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ORIGINAL ARTICLE

The apoptosis of osteoblasts and osteocytes in femoral head osteonecrosis: its specificity and its distribution

Eugène Mutijima · Viviane De Maertelaer · Manu Deprez · Michel Malaise · Jean-Philippe Hauzeur

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Abstract The pathogenesis of nontraumatic osteonecrosis (ON) remains unclear. Some studies have suggested that nontraumatic ON is attributed to increased osteocytic apoptosis. To test this hypothesis, a controlled study must compare the apoptosis of osteocytes and osteoblasts in cases of ON and osteoarthritis (OA). To assess either the localized or diffuse patterns of this increased osteocytic and osteoblastic apoptosis, we evaluated both the proximal and distal regions of necrotic areas. Femoral heads resected for total hip prosthesis were included for this study. Of these, 10 were ON cases—three were induced by corticosteroids, three by alcohol abuse, one resulted from trauma, one resulted from hyperlipemia, and two were idiopathic—10 were osteoarthritis cases, and 1 from a patient suffering from a subcapital fracture. The TUNEL reaction was used to detect the apoptosis in osteoblasts and osteocytes. A semi-quantitative evaluation was conducted, at both distal and proximal areas relative to the lesions, specifically in the area surrounding the necrotic region in the osteonecrosis cases, in the eburnated bone in the osteoarthritis cases, and in the subchondral bone fracture. The apoptosis of osteoblasts and osteocytes was statistically more frequent in the regions close to the necrotic areas in the ON group. No difference was found in the unpaired areas. In the ON group, no difference was found in terms of the etiological factors. During ON, the apoptosis of osteocytes and osteoblasts is increased proximally to the necrotic regions in the patients presenting with osteoarthritis and subcapital fractures. This increase was found not only in the corticosteroid-induced ON cases but also in the idiopathic and alcohol abuse- and trauma-induced ON cases.

Keywords Apoptosis · Femoral head · Osteoblast · Osteocytes · Osteonecrosis · TUNEL

E. Mutijima · M. Deprez
Department of Pathology, CHU Sart Tilman, Sart Tilman 4000, Liège, Belgium

E. Mutijima
e-mail: E.Mutijima@chu.ulg.ac.be

M. Deprez
e-mail: manuel.deprez@ulg.ac.be

V. De Maertelaer
Department of Biostatistics and Medical Informatics & IRIBHM, School of Medicine, Université Libre de Bruxelles, 808 route de Lennik, 1070 Brussels, Belgium
e-mail: vdemaert@ulb.ac.be

M. Malaise · J.-P. Hauzeur (✉)
Department of Rheumatology, CHU Sart Tilman, Sart Tilman 4000, Liège, Belgium
e-mail: jean-philippe@hauzeur.org

M. Malaise
e-mail: michel.malaise@ulg.ac.be

Introduction

Osteonecrosis (ON) of the femoral head is a bone disease that is characterized by the necrosis of trabecular bone and bone marrow, which can lead to subchondral bone weakness, followed by collapse and hip failure. This can also occur in young people suffering from ON who will consequently require a total hip prosthesis. The main nontraumatic etiological factors include alcohol abuse, corticosteroid use, and sickle cell anemia.

The physiopathology remains unclear, although vascular insufficiency, fat embolism, and bone fragility have been proposed. However, these factors alone cannot explain the entire pathogenesis from the onset of necrosis to the collapse of the femoral head.

A number of recent reports have suggested that ON might be related to abnormalities of the mesenchymal stem cells (MSCs) and/or osteoblastic cells. The number and activity of MSCs are decreased in ON [1]. The replicative capacity of osteoblastic cells is reduced in ON cases compared to osteoarthritis (OA) [2]. Osteocytic apoptosis is increased in mice after corticotherapy and in transiliac bone biopsies from patients treated with corticoids [3]. The same investigators reported that osteocyte apoptosis was more prevalent in glucocorticoid-induced ON than in alcohol-induced ON and absent in ON due to sickle cell disease or post traumatic ON [4]. They suggested that an increase of osteoblastic and/or osteocytic apoptosis in the femoral head might be one of the main features of the pathogenesis of ON. The controls used in this study were normal transiliac bone biopsies obtained from volunteers [4]. However, comparing femoral heads and iliac bones might not be the most appropriate comparison, as differences have been reported concerning the bone cell functions from both origins [2]. To clarify this point, we chose to compare femoral heads from ON and control patients, and we used femoral heads resected for total prosthesis in ON patients, OA patients, and a patient with a femoral neck fracture. To assess apoptosis, TUNEL method was choosing to have an opportunity to compare our data with the previous publications, although it was actually not the best method to detect cell apoptosis. A microscopic study and TUNEL assay were performed both in the most affected areas, which were proximal to the lesions—within the subchondral areas in OA samples and within the so-called creeping substitution areas in ON samples [5]—and in the less affected areas, within regions that were distal to the lesions [6].

