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Iron overload inhibits calcification and differentiation of ATDC5 cells

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There is a little information about the effects of iron overload on cartilage metabolism. In the present study, we examined the effects of excess iron on the differentiation and mineralization of cultured chondrocytes, ATDC5 cells. We used ferric ammonium citrate (FAC) as a ferric ion donor and desferrioxamine (DFO) as a ferric ion chelator. Neither chemical affected the production of proteoglycan, a marker of an early stage of ATDC5 differentiation. In contrast, FAC inhibited the deposition of calcium, a late-stage event in chondrocyte differentiation, by ATDC5 cells in a dose-dependent manner, and DFO accelerated it. Energy dispersive X-ray spectroscopy/scanning electron microscope analysis revealed that the levels of iron and calcium in cells treated with FAC were increased and decreased, respectively. Furthermore, FAC inhibited the expression of matrix metalloproteinase 13 mRNA, another marker of late-stage chondrocyte differentiation. In addition, we found that the heavy and light chains of ferritin were expressed specifically at a late stage of ATDC5 differentiation, and the levels of both proteins were enhanced by the addition of iron. These results suggest that iron overload might give rise to osteopenia and arthritis by inhibiting chondrocyte differentiation and mineralization.

Keywords: ATDC5 cells/calcification/differentiation/ferritin/iron.

Abbreviations: DFO, desferrioxamine; DMEM, Dulbecco's modified eagle medium; FAC, ferric ammonium citrate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H-ferritin, heavy chain of ferritin; L-ferritin, light chain of ferritin; α-MEM, α-minimum essential medium; MMP13, matrix metalloproteinase 13; NBT/BCIP, Nitro-blue tetrazolium chloride/5-bromo-4-chloro-3′-indolylphosphate *p*-toluidine salt; RT–PCR, reverse transcriptase–polymerase chain reaction; TBS, Tris-buffered saline.

The total iron pool of the body is composed of the iron in the red cell mass (2.5 g), tissue iron (1 g) and a small amount of iron circulating in the plasma (4 mg). The iron stores in tissues (predominantly the liver, spleen and bone marrow) are largely in the form of ferritin, a complex of ferric ion and the apoferritin protein; ferritin is a large multisubunit protein with eight Fe transport pores (*1*). Ferritin plays an important role in the control of iron concentration in cells. Iron deficiency anaemia is the most common nutritional deficiency worldwide (*2*, *3*). However, when the body absorbs too much iron, haemochromatosis occurs (*4*, *5*); this disease is caused by the gradual build up of excess iron in tissues and organs. The organs most susceptible to haemochromatosis include the liver, adrenal glands, heart and pancreas; haemochromatosis patients can present with cirrhosis, adrenal insufficiency, heart failure or diabetes (*4*, *5*). Osteoporosis and osteopenia have also been reported in patients with haemochromatosis (*6*, *7*). We recently reported that excess iron inhibited the proliferation, differentiation and mineralization of osteoblasts such as MC3T3-E1 cells and rat calvarial osteoblast-like (ROB) cells (*8*). Furthermore, arthritis has been found in patients with haemochromatosis (*4*). However, the effects of iron overload on chondrocyte metabolism are not fully understood.

The formation of bone occurs via two different processes, endochondral and membranous ossification. Longitudinal bone growth occurs via endochondral ossification in the cartilaginous growth plate. Endochondral ossification is a major mode of bone formation that occurs as chondrocytes undergo proliferation and condensation (early phase differentiation), hypertrophy (late phase differentiation), cell death, and osteoblastic replacement. The early-phase differentiation is characterized by inductive expression of the type II and IX collagen genes as well as the aggrecan gene (*9*, *10*). The late-phase differentiation is characterized by the expression of the type X collagen (*11*) and matrix metalloproteinase 13 (MMP13) (*12*, *13*) genes. Here, we used ATDC5, a clonal cell line that undergoes efficient chondrogenic differentiation, including the cellular condensation and calcification stages (*14*). ATDC5 cells were isolated from the feeder-independent teratocarcinoma stem cell line AT805 on the basis of chondrogenic potential in the presence of insulin (*15*), and they reproducibly undergo the early-phase differentiation of chondrocytes to form numerous cartilage nodules in the presence of insulin (*16*). Furthermore, under the appropriate culture conditions and with time, hypertrophic ATDC5

cells initiate calcium deposition in preformed cartilage matrix (*14*).

