribution pattern is reached. Normally, in the adult, haematopoietic marrow is scant in the femoral head but red marrow persists in the proximal shaft of the femur. This was demonstrated by Hashimoto and by MRI.

In spite of many studies since the first description by Pietrogrande and Mastromarino, the exact pathogenesis of femoral capital osteonecrosis related to corticosteroids remains uncertain. It has been suggested that the first lesions are seen in the marrow space and occur exclusively within fatty marrow. The distribution of haematopoietic marrow is related to various factors and may vary. MRI studies have indicated that the conversion of red to fatty marrow seems to occur prematurely in some patients with avascular necrosis at the upper end of the femur. Intramedullary vascularity is altered and this may be a predisposing factor for osteonecrosis in fatty marrow since bone-marrow changes and bone remodelling are linked.
Table I. Details of fibroblast colony-forming units (FCFUs) per $10^6$ bone-marrow cells in the three groups

<table>
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<tr>
<th>Group 1 (n=25) Necrosis (corticost)</th>
<th>Group 2 (n=20) Necrosis (SCD*)</th>
<th>Group 3 (n=13) Without necrosis</th>
<th>Group 3a (n=7) Normal patients</th>
<th>Group 3b (n=6) (SCD)</th>
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<tr>
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Mean ± sd 1.88 ± 4.02 1.20 ± 2.27 19.1 ± 11.5 28.2 ± 15.2 30.9 ± 33.1 37.8 ± 33.2 15.7 ± 7.34 27.3 ± 14.5 33.5 ± 21.7 43.7 ± 26.8 32.7 ± 15.9 36.8 ± 19.1 34.5 ± 28.7 51.8 ± 34.0

* sickle-cell disease
† volume of the femoral head involved by the osteonecrosis
Abnormalities in the bone marrow of the iliac crest in patients with steroid-induced osteonecrosis have already been reported and Cui et al. demonstrated in vitro the effects of steroids on cloned bone-marrow cells with the production of adipogenesis and the stimulation of fat-specific genes. These findings suggest that steroids have a direct action on marrow cells.

Little is known, however, about the relative numbers of marrow stromal stem cells in the normal femoral head and in steroid-induced osteonecrosis. The number of FCFUs in bone marrow is low and in vitro the entry of FCFUs into the cycle and the subsequent development of colonies depend on the growth factors present in the serum. In our experiments, we used fetal calf serum as the growth factor for the FCFU. This may be a limitation since this growth factor is not present in bone in vivo, and Shigeno and Ashton have recently shown that autologous serum supports greater cell proliferation than fetal calf serum.

Our findings failed to detect any difference in bone-marrow activity between osteonecrotic and non-osteonecrotic sickle-cell patients either in the femoral head or in the intertrochanteric region. We found, however, decreased activity of bone-marrow cells in the femoral head under the sequestrum and in the intertrochanteric region in patients with osteonecrosis related to corticosteroid therapy when compared with those with sickle-cell disease and with control patients without osteonecrosis. There may be several explanations for this phenomenon.

First, abnormal bone-marrow activity in the area of osteonecrosis in patients with corticosteroid treatment could be a non-specific response to severe insult to the subjacent bone, but there was no relation to the size of the area of osteonecrosis and this was not observed in patients with osteonecrosis related to sickle-cell disease.

Secondly, the decrease in bone-marrow activity may be related to corticosteroid treatment but not to the osteonecrosis, since several alternative mechanisms have been proposed for corticosteroid-induced osteonecrosis such as fat embolisation, pressure changes, circulatory impairment, coagulation disorders, and an effect on osteoblast function.

Thirdly, osteonecrosis in patients with corticosteroid therapy may correlate with the decrease in bone-marrow activity. Since adipocytes and osteoblasts share a common pool of stem cells and since steroids stimulate the differentiation of marrow stem cells into adipocytes, a decrease in the stem-cell pool as observed in our study may result in the number of pluripotential mesenchymal stem cells being insufficient to provide enough osteoblasts to meet the needs of bone remodelling; this, in turn, gives rise to bone necrosis. The implication is that the mechanism of steroid-induced osteonecrosis is related to the size of the osteoblastic cell pool. The lack of osteogenic cells could influence two different events in the pathogenesis of osteonecrosis; first the occurrence of osteonecrosis itself and then the bone repair which occurs after osteonecrosis. In so-called avascular osteonecrosis, ischaemia is considered to be the first phenomenon, but very little is known about the initial histological changes in the very early stage of osteonecrosis. Rutishauser and Tailland and Rutishauser, Rohner and Held showed that only medullary changes occur during the first days. At this point, ischaemia in combination with a sufficient capacity for repair makes the lesion still reversible by a mechanism of creeping substitution without a ‘reactive interface’ between living and dead bone. This is compatible with what we know about the initial phase of osteonecrosis in patients. At a very early stage of the osteonecrosis the first pathognomonic sign, i.e., the double line representing the reactive interface of vascular repair, is absent on conventional MRI. This initial phase can, however, be detected in the femoral head by bone scanning as a focal decreased uptake without the
marked increase in radioscintigraphic activity in the surrounding bone.\textsuperscript{36} It may also be detected as a reduced contrast enhancement in dynamic contrast-enhanced MRI\textsuperscript{37} or as non-specific patterns of osteonecrosis on conventional MRI. This early stage may represent an early ‘reversible’ osteonecrosis which has been observed on MR scans in some patients\textsuperscript{38} or a ‘reversible’ bone-marrow oedema syndrome.\textsuperscript{39} An insufficient repair mechanism related to a decrease in the osteoblastic cell pool produces a band of increased vascularity, a granulation tissue. This ‘reactive interface’ between living and dead bone may be considered as the unsuccessful attempt of viable tissue to repair the dead marrow and bone trabeculae. As a result, osteonecrosis becomes irreversible with the ‘reactive interface’ appearing on MRI as a band of low signal. At this stage, histological examination shows that there is active formation of new bone in the zone immediately adjacent to the necrotic segment associated with a marked increase in radioscintigraphic activity in the surrounding bone even in osteonecrosis related to corticosteroid therapy. We have found a decrease in osteogenic cells in the femoral head under the sequestrum and in the intertrochanteric region. This has also been confirmed by the observation of the extent of osteocyte death in the proximal femur of patients having total hip replacement for osteonecrosis.\textsuperscript{40} It suggests that FCFUs could appear in the reactive interface by revascularisation from another area of the proximal femur. Since we aspirated bone marrow from under the band-like hypointense line on the MR scan corresponding to the reactive interface demarcating the osteonecrosis, this could explain the absence of FCFUs observed in the femoral head. The lack of FCFUs, however, may influence the reparative response at this stage in which osteoblast differentiation by stromal progenitors in the granulation tissue of the reactive interface is probably a crucial event for the occurrence of the collapse and may explain why corticosteroid-induced osteonecrosis has a bad prognosis with frequent collapse.\textsuperscript{41}

We cannot confirm if this hypothesis is correct, but it may be possible to decrease the risk of osteonecrosis in patients having corticosteroid therapy or to improve the treatment by increasing the differentiation of the decreased number of mesenchymal marrow stem cells into the osteoblast lineage. Some growth factors, like transforming growth factor β, or other substances such as lovastatin\textsuperscript{42} or lipid-clearing agents can delay or decrease dexamethasone-induced adipogenesis. An alternative could be to increase the number of osteogenic stem cells in the femoral head by autologous bone-marrow grafting.\textsuperscript{13}

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References


