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Abnormalities in the Bone Marrow of the Iliac Crest in Patients Who Have Osteonecrosis Secondary to Corticosteroid Therapy or Alcohol Abuse

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ABSTRACT: The bone-marrow activity in the iliac crest of eleven patients who had idiopathic osteonecrosis of the hip and thirty patients who had osteonecrosis of the hip that was related to corticosteroid therapy (fourteen patients) or to alcohol abuse (sixteen patients) was compared with that in two groups of control subjects who did not have osteonecrosis (thirty-three healthy bone-marrow donors and thirty-four patients who had been managed with bone-marrow grafting for a non-union). Cultures of granulocyte-macrophage progenitor cells and fibroblast colony-forming units were performed to assess the activity of hematopoietic stem cells and stromal cells.

The activity of stem cells in both the hematopoietic and the stromal compartment of the bone marrow was decreased in the patients who were receiving corticosteroids or who abused alcohol, as compared with that in the two groups of control subjects. The patients who had idiopathic osteonecrosis also had a decrease in bone-marrow activity compared with the control subjects.

CLINICAL RELEVANCE: Our findings suggest that patients who are receiving corticosteroid therapy or who abuse alcohol have decreased activity of bone-marrow cells. Whether this decrease is related to the osteonecrosis could not be determined, as our study did not include control subjects who had a history of alcohol abuse or who were receiving corticosteroids but did not have osteonecrosis. However, it is possible that the reduced bone-marrow activity was related to the osteonecrosis, as patients who had idiopathic osteonecrosis also had decreased bone-marrow activity. The study of pathological alterations in the bone marrow outside the necrotic zone may provide important insights into the pathophysiology of osteonecrosis.

Despite the wealth of literature on the subject, the exact pathogenesis of osteonecrosis of the femoral head remains uncertain. It has been suggested that the first lesions are seen in the marrow space and, indeed, several authors have found abnormalities in the bone marrow at a distance from the sequestrum. As early as

1983 Arlot et al. reported abnormal findings on histological evaluation of bone from the iliac crest, and more recently Mitchell et al. reported abnormal findings on magnetic resonance imaging of the pelvis in patients who had osteonecrosis of the hip.

Corticosteroid therapy and alcohol abuse are among the most widely recognized risk factors for osteonecrosis. The introduction of techniques^(5,7,13) for the culturing of human hematopoietic and stromal progenitor cells has provided a unique opportunity to study the composition of bone marrow in several disease processes. We wished to examine the hypothesis that diffuse abnormalities in bone marrow are associated with the risk factors of corticosteroid therapy and a history of alcohol abuse in patients who have osteonecrosis. To do this, we compared the bone-marrow activity in specimens taken from the iliac crest of adults who had early osteonecrosis related to corticosteroid therapy or alcohol abuse with that in specimens from subjects who did not have osteonecrosis and from patients who had idiopathic osteonecrosis.

Materials and Methods

In order to measure bone-marrow activity, we used granulocyte-macrophage progenitor cells as indicators of hematopoietic stem-cell activity and fibroblast colony-forming units as indicators of stromal cell activity. The fibroblast is not an osteogenic cell; however, according to the theory developed by Owen et al.^(13,14) and by Friedenstein et al., osteocytes develop from fibroblast colony-forming units (progenitor cells) in the marrow. There seems little doubt that these colonies are clonal (that is, they originate from a single cell)⁽¹³⁾. Therefore, we performed *in vitro* cultures of the fibroblasts and the granulocyte-macrophage progenitor cells from samples of bone marrow that had been aspirated from the iliac crest during operative treatment for early osteonecrosis. The bone-marrow activity in these samples was compared with that in samples taken from the iliac crest of individuals who did not have osteonecrosis.

Subjects

Subjects Who Did Not Have Osteonecrosis (Control Groups)

Group 1 consisted of thirty-three healthy male subjects who had donated bone marrow and had no history of osteonecrosis. Group 2 consisted of thirty-four male patients who had been managed with bone-marrow grafting for a non-union⁽¹⁰⁾. This group was included to investigate whether patients who have an orthopaedic problem involving a lower limb have bone-marrow abnormalities in the iliac crest associated with the absence of weight-bearing or the presence of pain or osteopenia.