The objective of the present study was to elucidate the effects of iron overload on chondrocyte differentiation and calcification in ATDC5 cells.

Experimental procedures

Regents

Ferric ammonium citrate (FAC) and desferrioxamine (DFO) were purchased from Nacalai tesque Inc. (Kyoto, Japan) and Sigma Chemical Co. (St Louis, MO), respectively. Dulbecco's modified eagle medium (DMEM), Ham's F12, α-Minimum essential medium (α-MEM), and the penicillin/streptomycin antibiotic mixture were obtained from Life Technologies, Inc. (Grand Island, NY, USA). Fetal bovine serum was purchased from Moregate BioTech (Bulimba, Australia).

Culture of ATDC5 cells

ATDC5 cells were supplied by the RIKEN Cell Bank (Tsukuba, Japan). Cells were maintained in 55-cm^2 dishes in DMEM/Ham's F12 in a 1:1 ratio (v/v) supplemented with 5% fetal bovine serum, 10 μg/ml human transferrin (Roche Diagnostics K.K., Tokyo Japan), 3×10^{-8} M sodium selenite (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin, in a humidified atmosphere of 5% CO_2 in air at 37°C (*14*). After cells had reached 70% confluence, cells were detached by treatment with 0.05% trypsin solution, replated in 6-well plates (the area of each well was 9.4 cm^2) at a density of 6.4×10^3 cells/cm^2, and grown in DMEM/Ham's F12 in a 1:1 ratio (v/v) supplemented with 5% fetal bovine serum, 10 μg/ml bovine insulin (Sigma), 10 μg/ml human transferrin, 3×10^{-8} M sodium selenite (Sigma), 100 units/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere of 3% CO_2 in air at 37°C, for Day 21. After Day 21, cells were cultured in α-MEM supplemented with 5% fetal bovine serum, 10 μg/ml bovine insulin, 10 μg/ml human transferrin, 3×10^{-8} M sodium selenite, 100 units/ml penicillin, 100 μg/ml streptomycin and FAC (at 10^{-6}, 10^{-7} or 10^{-8} g/ml), DFO (at 10^{-5}, 10^{-6} or 10^{-7} M) or vehicle, in a humidified atmosphere of 3% CO_2 in air at 37°C. Fresh medium and compounds were supplied to cells at 2-day intervals.

Alcian Blue staining and Alizarin Red staining of cells

ATDC5 cells that had been cultured for 14–21 or 21–28 days in 6-well plates (9.4 cm^2/well) were washed twice with PBS and fixed with 95% methanol for 30 min. The fixed cells were stained with 0.1% Alcian Blue 8GX (Fluka, Milano, Italy)/0.1 M HCl overnight and washed twice with H_2O.

ATDC5 cells that had been cultured with FAC or DFO for 28–35 or 28–45 days in 6-well plates were washed twice with PBS and fixed with 95% ethanol for 30 min. The fixed cells were stained with 1% Alizarin Red S (Sigma) for 5 min and washed twice with H_2O.

Quantification of calcium deposition

ATDC5 cells were subcultured into 6-well plates (9.4 cm^2/well) for 28 days. The cells were then incubated with various concentrations of FAC or DFO for another 2 weeks (from Day 28 to Day 42). The amount of calcium, deposited as hydroxyapatite in the cell layer, was measured as follows. The layers of cells in 6-well plates were washed twice with PBS and incubated overnight with 1 ml of 2 N HCl with gentle shaking. The Ca^{2+} ions in the samples were quantified using the *o*-cresolphthalein complexone method with a Calcium C kit (Wako Pure Chemical Industries) according to the manufacturer's instructions. This kit is specific for Ca^{2+} ions and has a limit of detection of 1 μg/ml. We used the solution of Ca^{2+} ions (20 mg/dl) in the kit as the standard.

