Cryofibrinogen levels are increased in non-traumatic osteonecrosis: a new pathogenic clue to osteonecrosis?

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Abstract

Objective. To determine whether levels of cryofibrinogen are increased in non-traumatic osteonecrosis (ON) and could correlate with disease staging.

Methods. We prospectively analysed cryofibrinogen levels by immunofixation electrophoresis in 50 patients with non-traumatic ON, 50 healthy volunteers and 8 patients with traumatic ON. Staging of disease involving the femoral heads and the size of necrotic lesions were assessed by the Association Research Circulation Osseous (ARCO) classification system.

Results. Mean cryofibrinogen levels in patients with non-traumatic ON were significantly increased relative to healthy controls and to patients with traumatic ON (222.1 ± 20.6, 59.9 ± 5.6 and 52.3 ± 14.9 mg/dl, respectively, \( P < 0.001 \)). In the non-traumatic ON group, mean cryofibrinogen levels were significantly increased in patients with multifocal ON compared with patients with mono/bifocal ON (276.5 ± 56.5 and 149.3 ± 15.4 mg/dl, respectively, \( P = 0.03 \)). There were no significant differences in cryofibrinogen levels observed with respect to the size of the necrotic lesions involving the femoral heads. Moreover, cryofibrinogen levels in patients with ON of the femoral heads classified according to the stage of disease were not significantly different between patients with stage 1/2 and patients with stage 3 ON (179.2 ± 31.3 vs 204.1 ± 29.0 mg/dl, respectively; \( P = 0.813 \)).

Conclusion. Cryofibrinogen levels are increased in non-traumatic ON and, more importantly, in multifocal ON. The fact that cryofibrinogen levels are not correlated with the size of lesions and the stage of disease could imply systemic rather than local involvement characterizing the pathogenesis of ON.

Key words: osteonecrosis, cryofibrinogen, bone, biomarker, diagnostic tool.

Introduction

Non-traumatic osteonecrosis (ON) is a devastating disease that typically affects young patients and often leads to bone collapse [1]. It can occur at various bone sites, but mainly involves the femoral heads. The exact prevalence of ON of the femoral heads remains undetermined, but it is estimated that there are 10,000–20,000 new cases every year in the USA [1, 2].

ON is either idiopathic or associated with other risk factors including excessive alcohol intake, corticosteroid treatment, SLE and sickle cell disease [1, 3]. Even though tremendous progress has been made in deciphering the pathogenic mechanisms underlying ON, the exact trigger of the disease remains elusive. Different pathophysiological mechanisms leading to ischaemia have been postulated for this disease, but none can explain the occurrence of the lesion, the insufficient bone repair following the lesion and its evolution to bone collapse [1, 2, 4]. Recent works have shed light on the pivotal role of blood coagulation disorders in fostering ON, as portrayed by acquired or familial thrombophilia-hypofibrinolysis in patients suffering from ON, and that thrombosis in capillaries within the femoral head leads to increased intraosseous pressure and impaired arterial flow, osseous hypoxia and bone death [5–8].
Cryofibrinogenemia (CF) is defined by the presence of cryofibrinogen in the plasma [9] and is characterized by a precipitate composed of fibrinogen, fibronectin and smaller amounts of various proteins arising in plasma cooled at 4°C and which solubilizes when the temperature of the sample is brought back to 37°C [10]. The salient difference between cryofibrinogen and cryoglobulin relates to its sole detection in plasma and not in serum. CF can be classified as primary (or idiopathic) or secondary [11]. The secondary forms of CF are associated with several underlying diseases, including autoimmune diseases, malignancies, infections and thromboembolic events [12]. The pathogenesis of CF remains to be determined, but several clues implicate a role of plasma protease inhibitors (α1-antitrypsin and α2-macroglobulin) in the disease process [11]. This line of evidence has been fuelled by the fact that high plasma levels of these proteases have been found in patients with CF. These proteins inhibit plasmin activity, impede fibrinolysis, augment fibrinogen concentration and hence promote thromboembolic events. The clinical spectrum of CF ranges from cold sensitivity, purpura, thrombosis and neuropathy to ischaemic necrosis [13, 14].

