

Abnormalities in the Replicative Capacity of Osteoblastic Cells in the Proximal Femur of Patients with Osteonecrosis of the Femoral Head

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ABSTRACT. *Objective.* Aseptic nontraumatic osteonecrosis (ON) of the femoral head is a painful disorder that often leads to femoral head collapse due to subchondral fracture. We postulated that alteration of osteoblast function might play a role in the pathophysiology of ON. We evaluated the *ex vivo* proliferation rate and differentiation capacity of osteoblasts derived from the intertrochanteric region of the femur and of the iliac crest of patients with ON of the femoral head and compared it with patients with hip osteoarthritis (OA).

Methods. We examined the function of osteoblastic cells in cultures derived from bone biopsies of the intertrochanteric region of the femur and of the iliac crest obtained from 13 patients with ON of the femoral head and 8 patients with hip OA. The replicative capacity was assessed by the proliferation rate in secondary culture. The phenotypic characterization was evaluated by the level of alkaline phosphatase activity, the sensitivity to 1,25 (OH)₂ vitamin D₃, and collagen synthesis.

Results. The replicative capacity of the osteoblastic cells of the intertrochanteric area of the femur in ON patients was significantly reduced compared to patients with OA. The capacity of differentiation, however, was not different between ON and OA patients.

Conclusion. The replicative capacity of osteoblastic cells is significantly reduced in the femur of patients with ON. Our results confirm that altered osteoblastic function plays a role in the pathophysiology of ON of the femoral head. (J Rheumatol 2003;30:348–51)

Key Indexing Terms:
OSTEONECROSIS

OSTEOBLAST

FEMORAL HEAD

Aseptic nontraumatic osteonecrosis (ON) of the hip is a painful disorder that often leads at its late stage to femoral head collapse due to subchondral fracture. Glucocorticoids and alcohol abuse are among the most frequent etiological factors for ON in Caucasians¹. Although the causes of glucocorticoid-induced, alcohol abuse and idiopathic ON might not be the same, different pathophysiological mechanisms have been proposed for this disease including fat emboli², microfracture of trabecular bone³, microvascular tamponade of the blood vessels of the femoral head by hypertrophic marrow fat⁴, and retrograde embolization of the marrow fat⁵. Another hypothesis, however, could be that ON might also be a disease of osteoblastic cells. Weinstein, *et al* demonstrated that glucocorticoid-induced ON was characterized by an increase of osteocyte and cancellous lining cell apoptosis in

the femur⁶. Further, glucocorticoids induce an inhibition of osteoblastogenesis and promote apoptosis of osteoblasts and osteocytes. Defective osteoblastogenesis is linked to reduced bone formation and osteopenia⁷. Based on those findings, we postulated that osteoblast function could be altered in ON and play a role in its pathogenesis. To examine this hypothesis, we studied osteoblastic function in the intertrochanteric region of the femur and in the iliac crest of patients with ON and compared it with patients with osteoarthritis (OA) of the hip.

MATERIALS AND METHODS

Patients. Thirteen patients with ON of the femoral head and 8 patients with primary OA of the hip were included in this study. Informed consent was obtained from the patients in compliance with the Ethical Committee of the hospital. All patients with ON had Ficat stage 2 ON¹. Diagnostic criteria for ON were measured by radiography and magnetic resonance imaging (MRI)⁸. Demographic characteristics are given in Table 1. Among the 13 patients with stage 2 ON of the femoral head there were 8 men and 5 women. Nine patients had ON due to corticosteroid therapy; 2 patients had ON due to alcohol abuse. For 2 patients, no etiological factors for ON could be found. Their ages at diagnosis ranged from 25 to 68 years (mean 47 ± 3 yrs). The patients with glucocorticoid-induced ON had stopped taking glucocorticoids 7 ± 2.8 months prior to the study (range 3–12 mo). The time between the onset of hip pain and the core decompression procedure ranged from 4 to 24 months (mean 9 mo). Patients with primary OA of the hip who underwent total hip replacement were recruited as controls. OA was confirmed by radiography. Among the 8 patients with hip OA there were 5 men and 3 women with a mean age of 57 ± 6 years (range 30–79).