Patients Who Had Osteonecrosis

Forty-one men in whom osteonecrosis of the femoral head had been detected by magnetic resonance imaging, in a five-year period, were included in the study. The necrosis in all of these patients was considered to have been detected early,

as the signal in the area of the avascular necrosis was isointense with fat on T1 and T2-weighted images⁽⁶⁾. The findings on anteroposterior and lateral roentgenograms were normal, with no evidence of collapse. The forty-one men were divided into three groups: sixteen patients in whom the osteonecrosis was related to evident alcohol abuse (group 3), fourteen in whom the osteonecrosis was related to corticosteroid therapy (group 4), and eleven in whom the osteonecrosis was considered to be idiopathic (group 5). The lesion was categorized as idiopathic when there were no factors that are commonly associated with osteonecrosis, such as alcoholism, corticosteroid therapy, radiation therapy, sickle-cell disease, an injury of the hip, or abnormal steroid metabolism. Group 5 was included in order to investigate whether there are bone-marrow abnormalities of the iliac crest in patients who have osteonecrosis but do not have the risk factors of corticosteroid therapy or alcohol abuse.

Collection of Bone Marrow

Since 1991, we have treated early avascular necrosis (before collapse)⁽⁹⁾ with injection of autologous bone marrow into the necrotic area during core decompression. The bone marrow is obtained from the anterior iliac crest with use of a standard needle for aspiration of bone marrow⁽¹⁶⁾, rinsed with heparin solution, and introduced by hand. One hundred and fifty milliliters of bone marrow was taken for one hip. This method of collection was used for all of the patients who had osteonecrosis as well as for all of the subjects in the control groups.

Laboratory Studies

After heparin was added to each sample of bone marrow, nucleated cells were counted with use of a standard Malassez hemocytometer (Zintle, Germany) and buffy coats were collected after centrifugation of the specimens at 1300 times gravity for ten minutes^(3,4). The cells were washed once and then were resuspended in Hanks balanced salt solution without calcium and magnesium.

The same cell suspensions were used for the two culture techniques (to be described). All of the colony counts were performed in a blinded fashion.

Fibroblast Colony-Forming Units

Quadruplicate aliquots of 2×10^6 bone-marrow cells were inoculated in twenty-five-milliliter tissue-culture flasks containing ten milliliters of culture medium supplemented with 20 per cent fetal calf serum, penicillin (100 units per milliliter), and streptomycin (100 milligrams per milliliter). The flasks were placed in a humidified incubator with 5 per cent carbon dioxide and were maintained at 37 degrees Celsius. The medium was completely renewed every three to four days. Fibroblast colonies were stained with Giemsa stain and were counted at a magnification of twenty-five times with an inverted microscope. An aggregate of cells containing more than fifty fibroblasts was considered to be a colony. The results were expressed as the mean number of fibroblast colony-forming units per 10^6 bone-marrow

cells. In preliminary studies, the fibroblastic nature of the colonies was demonstrated by immunofluorescence staining with antibodies against fibronectin and type-I and type-III collagen.

Granulocyte-Macrophage Progenitor Cells

To obtain granulocyte-macrophage progenitor cells, aliquots of bone-marrow cells were plated in methylcellulose. Human-placenta-conditioned medium was added as a source of colony-stimulating factor. The assays were performed in quadruplicate in plastic culture plates, which then were incubated for ten days in 5 per cent carbon dioxide-humidified air at 37 degrees Celsius. Duplicate aliquots were plated in each Petri dish at 1×10^5 and 0.5×10^5 nucleated cells per milliliter, in order to control the potential inhibitor effect of red blood cells on culture. Colonies of granulocytes or macrophages, or both, containing more than fifty cells were considered to be granulocyte-macrophage progenitor cells. The counts for four Petri dishes were averaged, and the result was expressed as the mean number of granulocyte-macrophage progenitor cells per $10^{(5)}$ bone-marrow cells. The granulocyte-macrophage composition of the granulocyte-macrophage progenitor cells was confirmed on cytospin preparations.

Statistical Analysis

Because a normal model could not be assumed for the data for the control groups, non-parametric tests were used for statistical analysis. The Mann-Whitney U test (equivalent to the Wilcoxon rank-sum test) was used, with each group as an independent variable and the numbers of granulocyte-macrophage progenitor cells and fibroblast colony-forming units as the dependent variables. The differences were considered to be significant when p was less than 0.01. This conservative value was chosen because of the relatively small number of subjects in each of the five groups. We considered a p value of less than 0.05 but greater than 0.01 to be strongly suggestive of significance.

