Expression of the H- and L-subunits of ferritin in bone marrow macrophages of patients with osteoarthritis

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Abstract

Osteoarthritis is a disease characterized by an increase in the production of reactive oxygen species (ROS) in afflicted joints. Excess iron, due to its role in the production of ROS and crystal deposition in the joints, is implicated in the disease progression of osteoarthritis. Ferritin is a major regulator of the bioavailability of iron, and its functions are determined largely by the combination of H- and L-subunits present in its outer protein shell. The purpose of the study was to investigate the expression of the H- and L-subunits of ferritin in bone marrow macrophages of osteoarthritis patients. The cytokine profiles were assessed as cytokines play an important role in the expression of the ferritin subunits. The H-subunit of ferritin in the bone marrow macrophages was significantly higher (*P* value = 0.035) in the osteoarthritis patients compared with the controls (107.84; 69.25–167.94 counts/μm^2; *n*= 7 versus 71.07; 58.56–86.26 counts/μm^2; *n*= 19). A marginally significant increase (*P* value = 0.059) was shown for the expression of the L-subunit in the osteoarthritis patients compared with the controls (133.03; 104.04–170.10 counts/μm^2; *n*= 7 versus 104.23; 91.53–118.70 counts/μm^2; *n*= 19). The osteoarthritis and control groups had comparable C-reactive protein, as well as proinflammatory and anti-inflammatory cytokine concentrations. The major exception was for transforming growth factor-β (TGF-β), which was higher (*P* value = 0.014) in the plasma of the osteoarthritis patients (16.69; 13.09–21.28 ng/mL; *n*= 7 versus 8.60; 6.34–11.67 ng/mL; *n*= 19). Up-regulation of the ferritin subunits decreases the levels of bioavailable iron and provides protection against the unwarranted production of ROS and crystal deposition. A role for TGF-β in the up-regulation of the expression of the H-subunit, and possibly the L-subunit, of ferritin is postulated in osteoarthritis.

Keywords KeywordsH-subunit, L-subunit, ferritin, iron, oxidative stress, transforming growth factor-β

Introduction

Iron has been implicated long in the disease progression of osteoarthritis. Osteoarthritis-like changes in the joints of hemochromatosis patients were first described in 1964, but have since been reported in up to 50% of patients.[1] Iron accumulates in the joints of individuals with osteoarthritis. The synovia of osteoarthritis patients was seen to contain deposits of iron, although to a lesser extent than in that of rheumatoid arthritis patients.[2] A recent study by

Yazar *et al.*[3] reported increased concentrations of iron in the synovial fluid of osteoarthritis patients compared not only with that of healthy individuals, but also with that of rheumatoid arthritis patients. This increase in iron was found only in the synovial fluid and not in the plasma.[3] A potential cause of increased iron may be the compromised vasculature in the afflicted joints of osteoarthritis patients that could result in microbleeding and intra-articular iron loading.[4]

Iron is implicated in crystal deposition and joint deterioration in osteoarthritis. Ferric ions have been demonstrated to induce crystallization of the supersaturated synovial fluid through the process of nucleation.[4] However, iron may have a more damaging effect through its role in the production of oxygen radicals.

Abnormal chondrocyte metabolism in the afflicted joints contributes to excessive production of reactive oxygen species (ROS) in osteoarthritis patients.[5] Oxidative stress is an important contributor to joint deterioration, and iron is known to be a major contributor to the production of ROS by means of Fenton-type chemistry.[6] However, oxidative stress in osteoarthritis not only results from increased ROS generation, but also from a decrease in antioxidant substances.[7] The major catalytic antioxidant in joint fluid, extracellular superoxide dismutase, a scavenger of superoxide, was shown to be reduced in the later stages of osteoarthritis.[8] This pro-oxidant/antioxidant imbalance in the synovial fluid of osteoarthritis patients has also been reported for blood, where total peroxidase, lipid hydroperoxide, malondialdehyde and the oxidative stress index are significantly higher, while total antioxidant capacity, total thiol, glutathione, ascorbic acid, vitamin E, as well as catalase activity, are significantly lower than in controls.[7,9] This increase in oxidative stress is suggested to result in the decrease in collagen metabolism seen in osteoarthritis. The activity of prolidase, an enzyme involved in collagen production, was shown to negatively correlate with total peroxidase and with the oxidative stress index, and to positively correlate with total antioxidant capacity.[7]