Bone specimens from the femur of patients with ON were obtained from

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Table 1. Demographic characteristics of patients with osteonecrosis and osteoarthritis (controls).

Clinical Disorder	Patients, n	Age, yrs	Sex, M/F	Bone Mineral Density T Score	
				Lumbar Spine	Total Hip
Osteonecrosis	13	47 ± 3	8/5	-0.44 ± 0.32	-0.65 ± 0.30
Corticosteroid therapy	9	45 ± 5	6/3	-0.38 ± 0.40	-0.60 ± 0.41
Alcohol abuse	2	60 ± 5	1/1	-0.20 ± 1.21	-0.86 ± 0.91
Idiopathic	2	45 ± 3	2/0	-0.95 ± 0.68	-0.67 ± 0.34
Osteoarthritis	8	57 ± 6	5/3	-0.29 ± 0.54	-0.39 ± 0.29

the intertrochanteric region of the femur (2.5 mm diameter and 2–2.5 cm long biopsies) during a core decompression procedure for ON using a 3 mm trephine⁹. Iliac crest bone was obtained from transiliac bone biopsy (7.5 mm diameter and 2.5 cm long biopsies) using an 8 mm inner diameter trephine. Bone specimens from the femur (3 mm diameter and 2.5 cm long biopsies) and the iliac crest (7.5 mm diameter and 2.5 cm long biopsies) of OA patients were obtained during total hip replacement. A bone specimen from the intertrochanteric region of ON and OA patients was fixed in 10% formaldehyde.

Bone mineral density was measured by dual energy x-ray absorptiometry (Hologic) in ON and OA patients at the lumbar spine and the hip. All ON and OA patients had a normal bone mineral density. The mean T score was -0.44 ± 0.32 and -0.65 ± 0.30 at the lumbar spine and the hip, respectively, for ON patients and was -0.29 ± 0.54 and -0.39 ± 0.29 at the lumbar spine and the hip, respectively, for OA patients.

All ON and OA patients had normal laboratory tests including thyroid stimulating hormone, parathyroid hormone, and 25-OH vitamin D.

Bone cell culture. For each bone specimen, soft connective tissue and cortical bone were carefully removed and the remaining trabecular bone was minced into small fragments (1 mm³). The bone fragments were extensively washed with PBS (BioWhittaker, Verviers, Belgium) to remove the adherent marrow cells and seeded in 25 cm² tissue culture flasks in minimum essential medium (MEM, BioWhittaker) supplemented with 10% fetal calf serum (FCS) (Life Technologies, Gent, Belgium), 2 mM L-glutamine, 50 µg/ml gentamycin, and 2.5 µg/ml fungizone (Life Technologies). The medium was changed twice a week. After 4 weeks cells were released using trypsin-EDTA solution (BioWhittaker), counted, and replated for evaluation of proliferation and differentiation variables. Harvested cells were seeded at a density of 5 × 10³ cells per cm² in 24 well culture plates in MEM + 10% FCS.

Proliferation in secondary culture. Cells were counted after trypsinization following 2 and 7 days of culture (n = 4 wells per day). The population doubling time was calculated as follows: $\Delta t \times \log^2_{10} / \log_{10} n/n_0^{10}$.

Phenotypic characterization of the cells. Alkaline phosphatase response to 1,25 (OH)₂D₃. Five days after replating, the cells were incubated in MEM supplemented with 1 mg/ml of a lipid-rich bovine serum albumin (Albumax, Life Technologies) and stimulated with 10⁻⁸ M 1,25 (OH)₂ vitamin D₃ (a gift from Roche, Brussels, Belgium) for 48 h. Vehicle medium of the hormone was added in control cultures. At the end of the incubation period, the culture medium was removed, the cell layer was washed with PBS and then scraped off the dishes with a rubber scraper and sonicated in distilled water. After centrifugation, the supernatants were used for the determination of alkaline phosphatase activity (APA) (Kit MPR3, Boehringer-Mannheim, Brussels, Belgium) and protein content (Coomassie brilliant blue G-250 dye binding method). APA was expressed in mU/mg protein.