Energy dispersive X-ray spectroscopy/scanning electron microscope analysis (17)

ATDC5 cells in 6-well plates (9.4 cm^2/well) were cultured for 28 days. The cells were then incubated with 10^{-6} g/ml FAC for another 1 week (from Day 28 to Day 35). The cells were washed twice with PBS and fixed with 95% ethanol. The samples were coated with gold (400 A thick) by Quick auto coater (JFC-1500; JEOL Co., Tokyo, Japan). After observation with a scanning electron microscope (JSM-5500LV, JEOL Co.), iron and calcium were detected with Energy dispersive X-ray spectroscopy (JED-2200; JEOL).

Reverse transcriptase–polymerase chain reaction

We detected mRNA for the heavy chain of mouse ferritin (H-ferritin), the light chain of mouse ferritin (L-ferritin), mouse type II collagen, mouse type X collagen and mouse MMP13 in ATDC5 cells by reverse transcriptase–polymerase chain reaction (RT–PCR). The RNeasy Mini Kit (Qiagen K.K., Tokyo, Japan) was used to extract RNA from ATDC5 cells that had been exposed to FAC or DFO. Total RNA (3 μg) was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany) with random primers in a 20-μl reaction mixture according to the manufacturer's protocols. The resulting cDNA was amplified in 20 μl of Taq DNA polymerase mixture (Takara, Tokyo, Japan) that contained 1 μM sense primer, 5′-CATGCCGAGAAA CTGATGAA-3′, and antisense primer, 5′-TCTTGCGTAAGTTG GTCACG-3′, for mouse H-ferritin (accession number NM 010239; 280 bp); 1 μM sense primer, 5′-AATGGGGTAAAACCCAGG AG-3′ and antisense primer, 5′-GATGGTTGCCCATCTTCT TG-3′, for mouse L-ferritin (accession number NM 010240; 180 bp); 1 μM sense primer, 5′-CACACTGGTAAGTGGGGCAA GACCG-3′, and antisense primer, 5′-GGATTGTGTTGTTTCAG GGTTCGGG-3′, for mouse type II collagen (accession number BC051383; 210 bp); 1 μM sense primer, 5′-TGGGTAGGCCTGTA TAAAGAACG-3′ and antisense primer, 5′-CATGGGAGCCACTA GGAATCCTGAGA-3′, for mouse type X collagen (accession number NM 009925; 173 bp); 1 μM sense primer, 5′-TTGTTGAG TTGGACTCACTGT-3′ and antisense primer, 5′-GCGTGTGCCA GAAGACCA-3′, for mouse MMP13 (accession number NM 008607; 536 bp); or 1 μM sense primer, 5′-ATTGTTGCCATCAAC GACC-3′, and antisense primer, 5′-CATCGACTGTGGTCATGA GC-3′, for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (accession number M32599; 451 bp). Amplification cycles for L- and H-ferritin were repeated 25 times and consisted of incubation at 94°C for 30 s, 60°C for 30 s and 72°C for 45 s. For amplification of type II collagen, type X collagen, MMP13 and GAPDH, cycles consisting of incubation at 94°C for 30 s, 58°C for 30 s and 74°C for 45 s were repeated 30 times. The PCR products were subjected to electrophoresis on a 2% agarose gel and visualized by staining with ethidium bromide. DNA marker fragments (Molecular weight marker V; Boehringer Mannheim, Mannheim, Germany) were used as size markers.

