Apoptosis – a significant cause of bone cell death in osteonecrosis of the femoral head

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doi:10.1302/0301-620X.86B8.14834 \$2.00

J Bone Joint Surg [Br]
2004;86-B:1209-13.
Received 4 August 2003;
accepted, after revision,
15 January 2004

Osteonecrosis of the femoral head usually affects young individuals and is responsible for up to 12% of total hip arthroplasties. The underlying pathophysiology of the death of the bone cells remains uncertain. We have investigated nitric oxide mediated apoptosis as a potential mechanism and found that steroid- and alcohol-induced osteonecrosis is accompanied by widespread apoptosis of osteoblasts and osteocytes. Certain drugs or their metabolites may have a direct cytotoxic effect on cancellous bone of the femoral head leading to apoptosis rather than purely necrosis.

Non-traumatic osteonecrosis of the femoral head generally affects younger individuals and may lead to subsequent collapse and secondary degenerative arthritis of the hip. Osteonecrosis is responsible for up to 12% of total hip arthroplasties.¹ A number of different factors have been implicated in the development of osteonecrosis, but although it has been recognised for over 100 years, the biological mechanisms involved remain unclear.²

Recently, the free radical nitric oxide (NO) has been shown to play a central role as a mediator of bone cell function.³⁻⁶ It has also been implicated as an effector molecule of cytokines in the pathogenesis of various diseases of bone and as a mediator of apoptosis.⁷⁻¹⁰

We suggest that abnormal NO production in patients with osteonecrosis leads to defective osteoblastogenesis and that the initial bone cell death is apoptosis of osteoblasts and osteocytes rather than necrosis.

Methods

Patients and bone sampling. Following approval by the local ethical committee, we examined the femoral heads from 40 patients undergoing total hip arthroplasty. Of the 46 patients, 20 had advanced osteonecrosis (osteonecrosis group) and 20 had osteoarthritis (control group). Osteonecrosis was idiopathic in four patients and secondary to steroids in seven, sickle cell disease in six and alcohol in three. All specimens were coded to ensure that future examination was carried out in a blind manner. No specimens revealed evidence of metabolic bone disease. Histological examination excluded osteonecrosis in the control group

and confirmed stage III or IV changes, according to the Arlet and Durroux classification,¹¹ in the osteonecrosis group.

Examination of eNOS/iNOS expression. Standard immunohistochemical techniques were used to identify the expression of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) in decalcified specimens as a marker of NO production. The mean intensity of staining for eNOS and iNOS was measured by computerised image analysis (Seescan Symphony Analyzer, Seescan, Cambridge, UK). Standard western blotting techniques were also used and the intensity of the bands corresponding to eNOS and iNOS were calculated by computer image analysis (Bio-Rad, Hercules, California).

Examination for evidence of apoptosis. DNA laddering has become accepted as a major hallmark of apoptosis.¹² Its presence was detected using a commercially available DNA extraction kit (Oncogene Research Products, Cambridge, Massachusetts). We supported this method by identifying apoptotic cells using terminal deoxynucleotidyl transferase-mediated dUTP neck end labelling (TUNEL reaction) with TdT-FragEL (Oncogene Research Products) and the demonstration of Caspase-3 activation and the cleavage of Poly (ADP-ribose) polymerase (PARP).¹³⁻¹⁵

Statistical analysis. Statistical advice was sought from the Department of Medical Statistics, Imperial College School of Medicine. A power calculation was performed which showed that 15 patients in each group would be required to show a 50% difference between the two groups using a level of significance of 5%. Sta-

Table I. Production of eNOS and iNOS and evidence of apoptosis

		Osteonecrosis group (n = 20)	Control group (n = 20)	Mann-Whitney U test
Immunocytochemistry	eNOS	37.95 (19 to 62)	5.23 (3 to 8)	p < 0.001
Median intensity of antibody staining (interquartile range)				
	iNOS	18.20 (13 to 23)	6.18 (5 to 8)	p < 0.001
Western blotting	eNOS	0.57 (0.5 to 0.7)	0.29 (0.2 to 0.4)	p = 0.001
Median intensity of bands (interquartile range)				
	iNOS	0.720 (0.5 to 0.8)	0.145 (0.1 to 0.2)	p < 0.001
Apoptosis		13.14 (7 to 15)	0.55 (0.5 to 0.8)	p < 0.001
Median percentage of TUNEL positive cells (interquartile range)

Fig. 1a

Fig. 1b

Photomicrographs of a) immunostaining for eNOS in osteoclasts in a patient with osteonecrosis secondary to steroids and b) immunostaining for eNOS in a patient from the control group (avidin-biotin peroxidase complex method).