Because of the potential involvement of cryofibrinogen in promoting thromboembolic events, we hypothesized that it could be implicated in the pathophysiology of non-traumatic ON. In the present study we prospectively determined levels of cryofibrinogen in a series of patients with ON and correlated them with different parameters of disease severity.

Patients and methods

Patients

All patients were aged ≥18 years and had clinical evidence of non-traumatic ON. The findings of a thorough history and physical examination, anteroposterior and frog-leg lateral radiographs and MRI scans of the hips, knees, shoulders, ankles and other joints were used to confirm the diagnosis of ON. Non-traumatic ON was considered idiopathic when there was no known aetiological risk factors identified or related to risk factors when there was a history of corticosteroid use, alcohol abuse, SLE or HIV/AIDS. ON was considered traumatic when related to trauma, fracture or hip dislocation.

We prospectively studied 50 patients with multifocal and unifocal non-traumatic ON of a bone site and 8 patients with traumatic ON. Multifocal ON is defined as involving three or more anatomic sites. Inclusion criteria were traumatic or non-traumatic ON of at least one bone site. Exclusion criteria were patients diagnosed with sickle cell disease, hepatitis C, RP, SLE, cryoglobulinaemia, hypofibrinolysis or thrombophilia abnormalities and anti-coagulant treatments such warfarin and heparin. Patients were compared with 50 sex- and age-matched healthy control subjects. The local ethics committee board approved this study (Hopital Erasme ethics committee) and signed informed consent was obtained from each participant.

Laboratory methods

All patients were tested for cryofibrinogen and cryoglobulin. Patients who were positive for cryoglobulinaemia were excluded from the present study. For every patient, data were collected concerning clinical features, haemogram, presence of ANAs, fibrinogen, hepatitis C and HIV serology, α1-antitrypsin and α2-macroglobulin levels, lupus anticoagulant, aCLs, C protein, S protein, plasma homocysteine, mutations for Leiden factor, prothrombin and methylene tetrahydrofolate reductase.

Analysis of cryoprecipitate

By using previously warmed equipment, blood was collected into EDTA-containing tubes that were kept at 37°C until centrifugation (2000 g, 10 min). Then the plasma was immediately chilled at 4°C for 8 days, after which the presence of a precipitate or gel formation was evaluated. For the positive samples, the reversible nature of the cryoprecipitate was checked by heating the plasma at 37°C. The composition of the cryoprecipitate was then analysed by immunofixation electrophoresis (IFE) after a purification step consisting of two washes with ice-cold 0.9% NaCl and 5 mM EDTA. The washed precipitate was loaded on the gel and the immunofixation was performed using the Hydragel 9IF kit on a Hydrasys 2 instrument (Sebia, Vilvorde, Belgium) with the following tracks: fixative solution (total IFE), penta-valent antiserum (anti-human IgG, IgA, IgM, kappa light chains, lambda light chains) (Sebia), anti-human fibrinogen (Dako, Zebra Biosciences, Enschede, The Netherlands) and anti-human fibronectin (Dako). A second aliquot of the cooled plasma was washed in the same way, i.e. by two washes with ice-cold 0.9% NaCl, 5 mM EDTA, and the cryoprecipitate was then redissolved by adding 0.1 M NaOH, the added volume of 0.1 M NaOH being equivalent to the starting volume of plasma. The concentration of the proteins contained in the precipitate was finally determined by measuring the optical density at 280 nm and calculated using the equation (OD280/0.66) × 100, which gives the concentration in milligrams per decilitre. The cryoprecipitate was characterized according to the different constituents into fibrinogen, fibrinogen and fibronectin, and fibrinogen, fibronectin and immunoglobulins [13–15].

Characterization of osteonecrosis

Patients were included in the study if they suffered from epiphysyeal ON at one bone site (hip, shoulder, knee or ankle). The stage of ON was assessed using the Association Research Circulation Osseous (ARCO) classification system that is validated for ON of the femoral head. For ON of the femoral head, the location of the lesion was defined as medial, central or lateral and the area of involvement was quantified as <15% (minimal), 15–30% (moderate) and >30% (extensive) [16].

