

Down regulation by iron of prostaglandin E₂ production by human synovial fibroblasts

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Abstract

Objective—To examine the effect of iron on the prostaglandin (PG) E₂ production by human synovial fibroblasts in vitro.

Methods—Human synovial fibroblasts were isolated from synovial tissue of rheumatoid arthritis (RA) and osteoarthritis (OA) patients and cultured in medium. Synovial fibroblasts were stimulated by human recombinant interleukin (IL) 1 β (0.1–10 ng/ml) with or without ferric citrate (Fe-citrate, 0.01–1 mM). The amount of PGE₂ in the culture medium was measured by an enzyme linked immunosorbent assay.

Results—The production of PGE₂ by the synovial fibroblasts was increased by stimulation with IL1 β at all concentrations tested. Fe-citrate but not sodium citrate (Na-citrate) down regulated the production of PGE₂ by the synovial fibroblasts, both with and without stimulation by IL1 β . Fe-citrate inhibited the spontaneous PGE₂ production by the cells in a dose dependent manner, and a maximum inhibition by Fe-citrate was observed at the concentration of 0.1 mM with IL1 β stimulation. The down regulation by iron was reversed by the co-addition of desferrioxamine (100 μ g/ml), an iron chelator.

Conclusion—Iron down regulates the PGE₂ production by synovial fibroblasts in vitro. (*Ann Rheum Dis* 1998;57:742–746)

Since Muirden and Senator reported the deposition of iron in synovial tissue in rheumatoid arthritis (RA) patients,¹ the roles of iron in joint inflammation have been discussed extensively.² For example, iron catalyses oxidative radical reactions (Haber-Weiss reaction), which lead to the formation of the most toxic hydroxyl radical and subsequent lipid peroxidation and tissue damage.² Iron also changes the lymphocyte migration to the joint and modulates T cell surface molecule expression,³ macrophage phagocytic function,⁴ and natural killer cell activity.⁵ Synovial cell proliferation is a major pathological finding in RA synovia.⁶ We previously reported that iron by itself stimulated DNA synthesis by human synovial cells and did so synergistically with cytokines such as interleukin (IL)1 β , IL7, and tumour necrosis factor (TNF) α in vitro.⁷

Prostaglandin E₂ (PGE₂) is widely considered to have an important role in the inflammatory process in rheumatoid joints. PGE₂ has been demonstrated to be an important mediator of vascular permeability, hyperalgesia, and IL6 production in vivo, with

the use of a neutralising anti-PGE₂ monoclonal antibody.⁸ Furthermore, the effect of PGE₂ on the development of oedema and pain is shown by the marked therapeutic effect of non-steroidal anti-inflammatory drugs (NSAIDs), inhibitors of the production of prostaglandin metabolites.⁸ PGE₂ is also known to have a suppressive effect (as an internal regulator) on fibroblast proliferation in human dermal fibroblasts⁹ and IL1 production in murine peritoneal macrophages.¹⁰

In this study, we examined the effect of iron on the PGE₂ production by human synovial fibroblasts in vitro, and we found that iron may stimulate synovial fibroblast proliferation by down regulating PGE₂ production.

Methods

CULTURE OF SYNOVIAL FIBROBLASTS

Synovial tissues were obtained from 16 patients, eight with RA and eight with osteoarthritis (OA) who underwent total knee replacement surgery. All of the RA patients were women (age range 46 to 74, average: 63.4 years) who were at stage IV and class III. The eight patients with OA consisted of two men and six women, age range 66 to 77, average: 70.9 years. The synovial fibroblasts were isolated according to the method previously described.⁷ Immediately after surgery, the patients' synovial tissues were placed in plastic Petri dishes (Falcon No3003AJ, 100 \times 20 mm, Becton Dickinson Co, Lincoln Park, NY, USA) containing Hanks's balanced salt solution (HBSS, GIBCO Labs, Life Technologies Inc, Grand Island, NY, USA) without Ca⁺⁺ and Mg⁺⁺, and minced with scissors. The tissues in HBSS were incubated with collagenase (200 u/ml, Worthington Biomedical Co, Freehold, NJ, USA) and DNase (25 μ g/ml, Sigma Chemical Company, St Louis, MO, USA) with stirring for one hour at 37°C. The tissue solution was then passed through a layer of iron mesh. The cells were washed three times in Dulbecco's modified Eagle medium (DMEM, GIBCO). The cells (1 \times 10⁶/ml) were then resuspended in DMEM, which was supplemented with penicillin G (100 IU/ml), streptomycin (100 μ g/ml), amphotericin (2 μ g/ml, Squibb Co, Tokyo, Japan), and 10% fetal bovine serum (FBS, heat inactivated, GIBCO) and incubated in Petri dishes at 37°C in a 5% carbon dioxide incubator. When the cultured cells reached confluence in a single layer, they were detached with a solution containing one volume of trypsin (0.5 mg/ml, GIBCO Labs) and four volumes of EDTA (0.2 mg/ml, Dojindo, Kumamoto, Japan) and glucose (0.2 mg/ml, Nakarai Co, Kyoto, Japan), in