Results

Although all of the patients were matched for gender (male) and race (white), the age-range for the three groups of patients who had osteonecrosis (twenty-four to fifty-four years) differed from that for the two control groups (one to eighty-three years) (Table I). The differences with regard to age were not significant, although there was a strong suggestion of a significant difference between group 3 (patients who had osteonecrosis associated with alcohol abuse) and group 1 (bone-marrow donors who did not have osteonecrosis) ($p = 0.020$) (Table II). To account for a possible influence of age, two subgroups (groups 1a and 2a) that included only individuals who were twenty to sixty years old were created from groups 1 and 2. There were seventeen subjects in group 1a and twenty-eight patients in group 2a (Table I).

Groups 1 and 2: No Osteonecrosis

The bone-marrow samples that had been aspirated from the iliac crests of the thirty-three bone-marrow

donors (group 1) demonstrated a mean (and standard deviation) of 106.7 ± 67.6 granulocyte-macrophage progenitor cells per 10^5 bone-marrow cells and 19.4 ± 14.2 fibroblast colony-forming units per 10^6 bone-marrow cells (Table I). The corresponding values for group 2 (thirty-four patients who had a non-union) were 61.5 ± 17.8 per 10^5 bone-marrow cells and 13.0 ± 5.70 per 10^6 bone-marrow cells. When the two groups were compared, the difference in the number of granulocyte-macrophage progenitor cells was significant ($p = 0.007$) but the difference in the number of fibroblast colony-forming units was not ($p = 0.150$), with the numbers available (Tables III and IV).

There was no significant difference in the number of fibroblast colony-forming units or granulocyte-macrophage progenitor cells between groups 1 and 1a ($p = 0.825$ and $p = 0.822$) or between groups 2 and 2a ($p = 0.870$ and $p = 0.860$), with the numbers available (Tables III and IV). However, there was a possible suggestion of a significant difference between group 1a and groups 2 ($p = 0.047$) and 2a ($p = 0.065$) with regard to the number of granulocyte-macrophage progenitor cells. A similar possible suggestion of significance was found for the difference in the number of fibroblast colony-forming units ($p = 0.075$ [group 1a compared with group 2] and $p = 0.054$ [group 1a compared with group 2a]). As further differentiation of groups 1a and 2a would have required the addition of patients to the study, we followed a more conservative approach for the remainder of our analysis by comparing all of the groups of patients who had osteonecrosis (groups 3, 4, and 5) with the age-matched control groups (groups 1a and 2a).

Groups 3 and 4: Patients Who Had Osteonecrosis Related to Alcohol Abuse or Corticosteroid Therapy

The mean bone-marrow activity was lower in groups 3 and 4 than in the other groups (Table I). Group 4 had the lowest values of any of the groups, with 38.9 ± 15.1 granulocyte-macrophage progenitor cells per 10^5 bone-marrow cells and 2.29 ± 1.73 fibroblast colony-forming units per 10^6 bone-marrow cells. However, with the numbers available, there were no significant differences between groups 3 and 4 with regard to the number of fibroblast colony-forming units ($p = 0.813$) or granulocyte-macrophage progenitor cells ($p = 0.917$) (Tables III and IV).

The difference in bone-marrow activity (in terms of the number of both granulocyte-macrophage progenitor cells and fibroblast colony-forming units) was highly significant ($p \leq 0.0005$) between the patients who had osteonecrosis due to corticosteroid therapy or alcohol abuse and the control subjects who did not have osteonecrosis (groups 1, 1a, 2, and 2a) (Tables III and IV).

Group 5: Patients Who Had Idiopathic Osteonecrosis

In the bone-marrow samples from the eleven patients who had idiopathic osteonecrosis, there was a mean of 49.5 ± 19.9 granulocyte-macrophage progenitor cells per 10^5 bone-marrow cells. This value was significantly lower than that for group 1 (p

$= 0.004$), but there was only a strong suggestion of a significant difference between this value and the value for group 1a ($p = 0.012$) (Table III). With the numbers available, there were no other significant differences or strong suggestions of significant differences with regard to the number of granulocyte-macrophage progenitor cells between group 5 and the other groups. The mean number of fibroblast colony-forming units in group 5 (8.45 ± 4.61 per 10^6 bone-marrow cells) was lower than that for group 1 ($p = 0.011$), group 2 ($p = 0.029$), and group 2a ($p = 0.039$), but there was only a strong suggestion that these differences were significant. The value for group 5 was significantly greater than those for group 3 ($p = 0.0001$) and group 4 ($p = 0.0002$). The number of fibroblast colony-forming units also was significantly different ($p = 0.003$) from that for the bone-marrow donors who did not have osteonecrosis when the comparison was made with the age-matched subjects (group 1a) (Table IV).