Material and methods

The study protocol was approved by the ethical committee of the Faculty of Medicine of Liège. Data from 21 patients undergoing total hip prosthesis were prospectively collected. Ten femoral heads were removed for osteonecrosis, 10 for coxarthrosis, and one for a femoral neck fracture. All of the patients consented to the use of their tissues for research.

Osteonecrosis was confirmed in all cases using radiography and magnetic resonance images [7]. Osteoarthritis and femoral neck fracture were confirmed in all of the cases by radiography.

A band saw was used to cut the specimens into 5-mm-thick slices. The specimens were fixed in 10 % neutral buffered formalin, routinely decalcified and embedded in paraffin. Four-micron sections of each specimen were stained with hematoxylin and eosin for histopathological evaluation. Two representative blocks of each case were routinely deparaffinized using standard histological procedures. The apoptosis of the osteoblasts and osteocytes was assessed using TUNEL assays. The TUNEL assays (the "in situ cell death detection kit AP" from Roche reference 11684809910 (Roche, Mannheim, Germany)) were used for the qualitative analysis of DNA fragmentation on 4-μm-thick paraffin blocks. After deparaffinizing, rehydrating, and washing, the tissue sections were incubated in a buffer containing 20 μg/ml proteinase K (nuclease free) for 30 min at 37 °C and washed twice in a TRIS-buffered solution. The tissue sections were incubated with terminal deoxynucleotidyl transferase (TdT) and digoxigenin-dUTP a TUNEL reaction mixture of vial 1 (enzyme solution) and vial 2 (label solution), according with the manufacturer's protocols. Fifty microliters of vial 3 (converter alkaline phosphatase) was added to the slides for 30 min at 37 °C. Immunofluorescence staining was performed using the immunofluorescence marker included in the kit. The tissues were counterstained with Mayer's hematoxylin, and coverslips were applied. For the positive controls, the same procedure was performed on paraffin sections derived from human tonsils. For the negative control, the terminal transferase was omitted. To avoid any artifacts of TUNEL labeling, the presence of other classical apoptosis indices including nuclear and chromatin condensation was verified [8]. The whole sections were analyzed using a binocular microscope (Laborlux D) at medium power. The evaluation of the immunohistochemical staining was done using a modified Waltregny scoring [9]. The scores were defined, separately close to and distal from the lesion. The staining intensity of the apoptotic cells was recorded using an arbitrary ordinal scale as follows: 1=intense-to-moderate staining and 0=weak-to-absent staining. The extent of staining was, respectively for osteocytes and osteoblasts cells, the percentage of the positively stained cells to the total number of cells in the trabecular bones examined. The extent of staining was scored as follows: 1=<33 % of positive cells and 2=33–100 % of positive cells. A global score combined both score values: 1 = ≥1 positive score and 0=2 negative scores.

Statistical method

Exact chi-square tests were performed to compare the proportions between the ON and OA groups. The SPSS-IBM Version 20.0 statistical software was used.

Results

The data concerning the cases are summarized in Table 1. The mean age was 55.7 years (range, 48–68) in the ON group and 65.5 years (range, 51–81) in the OA group. The age of the patient with a femoral neck fracture was 78. An ON etiological factor was present in eight ON patients as follows: corticosteroid usage in three patients, alcohol abuse in three

Table 1 Patient data

Case number	Group	Etiological factors	Age	Gender	Side
1	ON	Corticosteroids	56	M	R
2	ON	Corticosteroids	68	M	L
3	ON	Corticosteroids	62	F	R
4	ON	Ethanol abuse	56	F	R
5	ON	Ethanol abuse	48	M	R
6	ON	Ethanol abuse	49	M	R
7	ON	Trauma	63	M	R
8	ON	Hyperlipemia	31	M	L
9	ON	no	63	M	L
10	ON	no	61	M	R
11	OA	no	81	F	R
12	OA	no	52	M	R
13	OA	no	51	M	R
14	OA	no	57	F	L
15	OA	no	65	M	L
16	OA	no	66	M	L
17	OA	no	76	F	R
18	OA	Ethanol abuse	54	F	R
19	OA	no	74	F	R
20	OA	no	79	F	L
21	F	no	78	F	L

ON osteonecrosis, *OA* osteoarthritis, *F* fracture

patients, trauma and hyperlipemia in one patient each. Alcohol abuse was reported by one patient from the OA group.