Controversial opinions exist about cytokine involvement in osteoarthritis. This is mostly due to the differences in circulating levels for cytokines. However, compelling evidence exists that subclinical inflammation is a common event, even in the absence of acute inflammatory flares[10], and that both catabolic and anabolic cytokines are involved in the progression of the disease.[11] The catabolic cytokines, interleukin-1β (Il-1β) and tumor necrosis factor-α (TNF-α), are said to be involved in primary cartilage damage, whereas the anabolic cytokine, transforming growth factor-β (TGF-β), is implicated in repair and the integrity of the cartilage.[11] The catabolic effects of Il-1β and TNF-α are exerted by enzymatic destruction through activation of metalloproteinases of cartilage matrix and by inadequate synthesis of inhibitors of the actions for Il-1β and TNF-α.[12] It is suggested that the balance between the signalling pathways of the catabolic and anabolic cytokines may be disrupted in osteoarthritis.[13]

Ferritin is a major determinant of the bioavailability of iron. Ferritin exists in different isoforms depending on the ratio of the H-subunit to L-subunit in the ferritin molecule. These ferritin subunits perform different functions in the mineralization process of iron.[14] Isoferritins are functionally distinct and it would appear that characteristic populations of isoferritins are found depending on the type of cell, the proliferation status of the cell and the presence of disease.[15] Bioavailable iron is a primary determinant for the up-regulation of ferritin, but in any condition marked by excessive production of toxic oxygen radicals, or by infectious and inflammatory processes, ferritin up-regulation is primarily stimulated by

increased reactive oxygen radical production and by cytokines.[16–18] The major function of ferritin in these conditions is to reduce the bioavailability of iron in order to stem uncontrolled cellular proliferation and excessive production of reactive oxygen radicals.[19]

The primary aim of this study was to examine the expression of the H- and L-subunits of ferritin in the bone marrow macrophages of patients with osteoarthritis.

Materials and methods

Patients

The study group consisted of seven osteoarthritis patients. The seven osteoarthritis patients were scheduled for hip replacement at the Department of Orthopaedics (Pretoria Academic Hospital, Pretoria, South Africa). Only osteoarthritis patients with no other infectious, inflammatory or chronic diseases were included in the study. Diagnosis of osteoarthritis was based on history, clinical examination and corresponding radiological features of osteoarthritis, mainly decreased joint space, subchondral sclerosis and osteophyte formation, as well as subchondral cyst formation. Most patients had advanced preoperative signs of osteoarthritis with obliteration of the total joint space. Patients had unilateral or bilateral hip osteoarthritis. None of the patients had osteoarthritis of the hands, Heberden's nodes or polyarticular disease. Nineteen patients with normal proinflammatory activity who attended the Department of Internal Medicine (Kalafong Hospital, Pretoria, South Africa) were included as a control group. With the exclusion of patients with proinflammatory or infectious conditions, controls were recruited in a consecutive manner from individuals for whom bone marrow aspiration/biopsies were indicated for clinical purposes. None of the controls had clinically overt arthritis (including osteoarthritis) and their X-ray investigations did not indicate it either. Ethical clearance for the study was obtained from the Faculty of Health Sciences Research Ethics Committee, University of Pretoria (ethical clearance numbers 118/2003 and 285/2003), and patients gave informed consent. Bone marrow and blood samples were obtained from all patients.

C-reactive protein

C-reactive protein (CRP) measurements were performed by a CRP enzyme-linked immunosorbent assay (ELISA) (DRG Diagnostics, Marburg, Germany and Orb Diagnostics, Modderfontein, South Africa).

Cytokines

Il-8, Il-1β, Il-6, Il-10, TNF-α and Il-12p70 were determined by the Human Inflammation Kit, BD™ Cytometric Bead Array (CBA; The Scientific Group, Midrand, South Africa). Il-2, Il-4, Il-5, Il-10, TNF-α and interferon-γ were determined by the BD™ Cytometric Human T-helper cell type-1/T-helper cell type-2 Cytokine Kit (The Scientific Group). With the T-helper cell type-1/T-helper cell type-2 CBA Cytokine Kit, the interferon-γ standards were lost and the measurement of interferon-γ was done by the human interferon-γ ELISA Kit, BD OptEIA™ test (The Scientific Group). TGF-β1 and granulocyte macrophage-colony stimulating factor were determined by their respective ELISAs (DRG Diagnostics and Orb Diagnostics).

