

Original Article

Expression of vaspin in the joint and the levels in the serum and synovial fluid of patients with osteoarthritis

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Received August 20, 2014; Accepted September 25, 2014; Epub October 15, 2014; Published October 30, 2014

Abstract: The aim of this study was to determine the expression of vaspin in the joint and investigate the distribution between paired serum and synovial fluid (SF) in osteoarthritis (OA) patients, and serum in healthy controls. The gene expression of vaspin was measured by quantitative real-time polymerase chain reaction (qPCR) in the OA joint tissues. The vaspin protein expression in the cartilage, synovium and osteophyte from OA patients who required total knee replacement (TKR) were detected by immunohistochemistry (IHC). Levels of vaspin in serum and SF were analyzed by enzyme-linked immunosorbent assay (ELISA), including 26 OA patients and 23 healthy controls. All the joint tissues including cartilage, synovium, meniscus, infrapatellar fat pad and osteophyte from OA patients expressed vaspin messenger RNA (mRNA), and the expression of vaspin protein was observed in OA cartilage, synovium and osteophyte. Furthermore, serum vaspin was reduced in OA patients compared to healthy controls, and serum vaspin levels from OA patients exceed those in the paired SF. Serum or SF vaspin were not related to age, gender, or body mass index (BMI). These results suggest that vaspin may be involved in the pathophysiology of OA and may have local effects in the joint during the process of OA.

Keywords: Vaspin, osteoarthritis, serum, synovial fluid, joint

Introduction

Vaspin (visceral adipose tissue-derived serine protease inhibitor), identified as a novel adipocytokine, has been found to be expressed in the visceral adipose tissue of Otsuka Long-Evans Tokushima Fatty rats at the age when obesity and insulin resistance peaks [1]. Several human tissues such as adipose tissue [2, 3], skin [4], stomach [5], liver and pancreas [6] have been found indicating an expression of vaspin. Overweight or obese humans show abundant vaspin gene expression in visceral adipose tissue, while lean individuals show undetectable levels of vaspin mRNA in visceral or subcutaneous fat [3]. It has been shown that vaspin expression decreases with the worsening of diabetes and body weight loss, whereas vaspin serum levels could be normalized by insulin or pioglitazone treatment, which has been suggested to be a compensatory mediator for abrogating obesity and its inflammatory complications [3]. Elevated serum vaspin levels have been associated with obesity, impaired

insulin sensitivity, and fitness level [7-9], while several other studies did not find an association between circulating vaspin and insulin sensitivity or parameters of obesity and fat distribution [10-13].

Recent studies have demonstrated novel links between vaspin and arthritis [14, 15]. Ozgen et al. [14] found serum vaspin levels were higher in RA compared to the healthy controls. Moreover, the serum vaspin level was increased after glucocorticoids (GCs)-treated but unaffected after adalimumab treatment in RA patients [15]. In addition, another study [16] demonstrated increased levels of vaspin in the synovial fluid of patients with RA compared with those with OA. These data suggested a possible role of vaspin in the pathophysiology of RA. Nevertheless, few studies have shown a correlation between vaspin expression and OA development.

In the present study, we analyzed the gene and protein expression of vaspin in joint tissues

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Table 1. Clinical and demographic characteristics of patients with osteoarthritis and control subjects

Characteristics	OA, n = 26	Controls, n = 23	p_1
Age, years	66.5 ± 4.8	62.4 ± 10.2	0.43
Sex, F/M	17/9	15/8	
BMI, kg/m ²	25.8 ± 3.1	25.1 ± 2.6	0.51
S-vaspin, pg/ml	18.5 ± 11.1	26.3 ± 9.6	0.01

OA, osteoarthritis; F, female; M, male; BMI, body mass index; S, serum; SF, synovial fluid; NM, not measured; p_1 , OA vs. Controls;

from OA patients, and also investigated the serum and SF vaspin concentrations in patients with OA and serum concentration in healthy controls. We demonstrated that all the joint tissues including cartilage, synovium, meniscus, infrapatellar fat pad and osteophyte from OA patients expressed vaspin gene, while the cartilage, synovium and osteophyte expressed vaspin protein. Additionally, the current study demonstrated that serum vaspin levels were reduced in OA patients compared to healthy controls, and the SF vaspin levels were less than those in the paired serum in OA patients, but not significantly correlated with each other. However, we failed to find that serum or SF vaspin levels were associated with age, gender or BMI in the current study.