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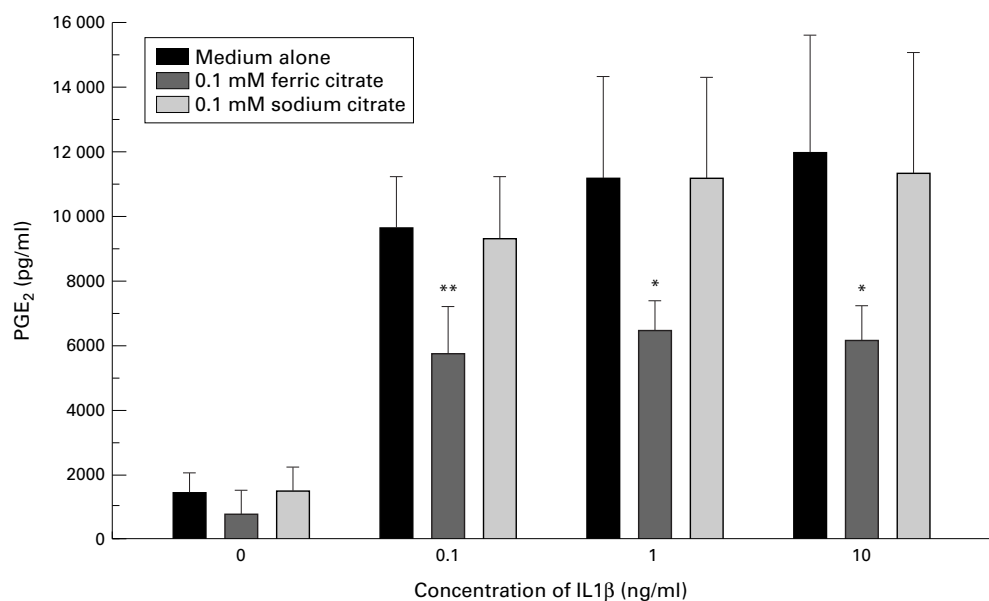


Figure 1 Effects of ferric citrate and sodium citrate on the PGE₂ production by human synovial cells stimulated without or with different concentrations of IL1β. Data are the mean (SEM) of six separate experiments using cells from different patients. * $p < 0.05$, ** $p < 0.01$ compared with medium alone.

phosphate buffered saline (0.1 M, pH 7.2). The detached cells were washed three times with DMEM and resuspended in DMEM with 10% FBS (2×10^4 /ml in a turtle shaped culture bottle, Falcon No 3013) to continue the culture. The material containing the synovial fibroblasts during the 3rd to 7th passage was used in the experiments.

PRODUCTION OF PGE₂

Synovial fibroblasts at two different cell concentrations, namely 1×10^4 and 1×10^5 cells/well, were added to each well of a 24 well macroplate (Falcon No 3047), and were cultured for 18, 24, 48, and 72 hours. After incubation for various periods, the supernatants were centrifuged at 1500 rpm for five minutes and stored at -20°C until required for the measurement of PGE₂. PGE₂ in the culture supernatant was measured by a commercially available enzyme linked immunosorbent assay (ELISA) kit (Cayman Chemical Co, Ann Arbor, MI, USA).

IRON SALTS

Ferric citrate (Fe-citrate, Sigma Co) or sodium citrate (Na-citrate, Ishizu Co, Osaka, Japan) was dissolved in HBSS (GIBCO) and adjusted to pH 7.2 with 1N HCl or 1N NaOH as described.⁷ The iron concentration in the 0.1 mM Fe citrate solution was 620 μg/dl.⁷ This in vitro iron concentration is comparable to the in vivo serum iron concentrations in patients with iron overload and the concentration of iron in synovial membrane from patients with RA.⁷ Desferrioxamine (DFX), an iron chelating agent was purchased from CIBA-GEIGY Ltd, Takarazuka, Japan.

