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Inflammatory focal bone destruction in femoral heads with end-stage haemophilic arthropathy: a study on clinic samples with micro-CT and histological analyses

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Abstract

Introduction—Focal bone destruction has a high prevalence in haemophilic arthropathy (HA) affected joints, but the mechanism remains unclear.

Aim—We undertook this study on clinic samples to explore the focal bone destruction in femoral heads suffered with end-stage HA.

Methods—Twenty-one femoral heads from HA patients and 19 femoral heads from rheumatoid arthritis (RA) patients were scanned by micro-CT. Histological analysis, including TRAP staining of subchondral bone were performed to evaluate the bone destruction and osteoclasts activity. RANKL, OPG as well as pro-inflammatory cytokines, such as TNF- α and IL-1 β in subchondral bone were detected by immunohistochemistry (IHC) method.

Results—Severe focal lesion was observed in all the HA and RA femoral heads by micro-CT imaging and histological analysis. The mean percentage of lesion volume to total volume of the femoral heads from HA patients was significantly higher than those from RA patients. There was no significant difference in osteoclasts numbers in subchondral bone between HA and RA groups. By IHC analysis, high expression of RANKL, TNF- α , IL-1 β and low expression of OPG and RANK were observed in subchondral bone, and there were no significant differences in the

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Authors contribution

Peijian Tong, Hongting Jin, Di Chen, and Shanxing Zhang designed the research. Shanxing Zhang, Chaofeng Lu and Jun Ying performed the research. Shanxing Zhang, Pinger Wang and Chaofeng Lu collected the data. Shanxing Zhang and Taotao Xu analysed the data. Shanxing Zhang and Chaofeng Lu wrote and revised the manuscript. Shanxing Zhang and Chaofeng Lu contributed equally to this work. Peijian Tong and Hongting Jin have same senior authority of this work.

Disclosures

The authors have no conflicts of interest.

expression of RANKL, OPG, RANK, TNF- α and IL-1 β in femoral heads derived from HA and RA patients.

Conclusion—Our findings demonstrated the focal bone destruction coupled with inflammatory osteoclastogenesis at subchondral bone in femoral heads from patients with end-stage HA, and that was similar to the changes in the femoral heads of RA patients.

Keywords

bone destruction; femoral head; haemophilia; haemophilic arthropathy; inflammation; osteoclastogenesis

Introduction

Haemophilia is hereditary X-chromosomal recessive disorder which due to deficiency or absence of coagulation factors VIII or IX [1]. Haemophilic arthropathy (HA), results from recurrent intra-articular bleeding, is very common in severe haemophilia patients, presenting as cartilage destruction, chronic synovitis and bone damage [2,3]. The major joints affected by the disease include knees, elbows, ankles, hips and shoulders, of which 80% of all are the knees, elbows and ankles [4]. In recent years, bone loss, in haemophiliacs attracted a lot of interests of clinic researchers. In HA patients, in addition to systemic osteoporosis, focal bone destruction presented as cystic change in radiographic imaging has a high prevalence in affected joints [3,5,6]. Focal bone destruction in HA affected femoral head presented as cystic change in radiographic imaging, and usually was misdiagnosed as osteonecrosis [7].

Inflammatory bone loss occurs in several rheumatic diseases, such as rheumatoid arthritis, systemic lupus erythematosus and axial spondylarthritis [8]. In RA, focal bone destruction also is very common in suffered joints [9,10]. It was confirmed that the focal bone destruction in RA joint occurs due to the synovitis, which can induce the expression of proinflammatory cytokines to facilitate osteoclasts activation and then to enhance bone resorption in affected joints [11,12].