Patients and methods

Characteristics of patients

Clinical and demographic data of the patient and control groups are shown in **Table 1**. There were no differences in term of age, gender or BMI. All OA patients required TKR. None of the 23 healthy controls showed clinical or lab measurement features of osteoarthritis before this study.

Patients and samples

Serum and SF was collected from 26 OA patients (mean age 66.5 ± 4.8 years) who required TKR in the study. Knee OA was diagnosed from clinical and radiologic evaluation, based on the American College of Rheumatology criteria [17]. Collected serum and SF samples were centrifuged at 2000 g for 15 min and stored at -80°C. Blood samples from 23 healthy subjects without radiographic knee OA (mean age 62.4 ± 10.2 years) were used as a control

group. Joint tissues including cartilage, synovium, meniscus, infrapatellar fat pad and osteophyte of knees were harvested from these OA patients during the operation, and these samples were immediately sampled and stored at -80°C for RNA extraction and qPCR analysis. Meanwhile, the remaining cartilage, synovium, and osteophyte from OA patients were collected and fixed for 48 hours in 4% paraformaldehyde immediately for IHC. All patients gave informed consent and the study was approved by the hospital ethics committee.

Laboratory measurements

The gene expression of vaspin in all joint tissues was analyzed by qPCR. Specimens were pulverized in liquid nitrogen, and total RNA was isolated using Tri reagent (Sigma-Aldrich, Shanghai, CHN) according to the manufacturer's instructions. One microgram of total RNA was used for synthesis of cDNA by reverse transcription, using Random Hexanucleotide Primers (Promega, Beijing, CHN). Then, qPCR analysis was performed using the iCycler apparatus (Bio-Rad system, California, USA). iQTM SYBR Green supermix PCR kit (Bio-Rad system) was used for real-time monitoring of amplification (5 ng of template cDNA, 45 cycles: 95°C/10 s, 62°C/25 s) with primers 5'-GGG CAA GCT GAA GCA CTT GGA G-3' and 5'-CCC GTC ATG TGG AGT CTG GGT-3' (NM 173850.2). Using 18S rRNA (5'-GAC TCA ACA CGG GAA ACC TCA C-3' and 5'-CCA GAC AAA TCG CTC CAC CAA C-3') primer, a parallel amplification of oyster 18S rRNA transcript (NR 003286) was carried out to normalize the expression data of the targeted gene transcripts. The relative levels of targeted gene expressions were calculated for 100 copies of the 18S rRNA housekeeping gene following the formula: $n = 100 \times 2^{-(\Delta CT_{\text{targeted gene}} - \Delta CT_{18S \text{ rRNA}})}$.

The immunohistochemical analysis of vaspin in the cartilage, synovium and osteophyte from OA patients (n = 9) was conducted as follows: Samples were fixed for 48 hours in 4% paraformaldehyde immediately after removal, decalcified with 10% ethylene diamine tetraacetic acid disodium salt, buffered at pH 7.4, for 2 months, and further fixed in 4% paraformaldehyde. The specimens were stained with EnVisionTM two-step strategy (DAKO). Four-micrometer-thick paraffin-embedded sections were deparaf-