CULTURE OF SYNOVIAL FIBROBLASTS WITH IRON OR SODIUM

Synovial fibroblasts (1×10^4 cells/well), suspended in DMEM with 10% FBS, were cultured for 48 hours with 0.1 mM Fe-citrate

or 0.1 mM Na-citrate in the presence or absence of various concentrations of recombinant human IL1β supplied by Otsuka Pharmaceutical Co (Tokushima, Japan). For the other experiments, various concentrations of Fe-citrate were added to synovial fibroblast cultures.

STATISTICAL ANALYSIS

The differences in PGE₂ production by synovial fibroblasts cultured with Fe-citrate compared with Na-citrate or medium alone were analysed with paired Student's *t* test. A level of $p < 0.05$ was accepted as significant.

Results

SPONTANEOUS PGE₂ PRODUCTION BY SYNOVIAL FIBROBLASTS

In a time course study, it was shown from four different experiments that the spontaneous PGE₂ production by synovial fibroblasts in culture medium reached a plateau after 24–48 hours of incubation at two different cell concentrations (mean (SEM) 1929 (256) pg/ml in 1×10^4 cells/well and 5831 (2005) pg/ml in 1×10^5 cells/well). From these results, the culture supernatants for the assay were collected after 48 hours of incubation at the cell number of 1×10^4 cells/well.

EFFECT OF FE- OR NA-CITRATE ON PGE₂ PRODUCTION BY SYNOVIAL FIBROBLASTS WITH OR WITHOUT STIMULATION BY IL1β

IL1β significantly increased the PGE₂ production by synovial fibroblasts after a 48 hour incubation at all concentrations tested (0.1 ng/ml: mean (SEM) 9544 (2139) pg/ml ($n=6$), 1 ng/ml: 11 107 (3025) pg/ml, 10 ng/ml: 11 896 (3169) pg/ml) compared with the value of 1400 (474) pg/ml without IL1β. Fe-citrate at the concentration of 0.1 mM but not Na-citrate downregulated the PGE₂ production by synovial fibroblasts, both with and without stimulation by IL1β (fig 1).