Discussion

Bone marrow is composed of a heterogeneous mixture of different populations of hematopoietic and stromal cells. However, little is known about the relative numbers of stromal stem cells in the marrow, either with respect to the age of the individual or to different disease states, or about the role of the cells in normal physiology or in pathological processes⁽¹¹⁾. The number of fibroblast colony-forming units in bone marrow is low, as indicated by the low number of fibroblast colonies obtained after a high number of bone-marrow cells has been plated. *In vitro*, the entry of fibroblast colony-forming units into the cell cycle and the subsequent development of colonies depend on the growth factor present in the serum. In our experiments, we used fetal calf serum as the growth factor for the fibroblast colony-forming units. This may represent a limitation in our methodology, as this growth factor is not present in human bone marrow and as it was recently demonstrated, by Shigeno and Ashton, that autologous serum supports greater cellular proliferation than fetal calf serum does. Nevertheless, the ability of the fibroblast colony-forming unit and the granulocyte-macrophage progenitor to develop colonies *in vitro* under our experimental conditions allowed us to quantitate their proliferative status in patients who had osteonecrosis and to compare these findings with the status of fibroblast colony-forming units and granulocyte-macrophage progenitor cells in control subjects.

Arlet, as well as Hauzeur et al., in biopsy specimens, and Mitchell et al., on magnetic resonance images, found abnormalities in bone marrow in regions remote from sites of osteonecrosis in the proximal part of the femur. It is not surprising that bone-marrow abnormalities are found under the sequestrum, in the deeper part of the femoral head. Although the pathogenesis of non-traumatic osteonecrosis of the femoral head is not well understood, vascular congestion and bone-marrow edema are thought to occur early in the course of the disease. Moreover, evidence of bone-marrow edema has been seen on magnetic resonance images

relatively early in the course of a variety of osseous conditions, including transient osteoporosis and occult intraosseous fracture⁽¹²⁾; thus, abnormal bone-marrow activity under the sequestrum may be a non-specific response to a severe insult.

In contrast, abnormal bone-marrow activity in the iliac crest is surprising. In our series, the bone-marrow activity in the patients who had osteonecrosis related to alcohol abuse (group 3) or to corticosteroid therapy (group 4) was abnormal, as compared with the activity in the control groups. These differences were highly significant, even when the comparison was with the control group of patients who had a non-union. This indicates that phenomena such as osteopenia and the absence of weight-bearing may not be the factors that led to the bone-marrow abnormalities.

Although we found decreased activity of bone-marrow cells in patients who were receiving corticosteroid therapy or who abused alcohol, this decreased activity may not have had any relationship to the osteonecrosis. To determine such a relationship, we would have needed to include a control group of patients who were receiving corticosteroids or who abused alcohol but did not have osteonecrosis. Such a control group was not available to us, and we found no data in the literature on such patients. The other theoretical explanation is that, in patients who are receiving corticosteroids or who abuse alcohol, the osteonecrosis is related to the decrease in bone-marrow activity. This hypothesis cannot be excluded, as our patients who had idiopathic osteonecrosis also had less bone-marrow activity than did the control subjects who did not have osteonecrosis. It may be that, in some patients at increased risk for the development of osteonecrosis, the risk is expressed as a result of external influences (alcohol abuse or corticosteroid therapy) on the bone-marrow activity.

Our results suggest that the abnormal bone-marrow activity associated with osteonecrosis of the hip is systemic rather than local. This theory may be in keeping with the findings of Arlot et al., who noted abnormal bone-marrow activity in the biopsy specimens from the iliac crests of nearly all of the patients in their study who had osteonecrosis. Additional investigations are needed to explore the possible relationship between these observations; the study of pathological alterations in the bone marrow outside the necrotic zone may provide important insights into the pathophysiology of osteonecrosis, as noted by Mitchell et al., if it is accepted that the first lesions occur in the bone marrow of the femoral head in osteonecrosis.