Statistical analysis

Statistical analysis was performed with GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). Comparisons between different groups were made using...
analysis of variance (ANOVA) with Dunn’s correction test and the Mann–Whitney test as appropriate. All values are expressed as the mean ± S.E.M. \( P \) values < 0.05 were considered statistically significant.

Results

Clinical characteristics and demography of the study population

Fifty patients with non-traumatic ON were tested for cryofibrinogen and 50 patients were used as healthy controls. Eight patients with traumatic ON were also included in the study as positive controls. The mean age of patients in the non-traumatic ON group was 47.6 ± 1.6 years. There were 25 male and 25 female patients. In the healthy control group, the mean age was 50.2 ± 1.6 years and there were 28 women and 22 men. In the traumatic ON group, the mean age was 38 ± 5.4 years and included eight men only.

In the non-traumatic ON study group, the most frequent aetiological factors were alcoholism, cigarette smoking and steroid use. In six cases the aetiology could not be determined and was therefore classified as idiopathic. The clinical and biological characteristics of the patients included are summarized in Table 1.

Cryofibrinogen levels are increased in ON and are significantly higher in multifocal ON

Patients suffering from ON had significantly higher cryofibrinogen levels compared with control groups (Fig. 1A). The mean cryoprecipitate levels were not significantly different following the causes of ON (Fig. 1B). Furthermore, the cryoprecipitate observed was qualitatively quantified into fibrinogen alone, fibrinogen and fibrinogen, and fibronectin and immunoglobulin. No significant differences were observed in the subtype of cryoprecipitate analysis (Fig. 1C).

Further subgroup analysis of patients with ON according to the number of sites of ON showed that the cryoprecipitate levels in patients with multifocal ON were significantly higher compared with control groups and patients with uni- or bifocal ON (Fig. 1D).

Cryoprecipitate levels in ON patients are not dependent on the size of the lesions of the femoral heads

To further assess the involvement of cryofibrinogen in ON, we next determined whether the levels of cryoprecipitate correlated with the size of necrotic lesions in ON of the femoral heads. Cryofibrinogen levels were determined in the subgroups of patients with involvement of the right and left femoral heads. The size of the necrotic lesions of the epiphyseal femoral regions was quantified as either involving <30% (moderate) or >30% (severe) of the surface of the femoral heads. In both right and left hips, there were no significant differences in the levels of cryofibrinogen observed independently of the size of the necrotic lesions (Fig. 2A and B).

CF levels are not correlated with the stage of ON of the femoral heads

We next compared cryofibrinogen levels in patients with ON of the femoral heads with stage 1/2 and patients with stage 3 at the time of diagnosis, according to the ARCO staging classification. Patients with stage 3 ON had higher CF levels relative to patients with stage 2 ONA, but they were not statistically significant \( P = 0.813 \).

Discussion

In the present study, we found significantly increased plasma levels of cryofibrinogen in patients with non-traumatic ON as compared with healthy controls and patients with traumatic ON. Furthermore, higher plasma levels of cryofibrinogen were observed in patients presenting multifocal ON relative to patients with uni- or bifocal ON.

Non-traumatic ON is a crippling disease affecting the quality of life of patients. The actual mainstay of treatment is arthroplastic surgery, and since most of the affected patients are of a younger age, many of them will outlive the existing prosthesis and will thus require revision surgery. Because of the potential side effects of arthroplastic surgery, identifying new potential players involved in the pathogenesis of non-traumatic ON could prove salutary as alternate therapeutic regimens. Different pathogenic mechanisms in non-traumatic ON have been described, but one of most recent concepts at the vanguard of the pathophysiology of the underlying disease relates to the fact that ON results from bone ischaemia and ensuing
osteocyte death. Different mechanisms leading to ischaemia have been postulated, including fat emboli, microvascular tamponade of the blood vessels of the femoral head by marrow fat, retrograde embolization of the marrow fat and intravascular coagulation [17–19]. However, none of those mechanisms explored the necrotic lesion as a bone disease.

