High serum alpha-2-macroglobulin level in patients with osteonecrosis of the femoral head

Running Title: Alpha-2-macroglobulin in osteonecrosis

This survey is performed in Ghaem and Imam Reza Hospitals, Mashhad University of Medical Sciences, Mashhad, Iran

Abstract

Background

Diagnosis of osteonecrosis of the femoral head (ONFH), a disabling and devastating condition, is complicated by the lack of reliable serum biomarkers. This study aimed to investigate whether the serum level of alpha-2-macroglobulin (A2M) can be used for ONFH diagnosis.

Methods

Blood samples from 36 ONFH patients were obtained. Serum protein capillary electrophoresis was performed on the sera of the patients. The serum levels of A2M were also subjected to be measured by A2M human enzyme-linked immunosorbent assay.

Results

Serum protein capillary electrophoresis of ONFH patients revealed that the level of alpha-2 subunit, composed of alpha-2-macroglobulin, ceruloplasmin and 2-2 haptoglobin phenotype, was increased significantly as compared to healthy subjects (p value: 0.0001). Moreover, alpha-2-macroglobulin ELISA assay proved that the A2M has been significantly raised (p value: 0.037).

Conclusion

Taken together, these findings suggest that avascular necrotic femur head presumably directly or indirectly elevates A2M into the bloodstream. Thus, measuring the serum level of A2M might be used as a reliable diagnostic tool in clinical practice.

Keywords: Avascular necrosis; Alpha-2-macroglobulin; Osteonecrosis; Femur.

INTRODUCTION

Osteonecrosis of the femoral head (ONFH) has been considered a really disable and devastating disease and is one of common causes of musculoskeletal disability (1). There are different names that contribute to this condition like avascular necrosis (AVN), aseptic necrosis of bone, osteochondritis dissecans, ischemic necrosis (2,3), which various names indicate that the etiology of the disease is not entirely known. Most of the ONFH patients are initially asymptomatic and have no changes on their plain radiography in the early stages of the disease, thus bone necrosis progresses to total destruction of the hip joint in a relatively short time, usually until the fifth decade of life (1). In most patients with a lesion less than 30% of the femoral head, ONFH remain asymptomatic for more than 5 years. However in a symptomatic painful ONFH, a poor prognosis with the rate of femoral head collapse greater than 85% at 2 years has been reported (4). Approximately 5% to 18% of the 500,000 total hip replacement (THR) procedures performed annually in the United States are related to ONFH (5). Although the actual prevalence of the disease remains undetermined, an estimated of 10,000 to 20,000 new cases were reported each year in the United States and three hundred thousand to six hundred thousand people suffer from this disease in total there (6). Noticeably, this disease is more common among the Asian population than other people. In Taiwan, 46.3% of all THR surgeries are because of ONFH. Several statistical reports show a high prevalence of the disease in Japan and Korea compared to Caucasian populations (7). In Japan 2500 to 3300 new ONFH cases are reported annually (1). A case series of 647 THR in Hong Kong revealed that ONFH accounted for 45.6% of all hip replacements. These data indicate that the disease is more prevalent in Asia and hence has been studied probably more thoroughly by Asian scientists and surgeons (7). Almost 75% of patients with ONFH are between 30 to 60 years of age (5). Since most patients with the disease are at the peak of their productive years, there is a harmful effect on the workforce with direct economic outcome on their families. Basically, this condition has a severe socioeconomic impact on all population in the world especially on poor/middle socioeconomic classes who the high cost of hip replacement procedures are far beyond the reach of them (8). Several previous studies have reported high failure rates in total hip replacements in short-term and mid-term follow up and the sufferance of patients from additional operations (9) although new modern designs and surgeon awareness in THR have relatively improved the outcomes (10). Core decompression is the most common available femoral head preserving technique, especially recommended in early stages (I and II) of the disease. It is usually employed before the collapse and fracture of

the femoral head and/or neck to delay or avoid reconstructive surgery of femur. Most patients ultimately require total hip arthroplasty (stage III or higher) (8) and no treatment method has proved to be completely effective in arresting the disease process before subchodral collapse or in slowing the progression of femoral head destruction and osteoarthritis after subchondral collapse. Based on these conditions, a quick and thorough investigation can lead to the formulation of a correct diagnosis in time for the application of therapeutic methods, less debilitating for the patients (1). Hip-preserving therapies especially core decompression are most effective in the earliest stages of ONFH. Thus, the key to successful treatment lies in identifying the individuals at risk and quantifying the risk in terms of clinical and pathophysiological characteristics so that early diagnosis can be made before femoral head collapse. One of the reliable methods providing a dynamic and powerful approach to understand the spectrum of a disease with applications in screening, diagnosis and prognosis is biomarker studies (11). A diagnostic biomarker for ONFH specifically in non-traumatic cases could lead to an early diagnosis and eventually an appropriate treatment.