Collagen and noncollagen protein synthesis. Two days after replating, the medium (MEM + 10% FCS) was changed and supplemented with 50 µg/ml ascorbic acid (Sigma-Aldrich, Bornem, Belgium). Five days later, collagen synthesis was evaluated by measuring the incorporation of 2,3,4,5-³H proline

(10 µCi/ml, specific activity 90 Ci/mmmole; American Radiolabeled Chemicals, Isobio, Fleurus, Belgium) into collagen for the last 2 h of culture. Cells were extracted and homogenized in 0.5% Triton X-100 (Sigma) in water, sonicated, and precipitated with 10% trichloroacetic acid (TCA, Sigma). The acid-precipitable material was collected by centrifugation. The pellets were acetone-extracted, dried, resuspended in 0.5 M acetic acid, and neutralized with NaOH. The labeled proline incorporated into collagenase-digestible protein (CDP) and noncollagen protein (NCP) was measured using bacterial collagenase (Sigma) according to the method of Peterkofsky and Diegelmann¹¹. The percentage of collagen synthesis was calculated after multiplying NCP by 5.4 to correct for relative abundance of proline in CDP and NCP.

Statistical methods. Data are expressed as mean ± SEM. Data were compared using paired and unpaired Student's t test. Statistical significance was determined at p < 0.05.

RESULTS

Proliferation rate of osteoblastic cells in secondary culture from the patients with ON. Cells were collected and counted at day 2 and day 7 after replating for the femur and iliac crest biopsies. The cell-doubling time calculated between 2 and 7 days was found to be significantly increased (10.9 ± 1.1 days) in the femur compared to the iliac crest (6.3 ± 0.8 days) (p = 0.003) of patients with glucocorticoid-induced ON and with ON due to other causes. For patients with glucocorticoid-induced ON, the cell doubling time was increased to 11.3 ± 1.6 days at the femur compared to the iliac crest (6.8 ± 1.1 days) (p = 0.01). For patients with ON due to other causes the cell-doubling time was also significantly prolonged at the femur (10.1 ± 1.5 days) compared to the iliac crest (5.2 ± 0.5 days) (p = 0.02).

Proliferation rate of osteoblastic cells in secondary culture from patients with OA (controls). The cell-doubling time was calculated between 2 and 7 days after replating and was not statistically different in the femur (6.8 ± 1.0 days) and in the iliac crest biopsies (8.0 ± 1.3 days).

The cell-doubling time of the osteoblastic cells of the femur was significantly increased in the femur of ON patients compared to OA patients (p = 0.031). For the iliac crest biopsy, the cell-doubling time was not statistically different in patients with ON and OA.

Phenotypic characteristics of osteoblastic cells from patients with ON. The mean basal alkaline phosphatase activities (APA) in ON patients for the femur and the iliac crest, respectively, were 799 ± 124 mU/mg protein and 1341 ± 340 mU/mg protein, with no significant difference between the 2 sites of biopsy (Table 2). The levels of APA after stimulation with 1,25 (OH)₂ vitamin D₃ were 936 ± 187 and 1610 ± 409 for the femur and the iliac crest, respectively (NS). The percentages of collagen synthesis were 23.4 ± 1.7% and 23.1 ± 1.2% for the femur and the iliac crest, respectively (NS). There were no differences in APA and collagen synthesis between patients with glucocorticoid-induced ON or with ON due to other causes.

Phenotypic characteristics of osteoblastic cells from the patients with OA (controls). The mean APA in OA patients in

Table 2. Proliferation rate and phenotypic characteristics of osteoblastic cells in secondary culture from the intertrochanteric region of the femur and the iliac crest of patients with osteonecrosis of the femoral head and hip OA.

	Proximal Femur	Iliac Crest	p
Osteonecrosis (n = 13)			
Cell-doubling time (d)	10.9 ± 1.1	6.3 ± 0.8	0.003*
Alkaline phosphatase activities (mU/mg protein)			
Basal level	799 ± 124	1341 ± 340	0.24
1,25 (OH) ₂ vit D3 response	936 ± 187	1610 ± 409	0.21
Collagen synthesis (%)	23.4 ± 1.7	23.1 ± 1.2	0.9
Hip OA (n = 8)			
Cell-doubling time (d)	6.8 ± 1.0	8.0 ± 1.3	0.56
Alkaline phosphatase activities (mU/mg protein)			
Basal level	919 ± 382	1024 ± 440	0.86
1,25 (OH) ₂ vit D3 response	1240 ± 340	1431 ± 565	0.78
Collagen synthesis (%)	21.7 ± 2.3	21.7 ± 4.3	0.3