In the early 1980s, the concept of accumulative cell stress was advanced, which is a theory that proposes that bone cells are exposed to multiple insults or stresses, the effects of which accumulate to the point that the cells cannot sustain themselves and die [20]. A better understanding of bone biology and the risk factors of ON indicates that those mechanisms should be revisited. Indeed, ON is characterized by apoptosis of the osteocytes and cancellous bone lining cells not only in the necrotic lesion, but also at a distance from the lesion, in the proximal femur [21]. The replicative capacities of osteoblastic cells

Fig. 1 Increased cryofibrinogen levels in patients with non-traumatic ON.

(A) Cryofibrinogen levels are significantly increased in patients with non-traumatic ON relative to controls. Values are represented as mean ± S.E.M. *P < 0.001 (ANOVA with Dunn’s correction test). (B) Cryofibrinogen levels in non-traumatic patients with ON following different aetiological factors. No significant differences in cryoprecipitate levels were observed in the different subgroups of non-traumatic ON-associated causes. P > 0.05 (ANOVA with Dunn’s correction test). (C) Qualitative analysis of cryoprecipitate in patients with non-traumatic ON. The cryoprecipitate observed was dichotomized into fibrinogen only, fibrinogen and fibronectin, and fibrinogen, fibronectin and immunoglobulins. No significant differences in the cryoprecipitate analysis were observed. P > 0.05 (ANOVA with Dunn’s correction test). Bars are representative of mean ± S.E.M. (D) Cryofibrinogen levels are significantly increased in patients with multifocal non-traumatic ON relative to patients with monofocal or bifocal ON. P = 0.03 (Mann–Whitney test).
obtained from the intertrochanteric area of the femur are reduced in ON patients compared with patients with OA [22]. The number and the activity of fibroblast colony-forming units, reflecting the number of mesenchymal stem cells that could potentially give rise to mature osteoblast, have been shown to be decreased in ON [23, 24]. Fundamental mechanisms of bone remodelling occur in what has been termed the basic multicellular unit (BMU), encompassing the osteoclasts, osteoblasts and osteocytes within the bone-remodelling cavity. Penetrating the canopy of bone-lining cells, and presumably serving as a conduit for the cells needed in the BMU, are capillaries. Moreover, the function of the capillaries serving as a conduit for the cells needed in the BMU and providing blood supply could be altered by emboli or thrombosis [17, 19]. This altered bone remodelling is responsible for three different events in the pathogenesis of ON: the appearance of ON itself, the bone repair that occurs after ON and its evolution to the subchondral fracture. First, glucocorticoids inhibit osteoblastogenesis and promote osteoblast and osteocyte apoptosis [25, 26]. Osteocyte apoptosis could disrupt the mechanosensory role of these cells and thus prevent the adaptation of bone to ischaemia and medullary changes seen in the very early stages of ON [26–28]. The decrease in osteoblast capacity to proliferate could therefore reflect the disruption of the mechanosensory role of the osteocytes’ canalicular network and may explain the evolution from marrow ischaemia and oedema to ON [22].

In the context of blood coagulation abnormalities, such as resistance to activated protein C or hypofibrinogenemia, it is easily conceivable to reconcile ON with underlying bone ischaemia. However, in the absence of characterized blood coagulation abnormalities, the role of bone ischaemia in fostering disease could be debated. In this particular setting, increased cryofibrinogen levels could prove to be the missing link strengthening the candidacy of bone ischaemia in the pathophysiology of ON. The significantly increased levels of cryofibrinogen observed in patients with non-traumatic ON relative to healthy volunteers and patients with traumatic ON support the fact that cryofibrinogen could be a potential player fostering bone ischaemia by promoting thrombosis of those capillaries.