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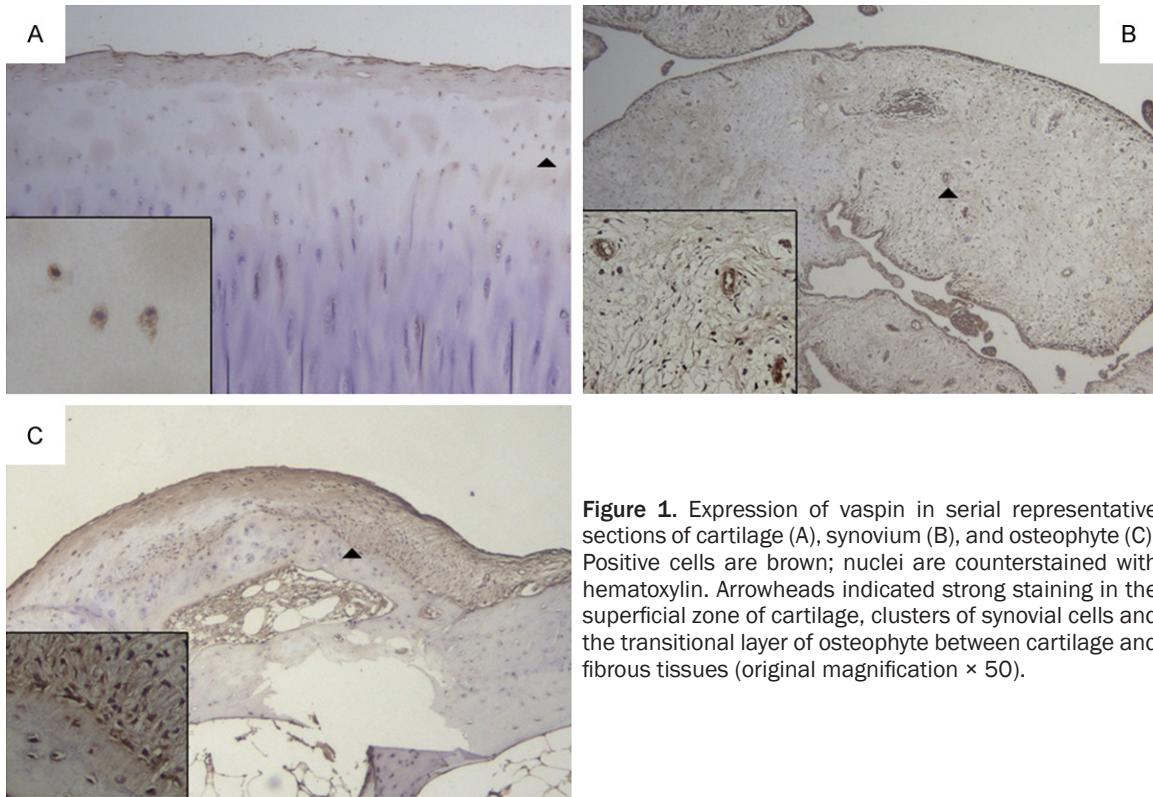


Figure 1. Expression of vaspin in serial representative sections of cartilage (A), synovium (B), and osteophyte (C). Positive cells are brown; nuclei are counterstained with hematoxylin. Arrowheads indicated strong staining in the superficial zone of cartilage, clusters of synovial cells and the transitional layer of osteophyte between cartilage and fibrous tissues (original magnification $\times 50$).

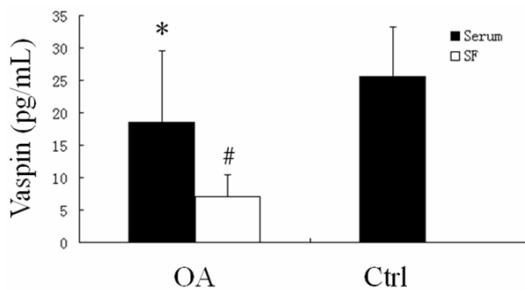


Figure 2. Vaspin levels in patients with osteoarthritis and healthy controls. Serum vaspin is decreased in OA patients than in healthy controls ($*p = 0.01$). Vaspin levels in serum are higher than in paired synovial fluid samples in OA patients ($\#p < 0.001$). Results are expressed as mean \pm SD.

finized twice, 10 minutes each in 100% xylene, and then hydrated with 100% ethanol for 5 minutes twice, 95% for 3 minutes, and 80% for 5 minutes. After 2-5 minutes soaking in distilled water, the slides were incubated with trypsin for 15 minutes at 37°C, and the slides were rinsed for 2-3 minutes with phosphate-buffered saline (PBS). Afterwards, the slides were incubated with 3% hydrogen peroxide for 10 minutes and rinsed in PBS for 2-3 minutes. Serum from non-immune animals was used as blocking buffer,

and the slides were incubated for 15 minutes. Then the primary antibody (bs-7536R, Biosynthesis Biotechnology, Beijing, CHN, diluted of 1:400) was applied to sections overnight in a moist chamber at 4°C to detect vaspin. After rinsing in PBS for 2-3 minutes, the slides were incubated with peroxidase (HRP)-conjugated secondary antibody for 30 minutes at 37°C. With DAB Chromagen added, the slides were observed and examined for color change under a light microscope. This was followed by counterstaining with hematoxylin for 1 minute, and rinsing in tap water for 1 minute.