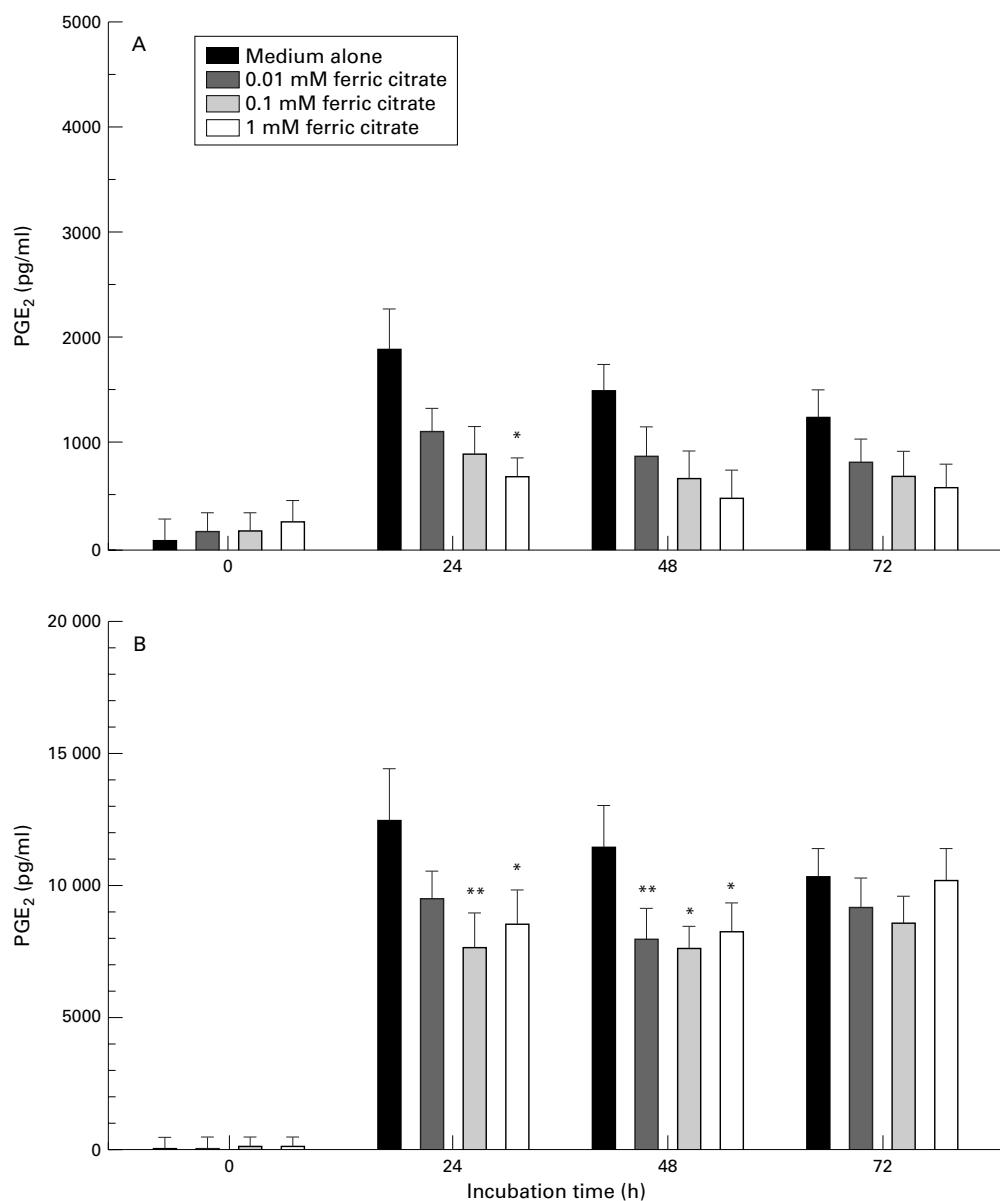


Figure 2 (A) Effects of three different concentrations of ferric citrate on the spontaneous PGE₂ production by human synovial cells in a time course study. Data are the mean (SEM) of five experiments. * $p < 0.05$ compared with medium alone. (B) Effects of three different concentrations of ferric citrate on the PGE₂ production by human synovial cells with stimulation by 1 ng/ml IL1 β in a time course study. Data are the mean (SEM) of five experiments. * $p < 0.05$, ** $p < 0.01$ compared with medium alone.

In a second set of experiments, we examined the effect of different concentrations of Fe-citrate on the PGE₂ production by synovial fibroblasts in a time course study. Fe-citrate significantly suppressed the PGE₂ production without stimulation by IL1 β , in a dose dependent manner (fig 2A). The stimulation by IL1 β (1 ng/ml) increased the PGE₂ production by synovial fibroblasts by about fivefold or sixfold in comparison with the values of spontaneous production at all incubation times examined (24, 48, and 72 hours) (fig 2A and 2B). The maximum inhibition by Fe-citrate of the PGE₂ production was obtained at the concentration of 0.1 mM after 24 or 48 hours with the stimulation by IL1 β (fig 2B).

No difference in PGE₂ production in response to iron treatment was observed using

the cells derived from either RA or OA patients.

EFFECT OF DESFERRIOXAMINE (DFX) ON THE FE INDUCED DOWNREGULATION OF PGE₂ PRODUCTION BY SYNOVIAL FIBROBLASTS
Desferrioxamine (DFX), an iron chelating agent, at the concentrations of 1, 10, and 100 μ g/ml was added to the synovial fibroblast culture system with or without Fe-citrate (0.1 mM) and IL1 β (1 ng/ml). DFX at 100 μ g/ml completely inhibited the down regulatory action of Fe-citrate on the PGE₂ production by synovial fibroblasts (table 1). DFX at the highest concentration tested significantly increased the spontaneous PGE₂ production by synovial fibroblasts. The same tendency by DFX was also observed in IL1 β stimulation (table 1).

Table 1 Effect of desferrioxamine (DFX) on the iron mediated down regulation of prostaglandin E₂ production by human synovial cells

Concentration of DFX (μ g/ml)	IL 1 β free		IL 1 β (1 ng/ml)	
	Ferric citrate (0.1 mM)		Ferric citrate (0.1 mM)	
	(-)	(+)	(-)	(+)
Medium alone	1043 (128) (pg/ml)	842 (100)*	7862 (871)	6211 (792)**
1	1253 (171)	857 (109)	8531 (915)	7168 (881)
10	1075 (131)	882 (67)	8317 (912)	7650 (740)
100	1548 (211)†	1224 (146)	12 486 (2893)	11 266 (2216)

The amount of PGE₂ in the culture medium was measured by an ELISA kit. Data are the mean (SEM) values of 8 experiments. *p<0.05 v medium alone. **p<0.01 v IL 1 β stimulated. †p<0.05 v medium alone.