2-D Electrophoresis analysis

Protein samples (1 mg/350 μl solution; 7 M urea, 2 M thiourea, 4% CHAPS and Bromophenol Blue) were prepared from ATDC5 cells that were subcultured for 12, 24 or 36 days with or without FAC or DFO. The protein samples were subjected to isoelectric focusing (IPGphor; Amersham Pharmacia Biotech, Uppsala, Sweden) using Immobiline DryStrip (pH4-7, 18 cm; Amersham Biosciences, Tokyo, Japan). Immobilized pH gradient strips were subjected to sodium dodecyl sulphate–polyacrylamide (12.5%) gel electrophoresis. The wet gels were stained with Coomassie brilliant blue R (KANTO Chemical Co. Inc., Tokyo, Japan) and were scanned, and the gel images were processed using the ImageMaster software (Amersham Pharmacia Biotech). The protein spots were excised from the gel, and the proteins were digested with a minimum amount of trypsin solution. The proteolytic peptide mixture was subjected to LC-MS/MS analysis. The peptide mass fingerprint was submitted to the MASCOT program (Matrix Science, Inc., Boston, MS, USA) for protein identification.

Western blotting analysis

Protein solution (20 μg) extracted from ATDC5 cells was subjected to sodium dodecyl sulphate–polyacrylamide (15%) gel electrophoresis and was then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Tokyo, Japan) with 25 V and 100 mA for 1 h. After the membrane was rinsed in TBS Tween Buffer (20 mM Tris/HCl, 0.5 M NaCl and 0.05% Tween 20, pH 7.2) containing 5% skim milk at room temperature for 1 h, the membrane was incubated with anti-ferritin light chain polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-ferritin heavy chain polyclonal antibody (Santa Cruz Biotechnology) at room temperature for 1–3 h. The membrane was washed twice with TBS Tween Buffer and was labelled with anti-goat IgG (H+L)

monoclonal antibody (Vector laboratories, Burlingam, CA, USA) as a secondary antibody. The membrane was washed three times with TBS Tween Buffer and was incubated with mixture of 20 ml of alkaline phosphatase solution (100 mM Tris/HCl, 100 mM NaCl, 5 mM $MgCl_2/6H_2O$, pH 9.5) and 350 µl NBT/BCIP (Nitro-blue tetrazolium chloride/5-bromo-4-chloro-3′-indolylphosphate *p*-toluidine salt) solution (Roche) 350 µl at room temperature for 5–15 min.

Statistical analysis

Numerical data were expressed as means ± SD of results from three or four individual cultures and the significance of differences were determined by analysis of variance (ANOVA) Dunnett's test. $P < 0.05$ were considered significant. Experiments were repeated independently in triplicate, and the results were qualitatively identical in every case. Results from representative experiments are shown.

Results

We evaluated the deposition of calcium by ATDC5 cells treated with excess iron and a chelator of iron. We used FAC as a ferric ion donor and DFO as a ferric ion chelator. As shown in Figure 1A, treatment with 1 µg/ml FAC decreased the calcification by ATDC5 cells at Day 35 and Day 42. In contrast, DFO accelerated the calcification by ATDC5 cells (Fig. 1B). Figure 2 shows that FAC inhibited the deposition of calcium and DFO accelerated it and that the effects of both treatments were dose dependent. The exposure of ATDC5 cells to 1 µg/ml FAC decreased the deposition of calcium by ~50% at 42 days relative to treatment with vehicle alone. In contrast, 10 µM DFO enhanced the deposition of calcium by 2-fold at 42 days relative to treatment with vehicle alone. However, FAC did not affect the production of proteoglycan, a marker for the early stages of chondrocytes differentiation in ATDC5 cells (Fig. 1C). As shown in Figure 3, energy dispersive X-ray spectroscopy/scanning electron microscope analysis revealed that the levels of iron and calcium in cells treated with 10^{-6} g/ml FAC for 1 week were increased and decreased, respectively.

To assess the effect of iron ion on differentiation and mineralization of ATDC5 cells, we added FAC or DFO to the culture medium of cells and assayed expression of type X collagen and MMP 13 mRNAs.