The TUNEL labeling results and the *p* values associated with the comparisons between the ON and OA groups (as there was only one case of femoral neck fracture, this patient was excluded from the statistical analysis) are shown in Table 2. The proportion of osteoblast and osteocyte apoptosis was statistically increased in the ON samples compared to those of the OA group within the lesion areas, with the exception of the osteoblast cell percentage ($p=0.057$). In the unpaired areas, no differences were found. In regard to the etiological ON factors, no difference in the frequency of apoptosis was detected between the corticoid-induced, ethanol abuse-induced, hyperlipemia-induced, trauma-induced, or idiopathic ON samples ($p=1.000$).

Table 2 A summary of the results with the *p* values associated with the comparisons between the ON and OA groups

	ON $n=10$	OA n=10	Fracture n=1	ON//OA *p* value
Lesion area				
OB global	9	1	0	0.001
OB %	6	1	0	0.057
OB intensity	9	1	0	0.001
OC global	9	2	0	0.005
OC %	6	0	0	0.011
OC intensity	8	1	0	0.005
Unimpaired area				
OB global	1	0	0	1.000
OB %	1	0	0	1.000
OB intensity	1	0	0	1.000
OC global	0	1	0	1.000
OC %	0	0	0	–
OC intensity	0	1	0	1.000

Discussion

This study shows that the apoptosis of osteoblasts and osteocytes is increased in cases of ON of the femoral head compared with OA or a case of subcapital fracture. However, this increase is only found within the so-called creeping substitution areas [5]. The creeping substitution areas comprise a zone of reinforced viable trabecular bone surrounding the necrotic trabeculae and bone marrow spaces, which are filled by vessel-rich fibrotic tissue. In the outer margin of the osteonecrotic zone, these changes produce a dense band that is often highly visible on radiography, as well as on low T1 and low T2 bands in MRI images [10]. This creeping substitution, which is mainly composed of reactive bone, is produced by the reparative process. Theoretically, when this process completely repairs the necrotic bone, no pathological sequela should be found [11]. In patients suffering from clinical ON however, this reparative process is defective. Failure in this repair process might result in a subchondral fracture that could lead to the collapse of the femoral head in two ways [11]. The main cause of this failure might be due to the focal resorption of necrotic trabeculae by the repair process, particularly within the lateral portion of the subchondral bone. Another cause might be excessive stress from pressure exerted on the femoral head, inducing internal deformation. The point of stress would be concentrated at the margin between the necrotic tissue and the area of active repair. This is a biomechanically weaker zone produced by an area of focal resorption surrounded by dense and rigid bone tissue. Thus, the increased apoptosis of osteoblasts and osteocytes in the repaired bone tissue might be a consequence of such insufficient healing because the slower repair process increases the period of stress.

In this study, we chose to take for control the reactive bone process exhibited by the subchondral bone in cases of hip OA and in a case of subcapital fracture. In the OA cases, the sclerotic bone, called bony eburnation, is associated with the deposition of new bone on pre-existing trabeculae [12].

Distal from the necrotic region, neither osteoblast nor osteocyte apoptosis was increased. This observation suggests that the disproportionally elevated levels of apoptosis

represent a specific problem with the repair tissue, although early-stage ON might present with diffuse impairment in the bone marrow [13].

Three studies investigating apoptosis in ON have been previously published. The first reported an increase in osteoblast and osteocyte apoptosis, as evidenced by TUNEL staining in only corticosteroid-induced ON cases but not alcohol-, sickle cell disease-, or trauma-induced ON cases [4]. We did not observe the same specificity of ON in terms of corticosteroid use. In this study [4], the area exhibiting the most frequent osteocytic apoptosis was adjacent to the subchondral fracture. The authors reported unusual histological features in the corticosteroid-induced ON femoral heads, including reduced cancellous bone areas, increased marrow adipocytes, and the absence of hyperemia and lipid cyst formation. Such histological features were not found in the corticosteroid-induced ON cases and other ON cases evaluated in the present study, as well as in a previous study [10]. The lesions included trabecular bone necrosis with fibrous tissue filling the intertrabecular spaces, surrounded by a creeping substitution reaction. We recommend therefore a diagnostic review of these ON cases to consider not only the diagnosis of ON but also the diagnosis of insufficiency fracture [14]. Another study has evaluated 40 femoral heads of 20 ON and 20 OA patients [15]. Although the osteocytic and osteoblastic apoptosis assessed by TUNEL staining was positive in all of the corticosteroid-induced and alcohol-induced ON samples, they were absent in the sickle cell disease-induced ON cases and in OA cases. No data were presented concerning the location of the apoptotic bone cells. The third and final publication presented a TUNEL labeling assessment of osteocytic apoptosis in the femoral heads from 58 ON cases. Among these, eight were induced by corticosteroid usage, 29 by alcohol, and six by trauma; the remaining 15 were idiopathic [16]. Significantly, more osteocytic apoptosis was detected proximal to the necrotic areas in corticosteroid- and alcohol-induced ON cases compared to the trauma-induced and idiopathic ON cases. Our results are partially consistent with these publications. In the present study, apoptosis was increased in all of the ON cases, regardless of etiological factor. However, sickle cell disease-induced ON cases were not assessed in our study. A further larger study is warranted to further elucidate the role of etiological factors in the induction of apoptosis in ON.