Receptor activator of nuclear factor- κ B ligand (RANKL) is a transmembrane ligand, which can induce osteoclasts differentiation and maturation by binding to its receptor RANK expressed on the cell surface of osteoclast precursors, leading to bone resorption. RANKL is produced by several kinds of cells, including osteoblasts, mesenchymal stem cells, immune cells and fibroblast-like synoviocytes (FLS) [8]. New evidences showed that osteocytes embedded in bone matrix control the osteoclasts formation by releasing the major source of RANKL and contribute to the rate-limiting step of bone resorption [13]. However, the FLS and immune cells are the major source for RANKL production under pathological conditions, such as RA [8]. In addition, pro-inflammatory cytokines, such as IL-1 β and TNF- α , are also highly expressed in the synovium of RA joints, promoting osteoclastogenesis through inducing RANKL expression. In contrast, OPG, the decoy ligand of RANK, has bone protective effect. It has been reported that the level of RANKL expression was up regulated, whereas the OPG expression was suppressed in the synovium of RA patients [9]. The investigation of synovial tissues of HA patients also revealed the high RANKL expression coupled with low OPG expression, indicating high

osteoclastogenesis activation in the affected joints [14]. However, the expression of bone resorption related factors in articular cartilage and subchondral bone of HA patients remains unclear.

In this study, we performed micro-CT and histological analyses on clinic samples to determine the mechanism of the focal bone destruction in femoral heads in patients with end-stage HA.

Materials and methods

Subjects

From 2011 to 2014, 21 femoral heads were obtained from 21 male haemophilia A patients (mean age 39 years, range 28–51 years) suffered with hip arthropathy when they underwent THR at Department of Orthopaedics, The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, Zhejiang, China. According to the coagulation factor activity present in blood before factor replacement therapy, four patients were staged in moderate and 17 patients were staged in severe [1]. Only three patients had got factor replacement therapy before THR surgery. During same time, 19 femoral heads from 19 relatively young female patients (young than 50 years, mean age 46 years, range 42–50 years), fulfilled the 1987 revised criteria of American college of rheumatology, were collected at the time of THR [15]. The study was approved by the Ethics Committee of The First Affiliated Hospital of Zhejiang Chinese Medical University according to World Medical Association Declaration of Helsinki. Written informed consent was obtained from each patient.

Micro-computed tomography imaging and analysis

All the femoral heads were scanned at 35 μ m isotropic resolution with Skyscan 1176 micro-computed tomography (micro-CT) scanner (BRUKER microCT N.V, Antwerpen, Belgium). Percentages of the lesion volume to total volume of femoral heads were calculated by CTan software (BRUKER microCT N.V), according to the manual provided by the manufacturer.

Histochemistry and immunohistochemistry analysis

After micro-CT scanning, the femoral heads were coronally sectioned into several pieces. The mid piece of the femoral heads was fixed in 10% buffered formalin followed with decalcification in 10% ethylenediaminetetraacetic acid. Then the samples were dehydrated, embedded in paraffin and cut in 3- μ m thick. The paraffin sections were stained with alcian blue/haematoxylin and eosin (ABH) to evaluate the cartilage and bone destruction, and tartrate-resistant acid phosphatase (TRAP) staining was employed to identify mature osteoclasts around the subchondral bone. The osteoclasts in each random field (magnification 5 $\times$) in subchondral bone were counted.

For immunohistochemistry analysis, PH 6.0 sodium citrate solution was used to perform the heat retrieval of antigen. Serial sections were stained with the following primary rabbit polyclonal antibodies: anti-RANKL (1:200; AbCam, Cambridge, UK), anti-OPG (1:200; Abcam), anti-RANK (1:100; Santa Cruz, Dallas, TX, USA), IL-1 β (1:500; AbCam), TNF- α

(1:500; AbCam). HRP conjugated goat anti-rabbit IgG (1:1000; AbCam) was used as secondary antibody, and a DAB kit (ComWin Biotech, Beijing, China) was used to detect the immunopositive products. Then the sections were counterstained with haematoxylin and observed under a light microscope (Zeiss Axio Scope A1; Carl Zeiss Co, Ltd, Jena, Germany) and then photographed with a digital camera (Zeiss AxioCam HRc). Negative controls were obtained by omitting the primary antibodies. The photos were quantitative analysed with the software named Image-Pro Plus 6.0 (Media Cybernetics, Rockville, Maryland, USA) by detecting the integral optical density of the tissue around the subchondral bone. The expression level of the proteins was presented as IOD/area.