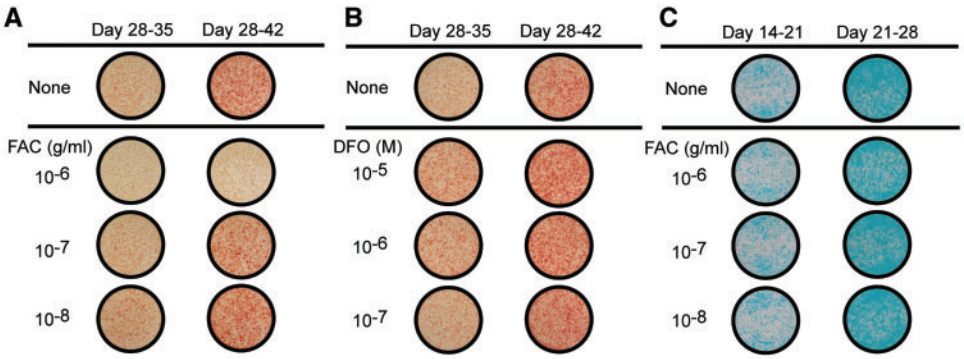

Fig. 1 Effects of iron on mineralization and proteoglycan production in cultures of differentiated ATDC5 cells. Cells were grown in 6-well plates (9.4 cm^2/well). The cells were subcultured with 10^{-6}, 10^{-7} or 10^{-8} g/ml FAC (A), 10^{-5}, 10^{-6} or 10^{-7} M DFO (B), or vehicle (*None*) for Day 28 to Day 35 or Day 28 to Day 42 for staining with Alizarin red. The cells were subcultured with 10^{-6}, 10^{-7} or 10^{-8} g/ml FAC (C) or vehicle (*None*) for Day 14 to Day 21 or Day 21 to Day 28 for staining with Alcian blue.

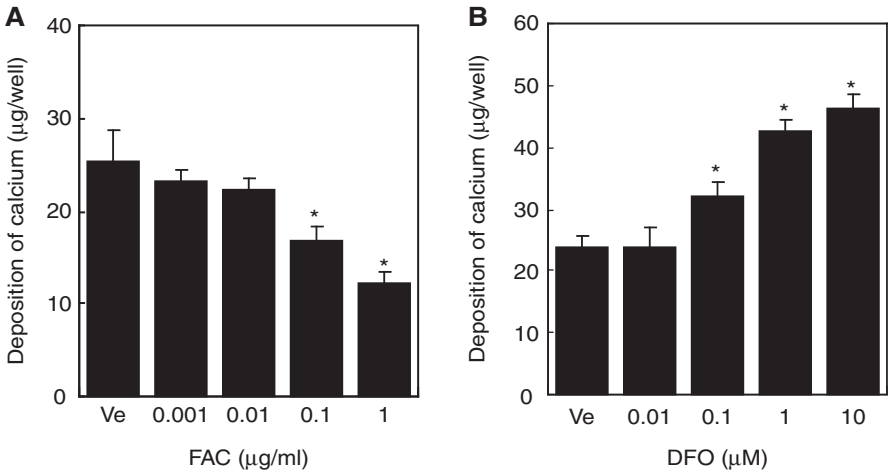

Fig. 2 Dose-dependent effects of FAC and DFO on the deposition of calcium by differentiated ATDC5 cells. ATDC5 cells in 6-well plates (9.4 cm^2/well) were cultured for 28 days. The cells were then incubated with various concentrations of FAC (A), DFO (B) or vehicle (*Ve*) for another 2 weeks (from Day 28 to Day 42). The deposition of Ca^{2+} was measured on Day 42. Quantitative analysis of Ca^{2+} was performed as described in 'Experimental procedures' section. Data are means ± SE of results from three determinations. Data are representative of results from three separate experiments. *$P < 0.05$ versus vehicle.