The apoptosis observed might be induced by cytotoxic factors such as the direct metabolic effects of alcohol [11], cortisone [4], nitric oxide [15], or the increased expression of Dickkoff-1 [17]. It is also possible that predisposition plays a role in certain cases of ON [reviewed in 18].

In conclusion, the incidence of osteocytic and osteoblastic apoptosis is significantly increased in the femoral head during ON, regardless of etiological factors, including corticosteroid or alcohol. The induction of apoptosis is localized around the necrotic areas. This increased apoptosis in the repair tissue surrounding the necrotic area might represent an important factor that promotes femoral head collapse.

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Disclosures None.

References

1. Hernigou P, Beaujean F, Lambotte JC (1999) Decrease in the mesenchymal stem-cell pool in the proximal femur in corticosteroid-induced osteonecrosis. J Bone Joint Surg (Br) 81:349–55
2. Gangji V, Hauzeur JP, Schoutens A, Hinsenkamp M, Appelboom T, Egrise D (2003) Abnormalities in the replicative capacity of osteoblastic cells in the proximal femur of patients with osteonecrosis of the femoral head. J Rheumatol 30:348–51
3. Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC (1998) Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. J Clin Investig 102:274–82
4. Weinstein RS, Nicholas RW, Manolagas SC (2000) Apoptosis of osteocytes in glucocorticoid-induced osteonecrosis of the hip. J Clin Endocrinol Metab 85:2907–12
5. Phemister DB (1949) Treatment of the femur in adults. J Bone Joint Surg 31-A:55–66
6. Kabata T, Kubo T, Matsumoto T, Nishino M, Tomita K, Katsuda S, Horii T, Uto N, Kitajima I (2000) Apoptotic cell death in steroid induced osteonecrosis: an experimental study in rabbits. J Rheumatol 27:2166–71
7. Hauzeur JP, Pasteels JL, Schoutens A, Hinsenkamp M, Appelboom T, Chochrad I, Perlmutter N (1989) The diagnostic value of magnetic resonance imaging in non-traumatic osteonecrosis of the femoral head. J Bone Joint Surg Am 71-A(5):641–9
8. Labat-Moleur F, Guillermet C, Lorimier P, Robert C, Lantuejoul S, Brambilla E, Negoescu A (1998) TUNEL apoptotic cell detection in tissue sections: critical evaluation and improvement. J Histochem Cytochem 46(3):327–34
9. Waltregny D, Bellahcène A, Van Riet I, Fisher LW, Young M, Fernandez P, Dewé W, de Leval J, Castronovo V (1998) Prognostic value of bone sialoprotein expression in clinically localized human prostate cancer. J Nat Cancer Inst 90:1000–8
10. Hauzeur JP, Sintzoff S Jr, De Maertelaer V, Appelboom T, Bentin J, Pasteels JL (1992) Relationship between magnetic resonance imaging and in vivo histology in non traumatic osteonecrosis of the femoral head. J Rheumatol 19:385–92
11. Glimcher MJ, Kenzora JE (1979) The biology of osteonecrosis of the human femoral head and its clinical implications. III. Discussion of the etiology and genesis of the pathological sequelae; comments on treatment. Clin Orthop Relat Res 140:273–312
12. Jeffery AK (1973) Osteogenesis in the osteoarthritic femoral head. A study using radioactive 32 P and tetracycline bone markers. J Bone Joint Surg (Br) 55(2):262–72
13. Hauzeur JP, Perlmutter N, Appelboom T, Pasteels JL (1991) Medullary impairment at early stage of non traumatic osteonecrosis of the femoral head. Rheumatol Int 11:215–7
14. Yamamoto T, Schneider R, Bullough PG (2000) Insufficiency subchondral fracture of the femoral head. Am J Surg Pathol 24(3): 464–8

15. Calder JD, Buttery L, Revell PA, Pearse M, Polak JM (2004) Apoptosis—a significant cause of bone cell death in osteonecrosis of the femoral head. J Bone Joint Surg (Br) 86:1209–13
16. Youm YS, Lee SY, Lee SH (2010) Apoptosis in the osteonecrosis of the femoral head. Clin Orthop Surg 2:250–5
17. Ko JY, Wang FS, Wang CJ, Wong T, Chou WY, Tseng SL (2010) Increased Dickkopf-1 expression accelerates bone cell apoptosis in femoral head osteonecrosis. Bone 46:584–91
18. Weinstein RS (2012) Glucocorticoid-induced osteonecrosis. Endocrine 41:183–90