Immunolabelling of the H- and L-subunits of ferritin

Fixation of bone marrow tissue and immunolabelling of the H- and L-subunits of ferritin were performed as described elsewhere.[20,21]

Statistical analysis

The osteoarthritis and control groups were compared using the Student's two-sample *t*-test. Data were logarithmically transformed due to the skewed distribution of the data. For descriptive statistics, as log-transformed data were considered, the geometric mean with a 95% confidence interval was reported. These comparisons were confirmed using the non-parametric Wilcoxon rank-sum test. Testing was done at the 0.05 level of significance.

Results

In the present study, CRP was determined as an overall indicator of the inflammatory status. The mean and SD for CRP for the osteoarthritis group was 2.2 ± 1.6 mg/L (*n*= 7), and that for the control group was 2.8 ± 2.8 mg/L (*n*= 19). Both groups were within the normal range (normal ranges for all ages and sexes: 0.1–7.5 mg/L; Chemical Pathology Laboratory, National Health Laboratory Services, University of Pretoria, Pretoria, South Africa). The results for the cytokines are presented in Table 1 and that for the expression of the ferritin subunits are presented in Table 2.

Table 1 Cytokine concentrations for osteoarthritis patients and controls

	Osteoarthritis (*n*= 7)	**Controls (*n*= 19)**	
	Geometric mean; 95% confidence interval	**Geometric mean; 95% confidence interval**	***P* value**
IFN-γ (pg/mL)	0.1; 0.1–0.1	0.18; 0.09–0.37	0.29
TNF-α (pg/mL)	2.05; 1.32–3.18	2.29; 1.88–2.78	0.56
Il-1β (pg/mL)	0.37; 0.14–1.01	0.49; 0.26–0.92	0.63
Il-6 (pg/mL)	4.37; 2.77–6.91	3.56; 2.08–6.09	0.64
Il-12 (pg/mL)	0.63; 0.36–1.10	2.73; 1.50–4.99	0.007
Il-2 (pg/mL)	4.07; 1.44–11.51	5.04; 2.45–10.37	0.73
Il-8 (pg/mL)	16.38; 11.42–23.49	14.20; 8.80–22.91	0.72
GM-CSF (pg/mL)	1.96; 0.52–7.46	2.58; 0.89–7.44	0.76
Il-4 (pg/mL)	1.24; 0.43–3.58	1.48; 0.87–2.50	0.72
Il-5 (pg/mL)	1.11; 0.23–5.37	2.23; 1.18–4.19	0.28
TGF-β (ng/mL)	16.69; 13.09–21.28	8.60; 6.34–11.67	0.014
Il-10 (pg/mL)	2.17; 0.99–4.75	4.56; 3.29–6.32	0.029

IFN, interferon; TNF, tumor necrosis factor; Il, interleukin; GM-CSF, granulocyte macrophage-colony stimulating factor; TGF, transforming growth factor

Table 2 Gold particle counts for the H- and L-subunits of ferritin, and the H-/L-subunit ratio of ferritin in osteoarthritis patients and controls

	Osteoarthritis (*n*= 7)	**Controls (*n*= 19)**	
	Geometric mean; 95% confidence interval	**Geometric mean; 95% confidence interval**	***P* value**
H-subunit (counts/μm^2)	107.84; 69.25–167.94	71.07; 58.56–86.26	0.035
L-subunit (counts/μm^2)	133.03; 104.04–170.10	104.23; 91.53–118.70	0.059
H-/L-subunit ratio	0.78; 0.56–1.08	0.68; 0.57–0.82	0.46

Patients with osteoarthritis showed a significantly higher (*P* value = 0.035) H-subunit expression in the macrophages (107.84; 69.25–167.94 counts/μm^2; *n*= 7 versus 71.07; 58.56–86.26 counts/μm^2; *n*= 19). A marginally significant higher expression of the L-subunit (*P* value = 0.059) was shown in the macrophages of the osteoarthritis patients (133.03; 104.04–170.10 counts/μm^2; *n*= 7 versus 104.23; 91.53–118.70 counts/μm^2; *n*= 19). No difference (*P* value = 0.46) was shown for the H-/L-subunit ratio of ferritin between the two groups (0.78; 0.56–1.08 versus 0.68; 0.57–0.82). The only cytokine found to be increased (*P* value = 0.014) in the group of osteoarthritis patients was TGF-β (16.69; 13.09–21.28 ng/mL; *n*= 7 versus 8.60; 6.34–11.67 ng/mL; *n*= 19).