These lines of evidence have been extensively highlighted in studies investigating the pathogenic roles of CF. High values of cryofibrinogen have been observed in patients with skin necrosis and gangrene and correlate with clinical severity of the disease [14, 15]. Furthermore, in a recent study by Saadoun et al. [13], CF contributed to thrombotic events in 40% of cases. The pathogenic role of CF could be explained by the plugging of small vessels, reducing blood flow and causing ischaemia. Moreover, a defect in the fibrinolysis process has been observed in patients with primary CF, further explaining the formation of blood clots and ischaemia. This defect of the fibrinolysis process is seen in the high serum levels of α1-antitrypsin and α2-macroglobulin and delayed euglobulin lysis time. Reflex vasospasm, vascular stasis and hyperviscosity in a vicious loop exacerbate the underlying process, further contributing to impaired blood flow and ischaemia. In our study, we did not observe increased serum levels of α1-antitrypsin and α2-macroglobulin in our cohort of patients. This observation raises the possibility that other causes than a sheer defect in the fibrinolysis pathway could be responsible for CF-associated thrombotic events.

**Fig. 2** Cryofibrinogen levels in patients with ON of the femoral heads and classified according to the size of the necrotic lesions (either <30% or >30% of the epiphyseal region).

(A) Cryofibrinogen levels in patients with ON of the right femoral head. No significant differences were observed regarding the size of the necrotic lesions. \( P > 0.05 \) by Mann-Whitney test. (B) Cryofibrinogen levels in patients with ON of the left femoral head. No significant differences were observed regarding the size of the necrotic lesions. \( P > 0.05 \) by Mann-Whitney test.

![Cryofibrinogen levels in patients with ON of the femoral heads and classified according to the size of the necrotic lesions](https://academic.oup.com/rheumatology/article-abstract/52/9/1694/1793451/1694/1793451)
Lending further support to the role of cryofibrinogen in the pathogenesis of non-traumatic ON was the fact that higher levels of cryoprecipitate were observed in multifocal ON relative to mono- or bifocal ON. To further substantiate the potential involvement of cryofibrinogen in ON, we quantified the size of the necrotic lesions affecting the femoral heads. We could not establish a well-defined correlation of hip lesions with levels of cryofibrinogen. Together, these results suggest that ON is characterized by systemic features whereby cryofibrinogen could play a pivotal role in triggering disease. How CF per se is involved in initiating or perpetuating disease is an interesting question that remains to be answered.

In conclusion, our study shows that cryofibrinogen levels are significantly increased in non-traumatic ON and could therefore be an interesting biomarker. Moreover, higher levels of cryofibrinogen were observed in patients with multifocal ON relative to mono- or bifocal ON, whereas no significant differences in cryoprecipitate levels were found in relation to the size of necrotic lesions or the stage of disease at the femoral heads. These results show that ON might reflect a systemic rather than local involvement whereby cryofibrinogen could play a cardinal role.

**Rheumatology key messages**
- Cryofibrinogen could be a potential biomarker of non-traumatic ON.
- Increased cryofibrinogen levels in non-traumatic ON reflect systemic rather local involvement of disease.

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Clinical vignette

Black and white: alkaptonuria and gout

A 49-year-old man was admitted to our hospital with an acute exacerbation of oligoarticular gouty arthritis. His medical history included alkaptonuria, SLE complicated with nephrotic syndrome, treated with bumetanide, metabolic syndrome and recently diagnosed crystal-proven tophaceous gout treated with urate-lowering therapy. A remarkable finding on physical examination was grey-black darkening of his auricular cartilage related to the alkaptonuria and also tophaceous gout deposits in the antihelix of his ear (Fig. 1). Aspiration of the ear tophi was omitted because of the clear clinical picture and recent crystal-proven tophaceous gout. His serum uric acid level was 0.68 mmol/l. The arthritis responded well to a higher dose of prednisolone, and subsequently urate-lowering therapy was re-initiated. Bumetanide was probably a contributing factor for induction of hyperuricaemia and gout as a result. Unfortunately, diuretic treatment could not be stopped because of the nephrotic syndrome. As far as we could determine, there is no association between the use of diuretics and the development of alkaptonuria. Tophaceous gout and alkaptonuria are both chronic disorders that can cause specific clinical features like subcutaneous depositions [1, 2]. Simultaneous occurrence of both diseases is very rare, and only a few cases have been reported. As our patient demonstrates, both diagnoses can be suspected based on specific findings following careful physical examination.

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