Fig. 2a

Fig. 2b

Photomicrograph of glucose oxidase DAB enhancement immunostaining for iNOS in a bone sample from a) a patient with osteonecrosis secondary to steroids and b) a patient from the control group.

OA, control group (osteoarthritis); ON, osteonecrotic group; S, steroids; SC, sickle cell disease; A, alcohol

Fig. 3

Image of Western blot demonstrating the presence of eNOS in the osteonecrosis and control groups.

OA, control group (osteoarthritis); ON, osteonecrotic group; S, steroids; SC, sickle cell disease; A, alcohol

Fig. 4

Image of Western blot demonstrating the presence of iNOS in the osteonecrosis and control groups.

tistical analysis was performed using the Mann-Whitney U and the Kruskal-Wallis tests. Statistical significance was accepted if $p < 0.05$.

Results

Evidence of eNOS/iNOS expression. eNOS was present in osteoblasts, osteocytes and marrow cells in both the osteonecrosis and control groups (Table I; Figs 1a and 1b). There was very little evidence of staining for iNOS in any cell types from the control group but it was found in osteoblasts, osteocytes and marrow cells in the osteonecrosis group (Figs 2a and 2b). The intensity of staining for both eNOS and iNOS was greater in the osteonecrosis group ($p < 0.001$). The intensity of bands in western blotting was greater in the osteonecrosis group for eNOS ($p = 0.001$) and for iNOS ($p < 0.001$) (Figs 3 and 4).

Evidence of apoptosis. There was no evidence of DNA laddering in any patient from the control group. Of the 20 patients in the osteonecrosis group, 15 had evidence of DNA laddering indicating that cell death was accompanied by apoptosis. The five patients from the osteonecrosis group who did not have evidence of DNA laddering all had sickle cell disease as the underlying cause of the osteonecrosis (Fig. 5). Western blotting for the cleavage of PARP and the activation of Caspase-3 confirmed these results.

The TUNEL reaction allowed identification of which cells were undergoing apoptosis (Fig. 6). Once again, there was very little evidence of apoptosis in sections from patients in the control group or in those patients with sickle cell disease (Tables I and II). The remaining 15 patients in the osteonecrosis group had marked evidence of apoptosis in osteoblasts, osteocytes and marrow cells. The proportion of apoptotic cells in the osteonecrosis group as a whole was greater than in the control group and the median percentage values were significantly different between the two groups ($p < 0.001$).

Discussion

The aim of this study was to investigate abnormalities in bone cell metabolism of NO and possible mechanisms of

cell death in osteonecrosis. We have demonstrated evidence of increased eNOS and iNOS expression in bone from the femoral heads of patients with osteonecrosis. The raised expression of eNOS may reflect the increased bone turnover in the areas of bone in which remodelling is occurring. However, the dramatic increase in iNOS expression seen in osteoblasts and osteocytes in the osteonecrosis group may be responsible for the levels of high NO production.⁴ This may have a deleterious effect on the process of repair and remodelling causing further harm to the already damaged bone and leading to the high proportion of apoptotic cells that were observed in this study.

When the sickle cell patients are analysed as a separate sub-group (Table II) there is a small but significant increase in the expression of eNOS compared with the control group (consistent with bone remodelling). However, unlike the remaining patients with osteonecrosis, the patients with sickle cell disease did not have a significant increase in expression of iNOS and showed little evidence of apoptosis despite the high bone cell turnover and increased expression of eNOS.

Mankin¹⁶ postulated four pathophysiological models of bone necrosis: mechanical vascular interruption; arterial thrombosis or embolism; injury to or pressure on a vessel wall and venous occlusion. Prethrombotic conditions such as sickle cell disease result in an intra-vascular blockage to arterial blood flow culminating in infarction of the end-organ unless an alternative source of oxygenated blood is available. There is no reason to expect any different mechanism in osteonecrosis secondary to sickle cell disease. The lack of evidence for apoptosis may be explained by infarction leading directly to necrosis of the cancellous bone cells with subsequent collapse of that segment and continuing osteonecrosis.