Discussion

Iron, due to its role in Fenton-type chemistry, can increase oxygen radical production. These oxygen radicals react with proteins, lipids and nucleic acids, resulting in degradation of cellular constituents.[1,22,23] Examples of the effect of excess iron in the joints of rheumatoid arthritis patients are the significant correlations found between the amount of iron in the synovial fluid, lipid peroxidation and the inflammatory activity, as well as the observation that increased lipid peroxidation and worsening of the synovial inflammation occur with iron supplementation.[1,24–26] The cartilage destruction as a result of collagen degradation in osteoarthritis was shown to be mediated by lipid peroxidation taking place in the chondrocytes.[27]

Ferritin is a major regulatory protein in the bio-availability of iron. The results of this study showed significant up-regulation of the H-subunit of ferritin in the bone marrow macrophages of patients with osteoarthritis. In addition, a marginally higher level of L-subunits of ferritin in the bone marrow macrophages was found for the osteoarthritis patients, compared with the control group. Although various studies indicate that H-subunit-rich ferritins are more suitable for protecting against oxidative damage, both H- and L-subunit-rich ferritins have been shown to reduce the accumulation of ROS.[28] However, H-subunit-rich ferritins accumulate and release iron faster than L-subunit-rich ferritins,[14,15,29,30] and it is suggested that the H-subunit-rich ferritins permit more dynamic intracellular traffic of iron,[15,31] while L-subunit-rich ferritins can accumulate more iron,[15,32] and retain iron more firmly than their H-subunit-rich counterparts.[33] It is thus feasible to accept that the increase in the H- and L-subunits of ferritin in osteoarthritis would be to sequester bioavailable iron and to protect against the unwarranted production of ROS. In addition, sequestration of free iron may alleviate the crystal deposition involved in the joint deterioration of osteoarthritis.

Various factors are responsible for the regulation of the expression of the H- and L-subunits of ferritin, including iron, ROS and cytokines. Metabolically available iron is a major determinant of the regulation of the expression of the H- and L-subunits of ferritin. The 5′-untranslated region (5′-UTR) of both the H- and L-subunit mRNA contains iron-responsive elements (IREs) sensitive to the metabolically active available iron.[30] These IREs are recognized by trans-acting cytosolic RNA-binding proteins required for the co-ordinated expression of the H- and L-subunits.[34] These cytosolic RNA-binding proteins, iron-responsive protein 1 (IRP1) and iron-responsive protein 2 (IRP2), cause a decrease in H- and L-subunit mRNA translation by binding to the IREs of the 5′-UTR of the respective mRNAs when iron concentrations are low.[17] When iron concentrations are high, the IRPs are bound to iron, resulting in diminished IRPs to bind IREs. Transcription of the H- and L-subunit genes can now take place. Although both the H- and L-subunit mRNA of ferritin contain an IRE, the IREs are regulated differentially. The IRE of the L-subunit mRNA was shown to be the primary responder to iron. ROS up-regulate the expression of the ferritin subunits to protect against oxidative stress. The genes for the H- and L-subunits of ferritin both contain an antioxidant responsive element (ARE) upstream to the transcription initiation site. These AREs are sensitive to the presence of oxidative stress, and when activated, can induce transcription of the H- and L-subunits of ferritin. The AREs of the H- and L-subunits are at different positions upstream from the transcription initiation site and are regulated differentially.[16,35,36] Oxidants can, furthermore, up-regulate the expression of ferritin through their influence on IRP1,[18,28,37] and by releasing iron from cellular proteins.[38] In addition to the regulation of the expression of the ferritin subunits by iron and ROS, cytokines can also influence the expression of these subunits. Cytokines modulate ferritin expression by both transcriptional and translational mechanisms,[39] but largely by an increase in the rate of transcription of the ferritin genes.[40,41] Furthermore, cytokines differentially regulate the expression of the ferritin subunits, and it is mostly the H-subunit of ferritin that is increased by cytokine induction at variance with the L-subunit.[40–44]