Statistics

All data were presented as mean $\pm$ SD. The unpaired Student's *t*-test for independent samples was used for comparison of groups. Calculations were carried out with GraphPad Prism V.5.03 (San Diego, California, USA) and SPSS V.22.0. (ArchimondeNew York, USA) *P* values less than 0.05 were considered significant.

Results

Gross anatomical changes and micro-CT imaging analysis

By gross anatomical analysis, articular cartilage damage was found at the loading zone of the surface in all femoral heads derived from HA and RA patients (Fig. 1a and b). By micro-CT analysis, one or more focal subchondral lesions were observed in both HA and RA femoral heads. Most of the focal lesions located at the weight-bearing area of the femoral heads (Fig. 1c and d). Quantitative analysis showed that the bone destruction in femoral heads from HA patients was more severe than those from RA patients. The mean percentage of lesion volume to total volume of the femoral heads from HA patients was significantly higher than that from RA patients ($8.2 \pm 3.0\%$ vs. $6.0 \pm 2.6\%$) (Fig. 1e).

Histological analysis

The results from ABH staining demonstrated that the articular cartilage of femoral heads from RA and HA patients was severely damaged. At where the cartilage completely damaged, the subchondral bone plate was covered by a layer of fibrous tissue or even the subchondral was substituted by fibrous tissue, which filled in the marrow cavity between the trabecular bones. The fibrous tissue was composed of fibroblast-like cells and inflammatory cells (Fig. 2a). Using TRAP staining, we examined the region away from the articular surface and found more mature osteoclasts around the focal lesion areas at where the subchondral bone was damaged (Fig. 2b). However, there was no significant difference in osteoclast numbers in subchondral bone areas between the femoral heads collected from HA and RA patients (Fig. 2c).

Expression of bone resorption-related factors and chemokines around subchondral bone

In IHC assay, RANKL was expressed by the fibroblast-like cells and inflammatory cell at the similar level (high expression) in the fibrous tissue around the subchondral bone in femoral heads from HA patients compared to those from RA patients. In contrast, low OPG and RANK expression was also observed at the same sites of the femoral heads from both

two groups compared to RANKL staining. Similar to RANKL, the chemokines, such as TNF- α and IL-1 β , were both expressed at the high level in the fibrous tissue around the subchondral bone areas of the femoral heads from HA and RA patients (Fig. 3a). Quantitative analysis of expression of above factors showed no significant differences between femoral heads collected from HA and RA patients (Fig. 3b). The ratio of RANKL/OPG also showed no difference between femoral heads derived from HA and RA patients (Fig. 3c).

Discussion

In this study, using micro-CT and histological analyses, we demonstrated severe focal lesions in all femoral heads collected from patients with end-stage HA or RA. The bone destruction in HA patients was more severe compared with that in RA patients. In addition, we observed high expression of RANKL, TNF- α , IL-1 β and low expression of OPG and RANK in the fibrous tissue around the damaged subchondral bone near the lesions in femoral heads derived from HA and RA patients.

In recent years, bone damage in haemophiliacs attracted a lot of attention of clinic researchers. A high prevalence of systematic osteoporosis or osteopenia in haemophilia patients has been reported [16–18]. Focal lesion of subchondral bone destruction was observed in CT and MR images of HA affected joints and the correlation between bone loss and severity of haemophilia arthropathy was also confirmed [5,6,17,19]. However, in this study, all of the femoral heads collected from patients with end-stage HA and RA had severe focal subchondral bone lesions. The incidences were higher than that in previous reports [5,6,20]. So the focal bone lesions in large joints may be inevitable during the arthropathy or arthritis progression.