Glucocorticoid (GC) administration is considered as the most common risk factor for non-traumatic ONFH (12) and so far, several candidate genes had been introduced to become up-regulated in GC induced animal models. Amongst the candidate genes, alpha-2-macroglobulin (A2M) is considered as a highly upregulated molecule in ONFH animal models. Taking into account the colorful roles of A2M in haemostasis, cartilogenesis and osteogenesis (13), in the present study, A2M is measured at the serum level in ONFH patients to assess its diagnostic biomarker applicability.

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MATERIALS AND METHODS

Population

The study was approved by the local ethical committee and signed informed consents were obtained from each study participant prior to blood collection. Patients with longstanding history of steroid consumption and established ONFH were recruited at two regional hospitals. The diagnosis of non-traumatic ONFH was established on the basis of the clinical history and a combination of radiology, computed tomography or magnetic resonance imaging. Patients whom were admitted for surgical treatment were classified into different stages based on Ficat and Arlet classification. Patients in stage 2 and 3 received surgical core decompression of the hip and patients with late stages and painful hip underwent total hip replacement. Our patients were recruited according to the following criteria: inclusion criteria

were glucocorticoid intake and exclusion criteria were a history of hip injury, rheumatoid arthritis and sickle cell anemia.

Blood Sample collection

Blood samples from 36 ONFH patients administered glucocorticoids were obtained from their brachial vein from two regional hospitals, recruited from February 2014 to December 2015. The venous blood samples were centrifuged at 1000 x g for 15 minutes and the resultant sera were extracted into labeled eppendorf tubes. The serum aliquots were stored at –80°C before use.

Capillary Electrophoresis of Serum Proteins

Automated multicapillary zone electrophoresis (CE) instrument (Capillarys®, 4.51 software version; Sebia) for human serum protein analysis was used. Briefly, with the Capillarys β1-β2+® reagent set (Sebia), proteins were separated at 7 kV for 4 min in 15.5 cm × 25 μm fused-silica capillaries (n = 8) at 35.5 °C in a pH 10 buffer with online detection at 200 nm.

Enzyme-linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) was performed against the A2M protein using Abcam's alpha 2 Macroglobulin Human *in vitro* competitive ELISA (Enzyme-Linked Immunosorbent Assay) kit (Abcam catalog# ab108888). The experiment was carried out following manufacturer's instructions. Briefly, the reagents (1X dilute M, 1X wash buffer, 1X biotinylated alpha 2 Macroglubolin and 1X conjugated SP) were initially prepared and the standards were made according to the Abcam standard dilution preparation table. Following preparing the ELISA plate, all experiments were performed in triplicate. Plate reader PerkinElmer (victor x5) was used to measure the absorbance of each well at 450 nm. Finally, data calculation was accomplished based on the standard curve.

Statistical Analysis

The mean concentration of A2M protein level in patients was compared with the normal mean values using one-tailed t-test. The correlation between A2M and alpha 2 was investigated with Pearson's Product-Moment Correlation test. The statistical evaluation of the results was performed by the "SPSS version 22.0" software package (SPSS Inc., Chicago, IL, USA). All values were expressed as the mean ± SD. A p-value of ≤ 0.05 was considered

as statistically significant. The normality of distribution was checked by the Kolmogorov-Smirnov test.

RESULTS

Clinical characteristics and demography of the study population

A total of 36 non-traumatic ONFH patients were enrolled in this study. There were 17 male and 19 female patients, 25 to 60 years of age (39 ± 10 years). According to the clinical history and a combination of radiology, computed tomography or magnetic resonance imaging, the femur head was 50% bilaterally and 50% unilaterally involved in ONFH patients. The majority of patients (69%) were located in stage 3 of Ficat and Arlet classification. 10 ml brachial blood was collected from each subject. All patients had received at least one sort of GC. The clinical and biological characteristics of the patients are summarized in Table 1.