Paired samples were centrifuged, and both the serum and SF were stored in aliquots at -80°C until use. Serum samples from healthy subjects were used as controls. Before analysis, SF samples were pretreated for 45 min at 37°C with 1 mg/mL of hyaluronidase. Vaspin concentrations were measured with a commercially available ELISA kit according to the manufacturer's protocol (AG-45A-0017EK-KI01, interassay CV was between 3 and 9% and intraassay CV was between 1 and 3.8%, detection limit 12 pg/mL, Adipogen, Liestal, Switzerland). Absorption was measured with 450 nm as the primary wavelength (Bio-Rad system).

Statistical analysis

The results are shown as mean \pm SD values. Statistical analysis was conducted with software SPSS 18.0 for Windows. The differences between patients with OA and healthy controls, female and male were analyzed using the Levene' Test and the independent samples *t*-test. Comparisons of the levels between matched pairs of SF and serum samples were made by using the paired samples *t*-test. The data normality was tested with the Kolmogorov-Smirnov test. The analysis of statistical correlation was performed by the Spearman test of rank correlation. A *p* value less than 0.05 was considered significant for differences and correlations.

Results

Gene expression of vaspin in joint tissues in OA patients

Total RNA was extracted from the joint tissues and analyzed by quantitative real-time PCR. The qPCR demonstrated that vaspin mRNA was expressed in all the joint tissues including cartilage, synovium, meniscus, infrapatellar fat pad and osteophyte, even though at low levels.

Protein expression of vaspin in joint tissues in OA patients

Samples of joint tissues were collected from OA patients who required TKR, and then vaspin protein expression was evaluated by IHC analysis. It was observed that joint tissues including cartilage, synovium, and osteophyte of knees expressed vaspin protein, especially in the degeneration region (**Figure 1**).

Comparison between serum and SF levels

Serum vaspin was markedly decreased in OA patients compared to healthy controls (18.5 ± 11.1 vs. 26.3 ± 9.6 pg/mL, *p* = 0.01) (**Figure 2**). Serum levels of vaspin from OA patients exceeded those of the paired SF (18.5 ± 11.1 vs. 7.1 ± 3.4 pg/mL, *p* < 0.001) (**Figure 2**). The SF vaspin levels did not correlate with the corresponding values in paired serum samples in OA patients (*r* = 0.256, *p* = 0.207).

Correlation of vaspin with clinical characteristics

For all subjects, there was no correlation between levels of vaspin in both serum and SF

with age or BMI (data not shown). In all subjects, the vaspin concentrations were found to be higher in males than in females, but with no statistical significance.

Discussion

Adipocytokines belong to a family secreted by white adipose tissue (WAT), which comprises leptin, adiponectin, resistin, visfatin, apelin, vaspin and others [18]. Recently, accumulating evidence has suggested that these adipokines play an important role during the pathophysiology of OA [19]. It has been shown that serum adipokine levels including visfatin and apelin were increased in OA patients compared to healthy controls in our previous studies [20, 21]. Furthermore, we found that leptin and apelin played a catabolic role on the articular cartilage by stimulating the expression of inflammatory and catabolic factors [22, 23]. However, the decrease of another important adipokine adiponectin in serum from an animal OA model suggested this adipokine played a protective role in the development of the disease [24]. Other studies suggested that adiponectin exhibited both protective and adverse roles in joints during OA [25-27]. The potential role of adipokines in OA pathogenesis is an important subject of study at present.

As a newly discovered cytokine, vaspin has been found to be associated with obesity, insulin resistance, and type 2 diabetes [1]. Several novel studies are concerned with the expression and precious role of vaspin in arthritis [14-16]. Senolt et al. [16] demonstrated increased SF vaspin levels in RA compared to OA patients for the first time, and their data also showed that SF vaspin levels had a tendency to correlate with disease activity score-28 (DAS28) but not to correlate with serum CRP levels or leucocyte counts in synovial fluid in RA patients. They also failed to find that SF vaspin levels correlated with serum levels of anti-citrullinated peptide antibodies (ACPA) or IgM-rheumatoid factor (RF) [16]. Moreover, SF vaspin levels were not affected by BMI or age in those RA or OA patients, and the mean SF concentration of vaspin was twice as high in women as in men but failed to reach statistical significance [16]. On the other hand, Ozgen et al. [14] found serum vaspin levels were higher in RA compared to healthy individuals but associated with neither insulin resistance (HOMA-IR) index

nor intima-media thickness (IMT). Interestingly, the serum vaspin levels in RA patients have been found to be increased after glucocorticoids (GCs) treatment but unaffected after adalimumab treatment [15].