In this study, we observed severe bone destruction by micro-CT and histological analyses in the femoral heads derived from HA and RA patients. The outcomes of the histological analyses of two groups were similar, and revealed typical inflammatory reaction at the lesion where the cartilage and subchondral bone severely damaged. Around the lesion, fibrous tissue infiltrated with fibroblast-like cells and inflammatory cells covered the subchondral bone plate and infilled the marrow cavity space. It is known that, in RA, articular bone damage is due to the over activation of osteoclasts, triggered by the synovitis induced by autoimmunity. The inflammatory reaction in the synovium of affected joints up-regulate the expression of pro-inflammatory cytokines and RANKL, which can result to over-active bone resorption [9,21]. Interestingly, synovitis was defined as the major changes in the HA, results from recurrent joint bleeding in haemophilia patient. Melchiorre D *et al.* demonstrated that the synovium in HA joint expresses high levels of RANKL, whereas OPG levels decreased [14]. Our study showed that the fibroblast-like cells and inflammatory cells in the fibrous tissue around the lesion also highly expressed highly levels of osteoclast specific and inflammation related genes in articular cartilage of femoral heads from HA and RA patients, similar to previous reports [8,9,14]. In addition, although the expression of RANK was low, more mature osteoclasts were found around the damaged subchondral trabeculae compared to those far away from articular surfaces. These findings confirmed the changes in inflammatory bone resorption in subchondral bone of femoral heads from HA

and RA patients, although we did not examine the inflammation in synovium as others reported.

In this study, more severe bone destruction was found in femoral heads of HA patients compared with femoral heads derived from RA patients, presented as more volume of subchondral bone destructed. However, the RANKL/OPG ratio and cytokines in subchondral bone in femoral heads of HA patients are at similar levels compared with those in femoral heads of RA patients. We cannot exactly explain why the focal lesion in HA patients was more severe than that in RA patients. Since focal bone lesions are usually located at the loading area of the femoral heads, mechanical stress may contribute to the severity of focal bone lesions. It is known that in HA patients the cartilage destruction is followed by bleeding and the synovitis [2]. To better understand the progression of long-term joint destruction in HA patients, long-term follow-up studies of HA and RA patients may be required.

One of limitations of this study is lacking a normal subject control so we would not be able to compare changes in gene expression of HA and RA patients with normal subjects. In addition, although we only analysed the volume of the lesions in femoral heads, gender difference between HA and RA patients is another concern regarding to the comparability of these two diseases. We are now trying to recruit more samples from different types of arthritis and normal control samples with the assistance of organ donation system.

In conclusion, in this study, we found the histological evidences of the focal bone lesion associated with inflammatory osteoclastogenesis in the femoral heads in patients with HA and that was similar to the changes in the femoral heads of RA patients. We also found that bone destruction in HA patients was more severe than that in RA patients. These findings illustrate that the cystic change in the radiographic images of the hips of haemophilia patients may be due to inflammatory focal lesion and proper therapeutic interventions are required.

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Fig. 1. Gross anatomy and micro-CT analysis of femoral heads derived from haemophilic arthropathy (HA) and rheumatoid arthritis (RA) patients. Severe cartilage damaged (black arrows) in femoral heads derived from the patients with HA (a) and RA (b). Focal subchondral bone destruction (red arrows) in femoral heads of HA (c) and RA (d) patients. The percentage of lesion volume to total volume of the femoral heads from HA and RA patients (e).

Fig. 2. Histological analysis of the subchondral bone in femoral heads from haemophilic arthropathy (HA) and rheumatoid arthritis (RA) patients. Alcian blue/haematoxylin and eosin (ABH) and tartrate-resistant acid phosphatase (TRAP) staining in subchondral bone area was performed and presented. Mature osteoclasts (black arrows) eroding the subchondral bone in femoral heads of HA and RA patients were observed (a and b). There was no significant difference in osteoclast density between subchondral bone of HA and RA patients (c).

Fig. 3. Immunohistochemistry (IHC) analysis of the subchondral bone in femoral heads derived from haemophilic arthropathy (HA) and rheumatoid arthritis (RA) patients. IHC staining of subchondral bone for RANKL, OPG, RANK, TNF- α and IL-1 β (a) was performed. Quantitative analysis of the IHC data showed no significant differences in the expression of proteins analysed (b). The ratio of RANKL/OPG was similar in HA and RA groups (c). Magnification 40 $\times$ (a).