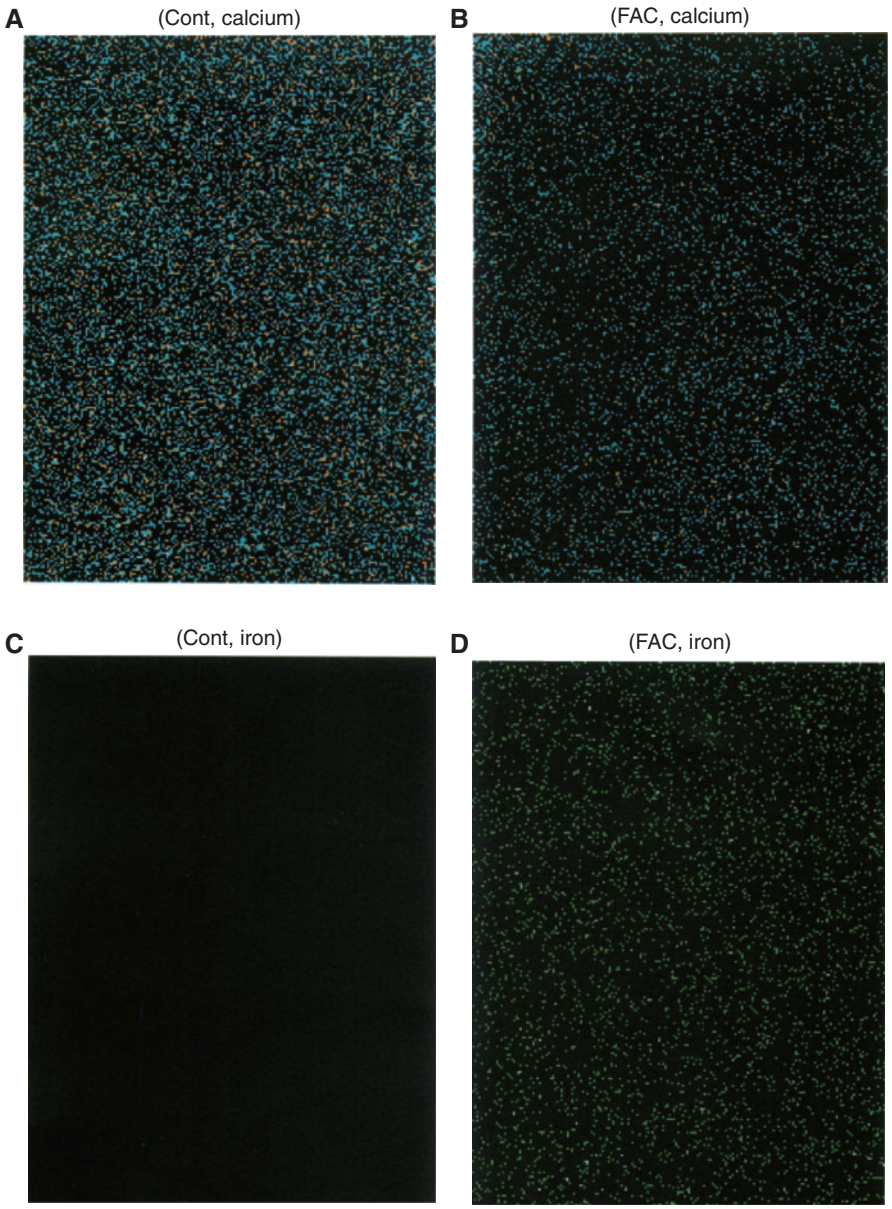

Fig. 3 Energy dispersive X-ray spectroscopy/scanning electron microscope analysis of ATDC5 cells treated with FAC. ATDC5 cells in 6-well plates (9.4 cm2/well) were cultured for 28 days. The cells were then incubated with 10^{-6} g/ml FAC (C and D) or vehicle (Cont; A and B) for another 1 week (from Day 28 to Day 35). Calcium (A and B) and iron (C and D) were detected. An analysis of iron and calcium was performed as described in 'Experimental procedures' section.

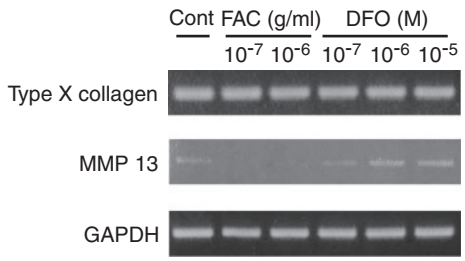

Fig. 4 Expression of Type X collagen and MMP13 mRNAs in ATDC5 cells treated with FAC or DFO. ATDC5 cells in 6-well plates (9.4 cm^2/well) were cultured for 28 days. The cells were then incubated with various concentrations of FAC, DFO or vehicle (*Cont*) for another 2 weeks (from Day 28 to Day 42). Total RNA was isolated from ATDC5 cells treated with FAC or DFO and levels of mRNAs for mouse type X collagen (173 bp) and mouse MMP13 (536 bp) were examined by RT-PCR with specific primers (see text for details). Amplification of mouse GAPDH mRNA (451 bp) was used as an internal control.