As the expression of the ferritin subunits are also influenced by proinflammatory cytokines,[21] the proinflammatory status of the patients was investigated by measuring CRP, and proinflammatory and anti-inflammatory cytokines in the plasma. CRP concentrations were not significantly different between the osteoarthritis and the control group and both were within normal limits for plasma CRP. In addition, none of the proinflammatory or anti-inflammatory cytokines were increased in the osteoarthritis patients, except for one anti-inflammatory cytokine. The cytokine results of this study are thus in agreement with those studies on osteoarthritis patients[45] where neither the plasma concentrations of the proinflammatory markers, nor the plasma cytokine concentrations, indicated increased proinflammatory activity. Although this does not exclude proinflammatory activity in the joints, it does exclude the presence of a systemic proinflammatory condition. The one cytokine found to be increased in the osteoarthritis patients was TGF-β.

The TGF-β family consists of over 35 members and includes, besides TGF-βs, activins and bone morphogenetic proteins (BMPs). They are known to be involved in the regulation of cell proliferation, differentiation, apoptosis and migration, in the control of extracellular matrix synthesis and degradation of various tissues.[46] Furthermore, they mediate cell and tissue responses to injury and modulate immune functions.[46] TGF-β plays an anabolic role in cartilage formation and it has been shown that TGF-β supplementation enhances cartilage repair.[46] In its anabolic role, TGF-β stimulates the synthesis of proteoglycans and other cartilage matrix components in osteoarthritis patients.[11] An increase in the expression of TGF-β has been reported early in osteoarthritis with an increase in extracellular matrix

production,[46] and TGF-β is said to counteract the effects of the catabolic cytokines in the later stages of disease progression. Although it has on occasion been used as a therapeutic tool, application of TGF-β causes other problems in tissues of the joints and is said to contribute to fibrosis and osteophyte formation.[46]

It is generally accepted that proinflammatory cytokines contribute to the up-regulation of the expression of the H-subunit of ferritin,[21] but very little is known about an association between ferritin subunits and TGF-β. In the present study, the anti-inflammatory cytokine, TGF-β, was significantly increased without any overt indication of proinflammatory activity. A possibility that TGF-β could have contributed to the increased H-subunit expression found in the osteoarthritis patients is inferred from results of a study on malignant H-ras transformed cells, where it was shown that TGF-β selectively increased the expression of the H-subunits of ferritin.[47] In addition, BMP-2, another member of the TGF-β family, has been shown to be involved in decreasing serum iron. BMP-2 increases hepcidin expression, resulting in a decrease in serum iron concentrations *in vivo*.[48] This increased expression of hepcidin has been shown to occur by the activation of the TGF-β/SMAD4 signalling pathway.[49] Hepcidin binds to ferroportin, the major transmembrane protein in macrophages responsible for the release of iron from the cell. Upon hepcidin binding, the ferroportin is internalized, resulting in accumulation of macrophage iron. The increase in the labile iron pool causes up-regulation of the H- and L-subunits of ferritin to sequester this iron.[34]

As this was a preliminary study, there are a number of weaknesses. One limitation of the study is the limited number of osteoarthritis subjects and the fact that controls and osteoarthritis patients were not matched with regard to age, gender and ethnicity. In addition, it would, in further studies, be of interest to collect and analyze synovial fluids. As this is the first study indicating an increase in the expression of the H-subunit of ferritin in the bone marrow macrophages in osteoarthritis, the results warrant further investigations.

Conclusions

The H- and the L-subunits of ferritin are up-regulated in the bone marrow macrophage of osteoarthritis patients. This increase in the H- and the L-subunits is involved in the sequestration of bioavailable iron, and thus in protection against the generation of ROS and probably against crystal deposition. It is suggested that TGF-β could have a possible role in the up-regulation of the H- and L-subunits of ferritin.

Author contributions: AMK conceived of the study, participated in the intellectual planning, carried out the immunolocalization of the H- and L-subunits and cytokine determinations, and participated in the intellectual analysis of the data and writing of the manuscript. PFL carried out the recruitment of patients, and participated in the intellectual analysis of the data and writing of the manuscript. ANH carried out sectioning of all embedded tissue and CFvdM carried out preparation of all micrographs. PJB performed the statistical analysis of the data. DJMF carried out the recruitment of osteoarthritis patients. MV participated in the intellectual planning of the study, intellectual analysis of the data and writing of the manuscript.

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