Capillary Electrophoresis of Serum Proteins

The alpha 2 zone measured by capillary electrophoresis increased significantly in ONFH patients in compared with the baseline (0.5-0.9 g/dL, 9.45%) of healthy individuals, (Mean:12.40, SD:2.97, p-value: 0.0001) (Figure 1).

Enzyme-linked Immunosorbent Assay (ELISA)

In case of testing for normality of A2M distribution, Kolmogorov-Smirnov test showed statistically no significant deviation from the normal curve (p-value: 0.977).

Serum concentration of A2M was determined using standard curve and expressed as miligrams per liter. Patients' A2M sera were compared with the mean of normal A2M level indicating a range of 1.49 – 1.79 g/L in the ELISA kit. The results demonstrated a significant increase in the level of A2M (p-value: 0.037) and it also significantly enhanced in compare to the upper limit of normal A2M level (p-value: 0.04). ELISA results for antibodies directed against A2M correlated to alpha 2 proteins were shown in Figure 2.

DISCUSSION

Today, researchers have encountered with some limitations regarding the etiology and treatment of ONFH, that are 1) lack of understanding the interaction between the disease and the bone cellular abnormalities especially in the early stages of the disease, 2) lack of suitable

animal models (13), and 3) lack of informative biomarker to evaluate the activity status or the prognosis of the disease (14). Finding an informative biomarker that can diagnose the disease especially in its early stages has been the interest of many researchers. In addition to diagnose, plasma biomarkers could be useful in screening, in routine assessment of disease recurrence and response to therapy without accompanying imaging studies or biopsies.

Upregulation of a number of genes such as A2M, collagen type II alpha 1 (Col2A1) and melanoma inhibitory activity (MIA) was previously reported in ONFH animal models (13). Since A2M could be measured in the blood, it has the potency to become a serum biomarker. Carli AV. and his colleagues showed elevated plasma A2M level during GC treatment in rodents. They suggested A2M raise could play an important role in the host reparative response to GC-associated effects (15). Ramadori G *et al.* showed GC could modulate A2M and apolipoprotein E gene expression by raising the steady-state levels of alpha-2-macroglobulin-specific messenger RNA and by reducing apolipoprotein E-specific transcript in cultured rat liver fat-storing (Ito) cells (16). Milosavljevic TC *et al*. showed GCs could mediate transcriptional activation of the A2M gene (17). This study and several similar studies denote the effect of GC in regulation of A2M especially in its up-expression. In this regard, capillary electrophoresis was performed on the serum of patients receiving GCs. For the six serum capillary electrophoresis accepted zones, all six peaks were observed (albumin, alpha 1, alpha 2, beta 1, beta 2 and gamma). Alpha 1 peak includes a combination of α1-antitrypsin, thyroxine-binding globulin (TBG), and transcortin. Alpha 2 peak contains haptoglobin, ceruloplasmin, and A2M. Measurement of alpha 2 in ONFH patients showed an elevation in comparison to normal values. The elevation could be due to an increase in each alpha 2 component especially A2M protein. Thus, A2M in ONFH patients' sera was measured, which demonstrated a significant increase in A2M protein level. Surprisingly, Soyfoo MS *et al*. did not observe an elevated serum level of A2M and α1-antitrypsin in their cohort of non-traumatic ONFH patients (18), however, they declared cryofibrinogenemia, a fibrinolysis defect, in their patients and, more importantly in their multifocal ONFH patients. Amdo TD *et al*. elucidated there was a high serum level of α1-antitrypsin and A2M and delayed euglobulinlysis time in cryofibrinogenemia (19) as opposed to Soyfoo's findings. In another study, Chen Y *et al.* reported that the levels of complement component 3 (C3), C4, inter-α-trypsin inhibitor heavy chain H4 and A2M in the serum of patients with ONFH have reduced (20), although several other studies did not confirm their results (13,21,22). The difference amongst the results could be related to various stages of disease and dissimilar environmental conditions as well as technical issues. Thus, in future studies it is

recommended to stage the disease and determine the size of necrotic lesions of the femoral head and then correlate them with A2M serum level. To minimize the co-founder effects, it is suggested to perform similar studies on different categories of ONFH patients such as glucocorticoid induced, alcohol induced or drug abused and idiopathic ONFH patients separately. Hence, the effect of each inducer could be compared to the A2M serum level of normal individuals and studied independently.

To sum up, the present study demonstrated that patients with nontraumatic ONFH have significantly higher serum levels of A2M than normal population.

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CONFLICT OF INTEREST DISCLOSURE

We declare that there are no conflicts of interest.

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