In the present study, we demonstrated that all the joint tissues including cartilage, synovium, meniscus, infrapatellar fat pad and osteophyte from OA patients expressed vaspin gene. This is the first time to our knowledge to report the expression of the vaspin gene in articular tissues. Furthermore, vaspin protein was observed in cartilage, synovium and osteophyte, especially in the superficial zone of cartilage, clusters of synovial cells and the transitional layer of osteophyte between cartilage and fibrous tissues, representing the most active region during the joint degeneration. These findings indicated that all joint tissues are responsible for the vaspin production into the joint cavity, and suggested vaspin may be involved in, and play an important role in the pathogenesis of OA. However, the precise role of vaspin in the development of OA should be further investigated.

An unexpected finding of this study was that serum vaspin levels decreased in OA patients compared to those of healthy controls. To the best of our knowledge, the serum levels of adipokines such as adiponectin, leptin, resistin, visfatin, and apelin were all distinctly higher in OA patients as compared to healthy controls [20, 21, 28]. It was interesting and difficult to explain why the variation tendency of serum vaspin levels was different from other adipokines in OA patients. The results of our finding could be explained by either different study populations or the relatively small numbers involved in the present study. Similar to the current study, Cantarini et al. [29] failed to find increased serum vaspin levels in juvenile idiopathic arthritis (JIA) children with active joints compared to those with no active joints. Indeed, most of adipocytokines had been shown to play a pro-inflammatory and catabolic role during the process of OA [19, 22, 23] except adiponectin mentioned above [24, 25], while the levels of this adipokine decreased in both plasma and SF associated with increased OA severity also indicated that it may play a protective role in OA [30]. On the other hand, Phalitakul et al. [31] demonstrated vaspin could play inhibitory roles on inflammatory states of vascular smooth

muscle cells, and also suggested that vaspin might exert anti-inflammatory effects by inhibiting expression of proinflammatory adipocytokines including resistin, leptin, and TNF- α in murine WAT [31]. It can be speculated that vaspin may play an opposing role from most other adipokines. Further investigations are required to characterize the precise role of vaspin throughout the course of OA.

On the other hand, we demonstrated that serum vaspin levels markedly exceeded those in the paired SF in OA patients, but there was no correlation. Presle et al. [32] demonstrated that resistin and adiponectin exceeded those in the paired SF but leptin SF concentrations were similar or higher than those measured in serum counterparts in OA patients. Besides, our previous study also suggested SF visfatin levels exceeded those in paired serum [20]. It was suggested that serum levels of adipokines were not predictive values for SF determination in OA patients, and also provide evidence for a specific local dysregulation of in the joint space. Actually, the expression of vaspin found in joint tissues in the current study suggested that vaspin may derive from local tissues and undergo specific metabolic pathways in the joint. It would be worthwhile researching the specific regulatory pathways of vaspin in the joint space during the process of OA to reveal the actual role of vaspin on the joint degeneration.

In line with our previous study [20], which found visfatin levels in serum (OA and healthy controls) and in SF (OA group) were not related to the age or BMI, the present study showed no relation between serum or SF vaspin levels with age, gender, or BMI. So we speculated that vaspin might be a less affected adipocytokine by other clinical characteristics in the development of OA.

Conclusions

Our study demonstrates for the first time that joint tissues including cartilage, synovium, meniscus, infrapatellar fat pad and osteophyte express the vaspin gene in OA patients, and that cartilage, synovium and osteophyte show positive vaspin protein. We also found relatively decreased serum vaspin levels in patients with OA compared to healthy controls, and we showed that serum vaspin levels were much higher than in SF in OA patients. However, at

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present we cannot rule out that the decreased level of serum vaspin in OA patients may be related to the modulation of inflammatory or may simply be an epiphenomenon. The precise role of vaspin during the development of osteoarthritis is still unclear, and further investigations are needed to determine whether vaspin performs a protective (anti-inflammatory) or a pro-inflammatory and catabolic role during the development of OA.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (No. 81401824).

Disclosure of conflict of interest

None.

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