* Statistically significant.

the femur and the iliac crest, respectively, were 919 ± 382 mU/mg protein and 1024 ± 440 mU/mg protein (NS). The levels of APA after stimulation with 1,25 (OH)₂ vitamin D₃ were 1240 ± 340 and 1431 ± 565 for the femur and the iliac crest, respectively (NS). The percentages of collagen synthesis were $21.7 \pm 2.3\%$ and $21.7 \pm 4.3\%$ for the femur and the iliac crest, respectively (NS). There were no statistical differences in APA or in collagen synthesis between ON and OA patients.

DISCUSSION

The present study revealed that the *in vitro* replicative capacity of osteoblastic cells obtained from the intertrochanteric area of the femur in ON was significantly reduced compared to patients with OA. This abnormality was confined to the intertrochanteric area and was not found in the iliac crest. However, the osteoblast phenotype was not modified. The decreased proliferative capacity was found not only in glucocorticoid-induced ON but also in ON due to other causes. We used OA patients as controls because of the ethical difficulties in obtaining bone biopsies from normal donors. Most clinical studies investigating the physiopathology of ON compared their results with OA¹². Further, the focal trabecular bone necrosis reported in OA is located under the eburnated surface or around cystic lesions and is not associated with extensive bone marrow necrosis¹³. Rare cases of deeper localization within the femoral head have been reported, but never in the intertrochanteric area^{14,15}. Therefore, bone biopsies were performed in the intertrochanteric region far away from the hip joint.

The osteoblastic replicative capacities have been shown to decrease with aging in patients with OA¹⁶, but we found no statistically significant differences in age in our study between ON and OA patients. Osteoblast proliferation can also be altered in involutional and glucocorticoid osteoporosis, but none of the patients reported had osteoporosis¹⁷. Abnormal osteoblast activities might be a nonspecific response due to severe insult to the adjacent dead bone, but bone biopsies

from the intertrochanteric area did not show signs of ON. The decrease in osteoblast proliferation might also have been due to corticosteroid treatment, but all patients had stopped corticosteroid therapy for an average of 7 months⁷. Moreover, this abnormality was confined to the proximal femur and could not be detected in the iliac crest.

The altered capacities of proliferation of the osteoblastic cell can be responsible for 2 different events in the pathogenesis of ON; the appearance of ON itself and the bone repair that occurs after ON. Glucocorticoids inhibit osteoblastogenesis and promote osteoblast and osteocyte apoptosis. Osteocytes descend from osteoblasts and remain connected by gap junctions after they are incorporated into the bone matrix. The glucocorticoid-induced osteocyte apoptosis could disrupt the mechanosensory role of these cells and thus prevent the adaptation of bone to ischemia and medullary changes seen in very early stages of ON^{6,7,14}. Abnormalities in bone marrow in regions remote from the site of ON in the proximal part of the femur have been described in bone biopsies^{18,19}. The decrease in osteoblast capacity to proliferate could therefore reflect the disruption of the mechanosensory role of the osteocyte-canalicular network and explain the evolution from marrow ischemia and edema to established ON. Secondly, at a very early stage, a sufficient repair capability would make the lesion reversible as described in pathological studies^{18,19}. An insufficient repair mechanism related to a decrease in bone formation might explain the evolution to a further stage of ON. The rate of bone formation is indeed largely determined by the number of osteoblasts, which, in turn, is determined by the rate of replication of progenitors²⁰⁻²². Hernigou, *et al* have described a decrease in the number of fibroblast colony-forming units leading to a reduction in the number of mature osteoblasts in the proximal femur in glucocorticoid-induced ON²³. This is confirmed by the extent of osteocyte death observed in the proximal femur of patients with ON¹².

We have shown that the replicative capacity of the osteoblastic cell is decreased in the proximal femur of patients

with ON, suggesting a potential role for the osteoblast in the pathophysiology of ON of the femoral head. Our results also confirm that stimulation of bone formation might be an effective treatment for ON.

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