Type X collagen and MMP 13 are markers for a late stage of chondrocyte differentiation, and as shown in Figure 4, cells cultured with FAC or DFO did not affect expression of type X collagen mRNA. However, FAC inhibited expression of MMP 13 mRNA and DFO enhanced it in a dose-dependent fashion.

We used 2D-electrophoresis to identify proteins that were induced or attenuated at specific time points during ATDC5 cell differentiation in culture. We found that H- and L-ferritin were expressed at a late stage of differentiation of ATDC5 cells, and the levels of both proteins were enhanced by the addition of iron (Figs 5A, 5B and 5C). Figure 5A shows the expression patterns of mRNAs for two known marker proteins, type X collagen and MMP 13 in ATDC5 cells. H- and L-ferritin mRNAs were also up-regulated on Day 21.

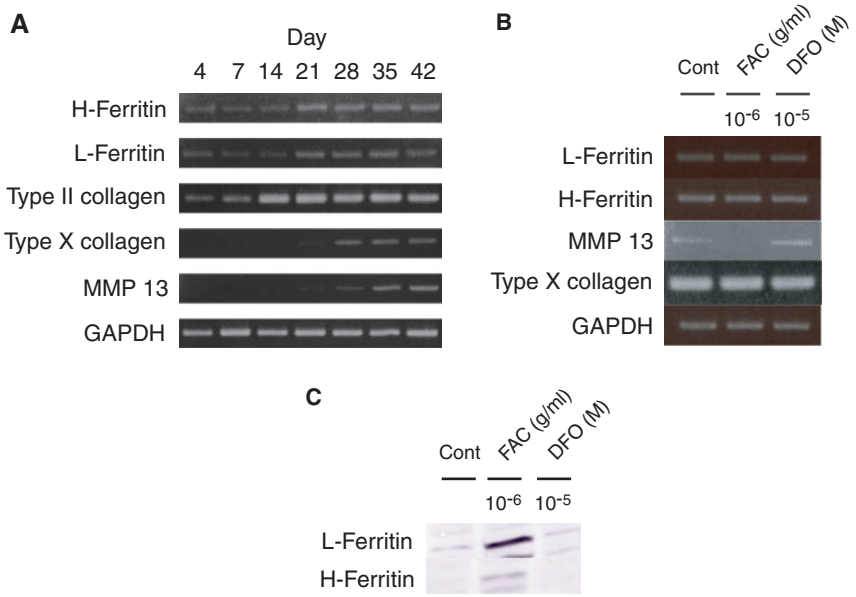

Fig. 5 Expression of ferritin mRNAs and proteins in ATDC5 cells. (A) Expression patterns of ferritin mRNAs during culture period of untreated ATDC5 cells. Total RNA was isolated from ATDC5 cells that had been subcultured for 4, 7, 14, 21, 28, 35 or 42 days, and the levels of mRNAs for mouse H-ferritin (280 bp), mouse L-ferritin (180 bp), mouse type II collagen (210 bp), mouse type X collagen (173 bp) or mouse MMP13 (536 bp) were examined by RT-PCR with specific primers (see text for details). Amplification of mouse GAPDH mRNA (451 bp) was used as an internal control. (B) Expression of marker mRNAs in treated and untreated ATDC5 cells. ATDC5 cells in 6-well plates (9.4 cm^2/well) were cultured for 28 days. The cells were then incubated with 10^{-6} g/ml FAC or 10^{-5} M DFO for another week (from Day 28 to Day 35). Levels of mRNAs for mouse L-ferritin (180 bp), mouse H-ferritin (280 bp), mouse MMP13 (536 bp) and mouse type X collagen (173 bp) were examined by RT-PCR with specific primers. Amplification of mouse GAPDH mRNA (451 bp) was used as an internal control. (C) ATDC5 cells in 6-well plates (9.4 cm^2/well) were cultured for 28 days. The cells were then incubated with 10^{-6} g/ml FAC or 10^{-5} M DFO for another week (from Day 28 to Day 35). L- and H-ferritin were detected by Western blot in treated and untreated ATDC5 cells.