However, for other causes of osteonecrosis such as alcohol or corticosteroids, which together may account for up to 60% of all cases, the mechanism may be more complex.¹⁷⁻¹⁹ Intravascular fat has been proposed as a potential factor following histological demonstration of fat in animal experiments and in humans taking steroids.²⁰ However,

SM, standard markers; OA, control group (osteoarthritis); ON, osteonecrotic group; S, steroids (patient 4); SC, sickle cell disease (patient 7); A, alcohol (patient 15); I, idiopathic (patient 20); +ve, positive control

evidence of fat emboli has not been found in histological studies of osteonecrosis in humans.²¹ Glimcher and Kenzora²² proposed that, in view of the lack of supporting evidence implicating fat emboli, there may be a direct cytotoxic effect of corticosteroids, alcohol or their metabolites on the bone cells leading to cell death. They suggested that a precarious blood supply in areas such as the femoral head, may predispose to an accumulation of these drugs or their metabolites, which may then initiate the process of bone cell death. This view was later supported by Spencer following histological analysis of steroid-induced osteonecrosis of the femoral head.^{23,24}

Our study supports the views of these authors and suggests that the process of cell death may involve the activation of iNOS. This leads to locally toxic levels of NO in osteoblasts and osteocytes with death of these cells, further activation of inflammatory mediators and continuing damage to bone.

Glucocorticoid-induced osteoporosis is characterised by a reduction in bone formation and death *in situ* of isolated

Fig. 5
Image of DNA electrophoresis gel demonstrating evidence of apoptosis in some patients in the osteonecrosis group.

Fig. 6
Photomicrograph of TUNEL reaction demonstrating evidence of apoptotic cells in a patient with osteonecrosis secondary to steroids.

Table II. Analysis of results in sickle cell patients and the remainder in the osteonecrosis group

	Apoptosis Median percentage of TUNEL positive cells (interquartile range)	eNOS Values of median intensity (interquartile range)		iNOS Values of median intensity (interquartile range)	
		Immunocytochemistry	Western blotting	Immunocytochemistry	Western blotting
Osteonecrosis group (excluding sickle cell)	14.24 (12 to 15)	40.50 (30 to 71)	0.59 (0.5 to 0.7)	20.25 (16 to 27)	0.770 (0.7 to 0.8)
Sickle cell group	0.90 (0.7 to 1.0)	12.60 (12 to 15)	0.41 (0.4 to 0.5)	7.22 (6 to 8)	0.16 (0.1 to 0.2)
Control group	0.55 (0.5 to 0.8)	5.23 (3 to 8)	0.29 (0.2 to 0.4)	6.18 (5 to 8)	0.15 (0.1 to 0.2)
Kruskal-Wallis test (All groups)	p < 0.001	p < 0.001	p < 0.001	p < 0.001	p < 0.001
Mann-Whitney U test (osteonecrosis and sickle cell groups)	p = 0.001	p = 0.001	p = 0.021	p = 0.001	p < 0.001
Mann-Whitney U test (sickle cell and control groups)	p = 0.144	p < 0.001	p = 0.023	p = 0.102	p = 0.708

segments of bone. The osteonecrotic process may continue in some patients on long-term therapy with collapse of the femoral head. Weinstein et al.²⁵ have recently demonstrated a possible mechanism for the adverse effects of glucocorticoids on bone. They have shown that they inhibit osteoblastogenesis and promote apoptosis of osteoblasts and osteocytes in mice with glucocorticoid-induced osteoporosis. Our study on human bone would appear to support this finding.

We have shown that nitric oxide metabolism is altered in bone cells during the development of osteonecrosis. The process leading to cell death in the femoral head of patients with osteonecrosis includes an increased rate of apoptosis rather than purely necrosis of bone cells. This study may help to explain a recent report which has demonstrated extension of osteonecrosis beyond the femoral head into the proximal femur.²⁶ These findings may also provide an avenue for treatment during the earliest stages of the disease by focussing on blocking the mechanisms responsible for triggering apoptosis, perhaps through oxygen free radical scavengers, and thereby prevent progression of the disease with development of disabling secondary osteoarthritis.

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.

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