Discussion

It was shown in this study that iron in the form of ferric citrate (Fe-citrate) suppressed the PGE₂ production by human synovial fibroblasts in vitro. In contrast, Okazaki *et al* reported that iron in the form of ferric nitrilotriacetate (Fe-NTA) increased the PGE₂ production by rabbit synovial fibroblasts.¹¹ This discrepancy might be derived from (1) the different forms of iron salts used; Fe-citrate is physiological and Fe-NTA is carcinogenic, and (2) the cells used are from different species (human and rabbit synovial fibroblasts). Iron is present in the rheumatoid synovial tissue mainly in the form of ferritin, iron storage protein or Perl positive stainable iron as haemosiderin. Each of these forms might be able to release iron to form hydroxyl radicals.^{2,12} Moreover, iron exists in the form of citrate as non-transferrin bound iron, one of the low molecular weight chelators in RA synovia, being a donor for iron in the Haber-Weiss reaction.² Interestingly, it was recently demonstrated that K562 cells, an erythroid leukaemia cell line, have two iron transport systems: (1) the classic transferrin (Tf) mediated iron uptake via the Tf receptors, and (2) a non-Tf (commonly citrate) pathway via cell surface integrin.¹³ Human synovial fibroblasts may also utilise the non-Tf pathway to take up iron for their proliferation, as described in our previous report.⁷

PGE₂, the major arachidonic metabolite, is a potent mediator of pain and oedema at sites of inflammation.⁸ In addition, PGE₂ has many immunoregulatory functions including the inhibitions of Ia expression, the IL1 and TNF production by macrophages and antigen induced T cell proliferation, and the promotion of the differentiation of suppressor T cells and immunoglobulin secreting cells.¹⁴ PGE₂ plays a part as an internal regulator of the growth of fibroblasts⁹ and of IL1 production in macrophages.¹⁰ Our results demonstrate that iron downregulates the PGE₂ production by human synovial fibroblasts in culture, although the possibility that iron stimulates PGE₂ metabolism remains. This indicates that the stimulation of synovial fibroblast DNA synthesis by iron⁷ might be caused by the suppression of PGE₂ production.^{9,10} The concentration of 0.1 mM Fe-citrate was most effective in the suppression of PGE₂ production (fig 2B). This phenomenon was also observed in the stimulation of synovial fibroblast DNA synthesis⁷ and the modulation of cell surface molecule expression,³ which may also support the above mentioned mechanism.

Prostanoid production including PGE₂ is mediated through cyclooxygenase (COX), a classic heme peroxidase in the prostaglandin cascade.¹⁵ There are two isoforms of COX. COX I is constitutively expressed in most tissues, and COX II is inducible by various proinflammatory agents including IL1 β .¹⁵ Iron may suppress the PGE₂ production by directly influencing the activity, protein synthesis or mRNA expression of COX. Salvemini *et al* demonstrated that nitric oxide (NO) increase PGE₂ production via the activation of COX activity in a mouse macrophage cell line.¹⁶ Weiss *et al* reported that iron suppresses the NO production of a murine macrophage cell line by decreasing NO synthase activity.¹⁷ Thus, iron may downregulate PGE₂ production indirectly by decreasing the NO production via suppressed NO synthase activity.

The synovitis caused by the actions of PGE₂⁸ may be diminished by the down regulation by iron of PGE₂ production. Agro *et al* recently demonstrated that PGE₂ increases IL6 and IL8 but not granulocyte macrophage colony stimulating factor (GM-CSF) production in human synovial fibroblasts.¹⁸ Thus, the production of IL6, one of the most potent inflammatory mediators⁸ could be decreased by iron through the suppressed production of PGE₂, which is an enhancer of IL6 production.¹⁸ However, IL6 has the ability to increase the production of tissue inhibitor of matrix metalloproteinase, which is involved in the restoration of tissue at the site of inflammation.¹⁹ From this aspect, iron may delay the tissue reconstruction after inflammation by decreasing the IL6 production. Thus, iron does not necessarily work in the anti-inflammatory direction only by suppressing PGE₂ production. In fact, tetradecanoyl phorbol acetate induced inflammation still occurs in the COX-II gene knock out mice without PGE₂.²⁰ In addition, PGE₂ inhibits the rheumatoid factor production by B cells from RA patients.¹⁴ Rheumatoid factor production could be increased by the suppression of PGE₂ production. Taken together, these findings indicate that down regulation of PGE₂ production by iron could increase the chronic inflammation in RA joints.

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