While neither FAC nor DFO affected expression of H-ferritin or L-ferritin mRNAs (Fig. 5B), FAC did enhance the expression of H- and L-ferritin proteins (Fig. 5C).

Discussion

The majority of this study focused on the effects of iron on the metabolism and differentiation of cultured chondrocytes, ATDC5 cells. Haemochromatosis is an iron overload disorder associated with osteopenia and arthritis. However, there is a little information about the effects of iron on the cells, such as chondrocytes and osteoblasts, of the hard tissues that are affected by these diseases. Recently, we reported that excess iron ions inhibited cell proliferation and differentiation (as evidenced by type I collagen production, alkaline phosphatase activity and the deposition of calcium) of well-characterized mouse calvarial preosteoblastic cells (MC3T3-E1 cells) and ROB cells (*8*). Furthermore, it had been reported that lactoferrin inhibited the hypertrophic differentiation of ATDC5 cells (*18*). In the present study, we found that FAC, an iron ion donor, inhibited the later stages of chondrocyte differentiation and that DFO, a chelator of ferric ion, accelerated chondrocyte differentiation in the later stages. Furthermore, we found that H- and L-ferritin were expressed at a late stage of chondrocyte differentiation and that iron overload accelerated the production of ferritin proteins. Taken together, our results suggest that iron overload down-regulates metabolisms of osteoblasts and chondrocytes and might inhibit the formation of hard tissues.

When ATDC5 cells were treated with 10^{-6} g/ml FAC for 1 week (Day 28 to Day 35), the level of iron increased in the cells (Figs 3C and 3D). In contrast, iron overload decreased in the level of calcium in the cells, although its action mechanism is unclear yet. These results indicated that iron overload participated in calcium mobility in the cells.

Changes in iron ion availability did not affect the production of proteoglycan, a marker of the early stages of chondrocyte differentiation, but they did inhibited indices of late-stage differentiation, such as mineralization (Figs 1 and 2) and MMP 13 mRNA expression (Fig. 4) by ATDC5 cells. Thus, iron overload specifically inhibited late-stage chondrocyte differentiation. It has been well known that MMP 13 and type X collagen expression were restricted to chondrocytes of the lower zone of hypertrophic cartilage (*12*). Interestingly, iron overload inhibited MMP 13 mRNA expression but did not affect type X collagen mRNA expression (Fig. 4). These mean that iron do not involve in transcription of type X collagen gene.

We found that H- and L-ferritin mRNAs were expressed in ATDC5 cells at a late stage of differentiation (Fig. 5A). Ferritin might be a new index of late-stage differentiation of ATDC5 cells. Ferritin has an ability to store 2,000–5,000 atoms of iron, thereby protecting the cells from cytotoxicity by hydroxyradical produced from the fenton reaction. It has been known that translation of ferritin mRNA is accelerated by iron overload (*19*) and this process is

controlled with the IRP (iron regulatory protein)/IRE (iron responsive element) system (*20*, *21*). In our study, it was also confirmed that the production of ferritin protein, but not the expression of ferritin mRNA, was enhanced in chondrocytes exposed to excess iron (Fig. 5B and C). Chondrocytes might circumvent the stress of excess iron ion by up-regulating ferritin protein production.

In the present study, we revealed that the calcification of chondrocytes in culture, ATDC5 cells, was affected by exposure to excess iron ions. These results might support the hypothesis that iron overload results in osteopenia and arthritis by inhibiting chondrocyte differentiation and mineralization.

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Conflict of interest

None declared.

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