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TABLE I
DATA ON THE SUBJECTS*

	Group 1/Group 1a†		Age (Yrs.)
	GMP Cells§	FCF Units¶	
	NA	31	1
	79	12	17
	70	5	32
	46	9	20
	217	14	23
	177	22	45
	177	36	54
	79	18	36
	75	48	18
	61	20	47
	51	4	12
	86	12	36
	78	19	22
	57	19	28
	41	35	26
	NA	7	31
	44	3	4
	76	8	12
	40	16	36
	229	NA	13
	48	12	32
	203	9	55
	194	19	4
	85	51	44
	NA	21	7
	62	50	5
	189	17	36
	NA	40	70
	110	2	79
	266	6	70
	30	5	72
	116	38	83
	108	14	73
Mean (and stand. dev.)	106.7 ± 67.6	19.4 ± 14.2	34.6 ± 23.8
	103.4 ± 64.4	18.9 ± 11.9	35.5 ± 10.5

*Group 1 included thirty-three male bone-marrow donors; group 2, thirty-four male patients who had been managed with bone-marrow grafting for a non-union; group 3, sixteen men who had osteonecrosis related to alcohol abuse; group 4, fourteen men who had osteonecrosis related to corticosteroid therapy; and group 5, eleven men who had early idiopathic osteonecrosis. NA = not available.

†The values in boldface are those for group 1a, which was created to include only patients who were twenty to sixty years old.

‡The values in boldface are those for group 2a, which was created to include only patients who were twenty to sixty years old.

§The values are given as the number of granulocyte-macrophage progenitor (GMP) cells per 10⁵ bone-marrow cells.

¶The values are given as the number of fibroblast colony-forming (FCF) units per 10⁶ bone-marrow cells.

Group 2/Group 2a‡			
GMP Cells§	FCF Units¶	Age (Yrs.)	GMP Cells§
55	10	29	53
42	7	42	27
93	11	43	35
61	8	30	39
66	8	33	46
68	8	36	27
65	25	36	34
59	20	37	64
79	14	24	25
58	23	78	21
76	11	44	78
73	9	26	21
48	10	54	43
57	16	74	42
57	19	22	44
50	10	41	34
38	15	17	
58	18	20	
46	11	38	
64	23	39	
64	12	30	
61	25	13	
62	19	26	
70	12	29	
24	8	44	
77	6	71	
63	10	60	
45	15	47	
61	9	38	
31	11	50	
126	18	51	
67	7	29	
63	5	75	
65	8	58	
61.5 ± 17.8	13.0 ± 5.70	40.7 ± 16.8	39.6 ± 15.6
62.1 ± 18.9	12.5 ± 5.08	37.7 ± 10.8	

Age (Yrs.)	GMP Cells§	FCF Units¶
47	38	1
41	22	1
38	41	2
54	72	1
46	31	7
43	27	2
50	37	4
37	38	0
43	29	3
39	66	3
41	26	2
53	48	3
39	46	1
45	24	2
52		
47		
44.7 ± 5.48	38.9 ± 15.1	2.29 ± 1.73

TABLE IV
P VALUES FOR COMPARISON OF THE NUMBER OF FIBROBLAST COLONY-FORMING UNITS*

	Group 1	Group 1a	Group 2	Group 2a
Group 1 (19.4 ± 14.2 per 10 ⁶ bone-marrow cells)	—	NS	NS	NS
Group 1a (18.9 ± 11.9 per 10 ⁶ bone-marrow cells)	0.825	—	NS	NS
Group 2 (13.0 ± 5.70 per 10 ⁶ bone-marrow cells)	0.150	0.075	—	NS
Group 2a (12.5 ± 5.08 per 10 ⁶ bone-marrow cells)	0.123	0.054	0.870	—
Group 3 (2.31 ± 1.35 per 10 ⁶ bone-marrow cells)	0.0000	0.0000	0.0000	0.0000
Group 4 (2.29 ± 1.73 per 10 ⁶ bone-marrow cells)	0.0000	0.0000	0.0000	0.0000
Group 5 (8.45 ± 4.61 per 10 ⁶ bone-marrow cells)	0.011	0.003	0.029	0.039

*NS = not significant, S = significant ($p > 0.05$), S = significant ($p < 0.01$), and SS = strongly suggestive of significance ($0.01 < p < 0.05$), according to the Mann-Whitney U test.

Group 3	Group 4	Group 5
S	S	SS
S	S	S
S	S	SS
S	S	SS
—	NS	S
0.813	—	S
0.0001	0.0002	